

# **The Neurobiology of Stress-Induced Hyperthermia**

## **Colofon**

Cover design: The Dotted Line, Antwerp, Belgium

Printed by: Gildeprint Drukkerijen B.V., Enschede, The Netherlands

ISBN: 978-90-393-5222-9

# **The Neurobiology of Stress-Induced Hyperthermia**

De Neurobiologie van Stress-Geïnduceerde Hyperthermie  
(met een samenvatting in het Nederlands)

## **Proefschrift**

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op  
gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit  
van het college voor promoties in het openbaar te verdedigen op  
woensdag 23 december 2009 des ochtends te 10.30 uur

door

**Christiaan Hendrik Vinkers**

geboren op 6 september 1980  
te Wageningen

Promotoren: Prof. dr. B. Olivier  
Prof. dr. C.J. Kalkman

Co-promotoren: Dr. L. Groenink  
Dr. S.M. Korte

Printing of this thesis was financially supported by:

*DataSciences Int. St. Paul, MN, USA*

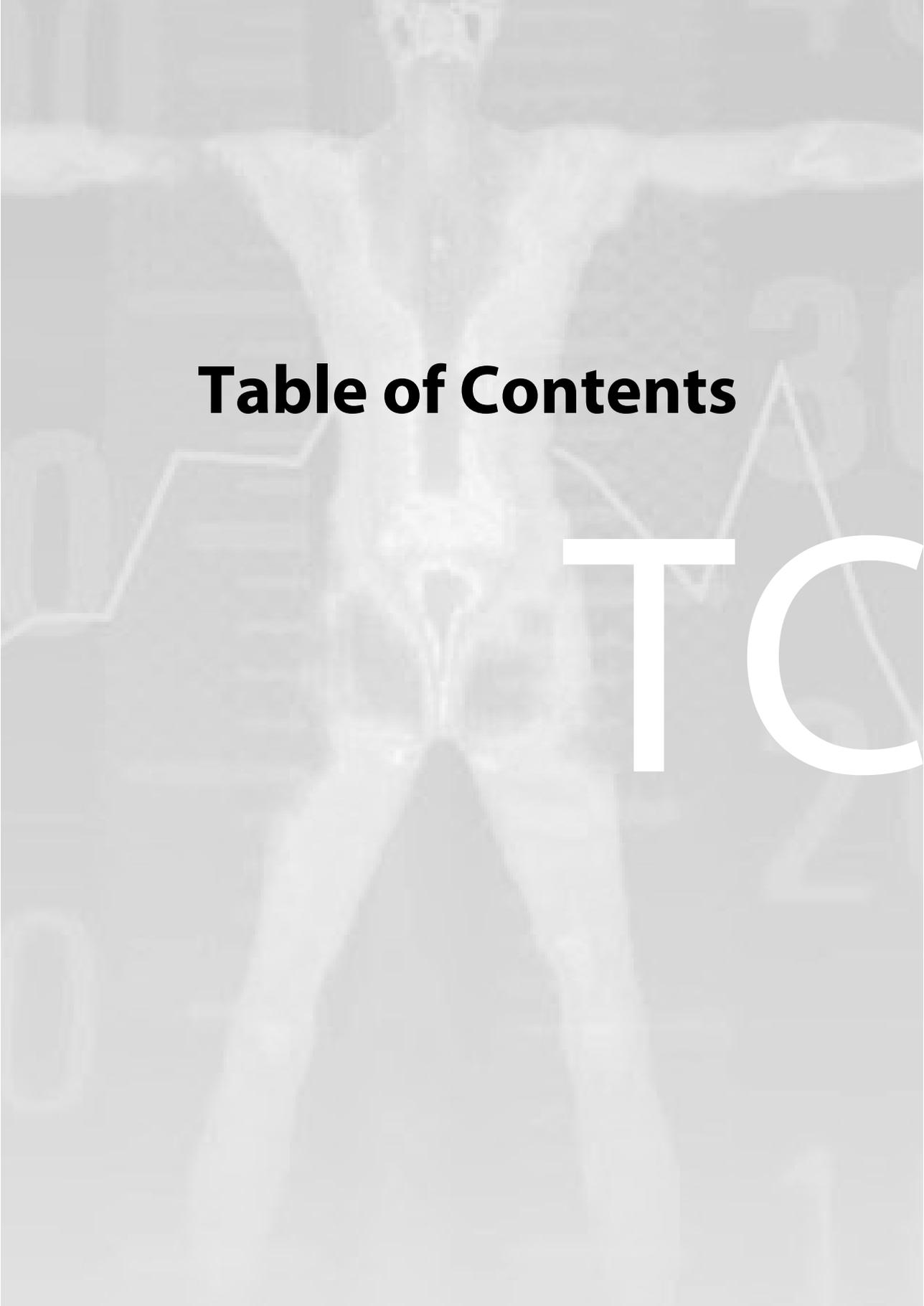
*DaACRO, Trier, Germany*

*J.E. Jurriaanse Stichting, Rotterdam, The Netherlands*

*Neurosearch A/S, Ballerup, Denmark*

*PsychoGenics Inc., Tarrytown, NY, USA*

*Schering-Plough, Houten, The Netherlands*

The background of the page features a faint, light gray illustration of a human figure with arms and legs spread wide. Overlaid on this figure are various data visualization elements: a line graph on the left side, a bar chart in the center, and several large, semi-transparent numbers (0, 1, 2, 3) scattered across the scene. The overall aesthetic is clean and modern, suggesting a focus on data and human performance.

# **Table of Contents**

# TC

# Table of contents

## Part I: General introduction

<b>Preface</b>	<b>9</b>
<b>Chapter 1</b> Pharmacological research into anxiety disorders: the stress-induced hyperthermia (SIH) paradigm	<b>13</b>
<b>Chapter 2</b> The basic protocol to conduct SIH research	<b>43</b>

## Part II: Pharmacological applications of the SIH paradigm

<b>Chapter 3</b> Dissociating anxiolytic and sedative effects of subunit selective GABA <sub>A</sub> ergic drugs using the SIH paradigm	<b>57</b>
<b>Chapter 4</b> 5-HT <sub>1A</sub> receptor blockade reverses anxiolytic effects mediated by the GABA <sub>A</sub> receptor $\alpha_3$ subunit	<b>75</b>
<b>Chapter 5</b> Differential tolerance after chronic treatment with GABA <sub>A</sub> receptor $\alpha$ subunit-selective compounds	<b>89</b>
<b>Chapter 6</b> Early-life blockade of 5-HT <sub>1A</sub> receptors alters adult anxiety behavior and benzodiazepine sensitivity	<b>107</b>
<b>Chapter 7</b> Stress-induced hyperthermia and fever: two of a kind?	<b>123</b>

**Chapter 8** 141  
Stress-induced hyperthermia is reduced by rapid-acting anxiolytic drugs independent of injection stress in rats

**Chapter 9** 151  
The rapid hydrolysis of chlordiazepoxide to demoxepam has consequences for chronic minipump applications

### **Part III: Genetic applications of the SIH paradigm**

**Chapter 10** 165  
Corticotropin-releasing factor overexpression modulates GABA<sub>A</sub> and glutamate<sub>2/3</sub> receptor systems

**Chapter 11** 181  
GABA<sub>A</sub> and serotonin receptor sensitivity in chromosome substitution strains of mice using stress-induced hyperthermia

### **Part IV: CNS mechanisms in the SIH paradigm**

**Chapter 12** 195  
Medial amygdala lesions differentially influence stress responsivity and sensorimotor gating in rats

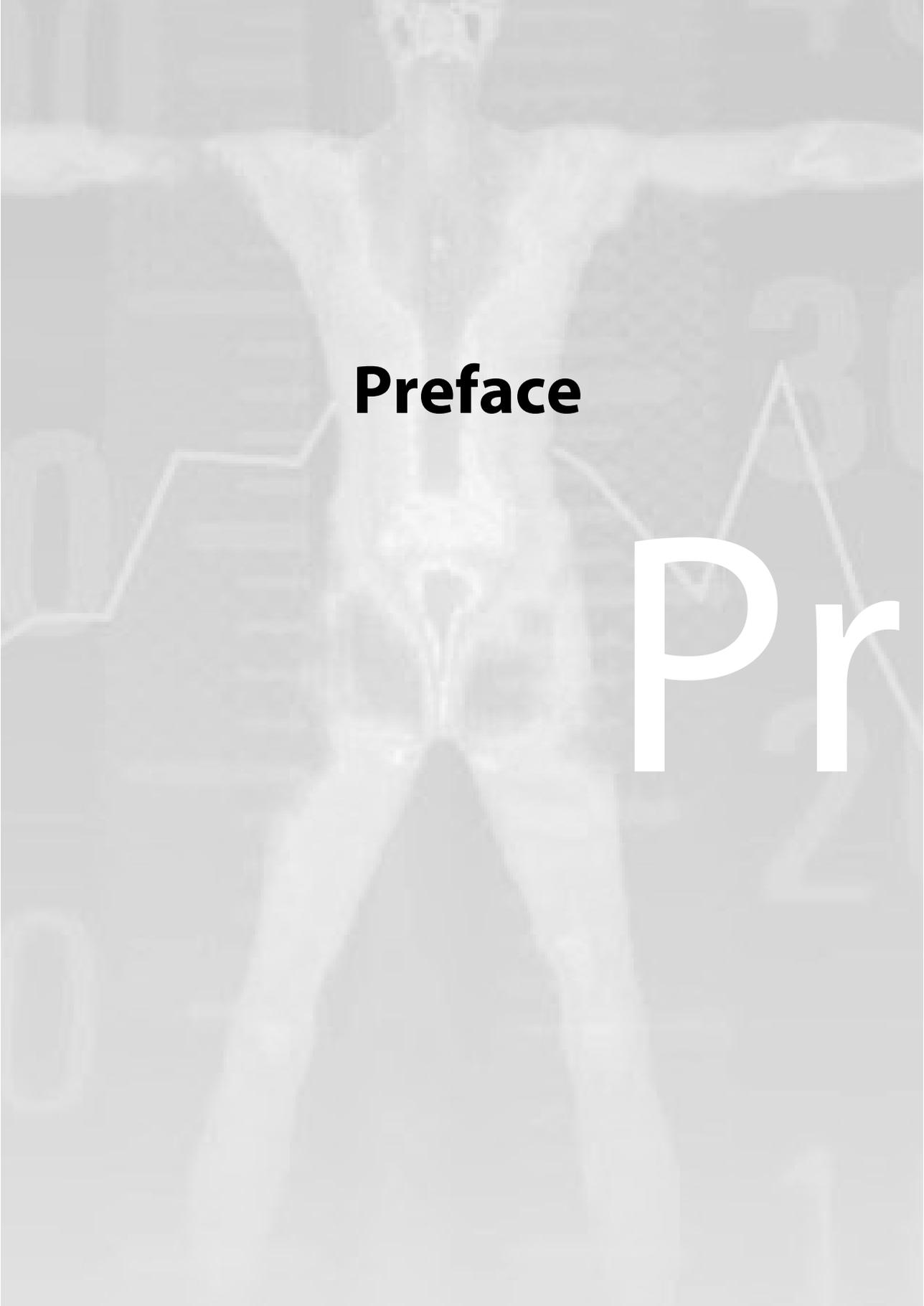
**Chapter 13** 209  
Olfactory bulbectomy induces rapid and stable changes in basal and stress-induced locomotor activity, heart rate and body temperature responses in the home cage

### **Part V: The SIH paradigm as a translational anxiety model**

**Chapter 14** 223  
Exposure to stress differentially affects central and peripheral body temperature in human subjects

## **Part VI: Discussion**

<b>Chapter 15: Discussion</b>	<b>237</b>
<b>Part 15.1:</b> General discussion	237
<b>Part 15.2:</b> Elucidating GABA <sub>A</sub> and GABA <sub>B</sub> receptor functions in anxiety using the stress-induced hyperthermia model: a review	241
<b>Part 15.3:</b> Stress-induced hyperthermia, the serotonin system and anxiety	263
<b>Part 15.4:</b> Stress-induced hyperthermia in translational stress research	285
<b>References</b>	<b>291</b>
<b>Summary</b>	<b>335</b>
<b>Samenvatting (Nederlands)</b>	<b>338</b>
<b>List of publications</b>	<b>342</b>
<b>Author affiliations</b>	<b>344</b>
<b>Acknowledgements / Dankwoord</b>	<b>349</b>
<b>About the author</b>	<b>352</b>



**Preface**

Pr

## Preface

Every individual who has experienced a stressful situation, whether it is being attacked by a wild animal, the moment right before an important presentation or just finding yourself in an awkward situation, is familiar with the warm and aroused feeling that one can experience at that moment. This change in body temperature in response to stress is referred to as stress-induced hyperthermia (SIH), and is the main subject of the research that has led to this thesis. This response is a consistent phenomenon that occurs in reaction to a stressful experience and is mediated by the autonomic nervous system. It is used throughout this thesis as a quantitative read-out parameter for the experience of stress. The principal aim of this thesis is to show diverse applications of the SIH paradigm, and we propose that it constitutes a robust and reproducible across-species phenomenon with translational potential that is very suitable for drug screening. Moreover, this paradigm can be used to investigate the neurobiological mechanisms underlying the stress response as well as to examine the impact of genetic modifications or naturally occurring genetic variation such as strain differences. To enhance the readability of this thesis, a schematic (Figure 1) will be displayed at the beginning of each experimental chapter with the relevant part of the figure highlighted.

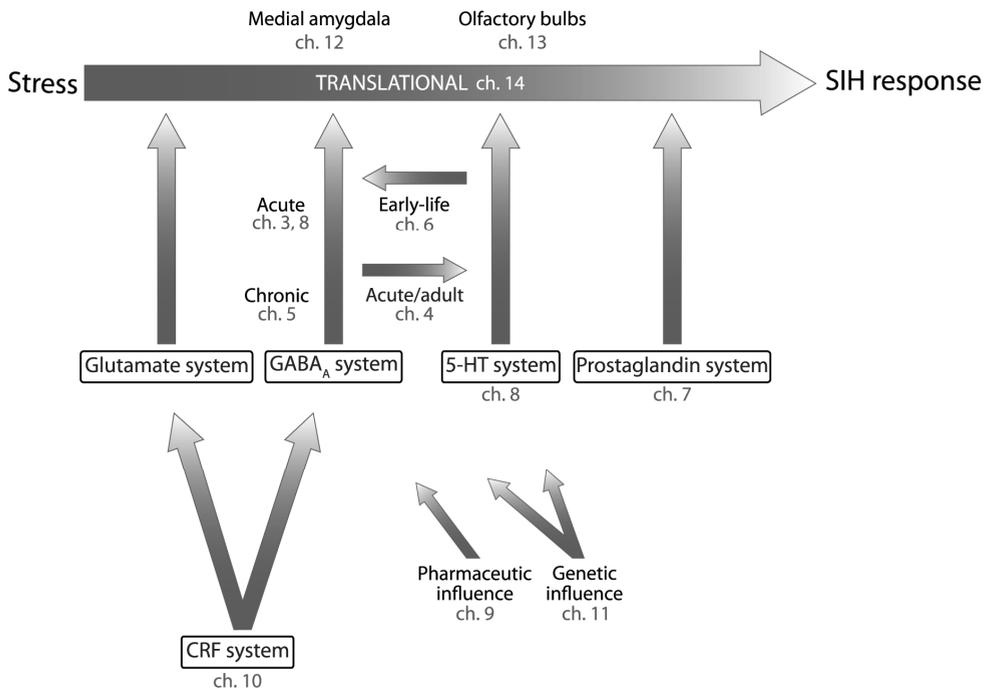


Figure 1: Schematic overview of the research described in this thesis. The numbers indicate the relevant thesis chapter. The different receptor systems (GABA<sub>A</sub>, glutamate, 5-HT, CRF) concern the CNS receptor systems and include ligands acting via these receptors.

This thesis consists of six parts. The first part (**chapter 1 and 2**) describes the background of the SIH paradigm, its neurobiological basis and the putative genetic and pharmacological applications. Specifically, the different drug classes that modify the SIH response are discussed. This introductory part also includes a chapter on the practical requirements to carry out SIH studies in mice.

Part two focuses on a variety of pharmacological applications of the SIH response and consists of seven chapters. **Chapter 3** describes the contribution of the different GABA<sub>A</sub> receptor subunits to the benzodiazepine-mediated reduction of the SIH response, whereas **chapter 4** examines the role of the serotonergic (5-HT) system in these GABA<sub>A</sub> receptor mediated actions. **Chapter 5** discusses the effects of chronic (subunit-selective) GABA<sub>A</sub> receptor activation and the consequences for the development of tolerance. The effects of early-life disruption of the serotonergic system on adult benzodiazepine sensitivity and anxiety-related behavior in mice are the main subject of **chapter 6**. **Chapter 7** investigates whether the SIH response should be regarded as a form of fever or not, and **chapter 8** studies the effects of injection stress on the SIH-reducing effects of various rapid-acting anxiolytic drugs. The importance of an in-depth pharmaceutical analysis during chronic drug studies is described in **chapter 9**.

Part three of the thesis comprises two studies with a genetic approach. The effects of genetic CRF overactivation on the GABA<sub>A</sub> and metabotropic glutamate receptor systems are discussed in **chapter 10**. **Chapter 11** investigates the genetic basis of drug sensitivity using chromosome substitution mice. In part four, the involvement of two brain structures in the SIH response is considered. Specifically, **chapter 12** discusses the effects of bilateral medial amygdala lesions, whereas in **chapter 13**, the impact of olfactory bulbectomy on basal and stress-induced autonomic parameters is the central topic. Part five contains a study on the human SIH response in healthy male and female volunteers (**chapter 14**). The thesis will be concluded with a general discussion which also contains the future directions for SIH research (**chapter 15.1**). Moreover, three separate reviews discuss the body of SIH literature (also based on the research in this thesis) on the GABA<sub>A</sub> and GABA<sub>B</sub> receptor systems (**chapter 15.2**), the serotonin system (chapter 15.3) and the translational potential of SIH applications (**chapter 15.4**).



# Chapter 1

## **Pharmacological research into anxiety disorders: the stress-induced hyperthermia paradigm**

Christiaan H. Vinkers

Meg J. van Bogaert

Marianne Klanker

S. Mechiel Korte

Ronald S. Oosting

Taleen Hanania

Seth C. Hopkins

Berend Olivier

Lucianne Groenink

1

*European Journal of Pharmacology (2008) 585:407-425*

## **Abstract**

In anxiety research, the search for a model with sufficient clinical predictive validity to support the translation of animal studies on anxiolytic drugs to clinical research is often challenging. This review describes the stress-induced hyperthermia (SIH) paradigm, a model that studies the omnipresent activation of the autonomic nervous system by measuring body temperature in response to stress. The reproducible and robust SIH effects, combined with the relatively easy way of testing, make the SIH paradigm very suitable for drug screening. We will review the current knowledge on the neurobiology of the SIH response, and discuss the role of GABA<sub>A</sub> and serotonin (5-HT) pharmacology as well as how the SIH response relates to infectious fever. Furthermore, we will present novel data on the SIH response variance in different mice strains and their sensitivity to anxiolytic drugs. The SIH response is an autonomic stress response that can be successfully studied at the level of its physiology, pharmacology, neurobiology and genetics and possesses excellent animal-to-human translational properties.

## 1. Introduction

Anxiety is characterized by typical cognitive, behavioral, somatic and emotional responses. Typically these include psychological symptoms like worrying, restlessness, fear, in addition to somatic symptoms like sweating, elevated heart rate, and trembling. As part of an immediate response to external stimuli, this is a functional response and falls within the range of normal physiological states. However, when chronically present or out of relevant context, these reactions become maladaptive. An inappropriate activation of the autonomic nervous system is critical in anxiety disorders, resulting in heightened physiological responses of heart rate, blood pressure and temperature (Stockmeier et al 2009).

Anxiety disorders are the most prevalent mental disorder with very high co-morbidity (Grant et al 2005; Kessler et al 2005; Merikangas et al 1996). Anxiety disorders are multifactorial with a complex etiology that is not fully known (Craske and Waters 2005; Hettema et al 2001; Schneiderman et al 2005). Under DSM-IV, anxiety disorders include generalized anxiety disorder, panic disorder, phobias and post-traumatic stress-disorder. These disorders have severe impact on quality of life and are generally underrecognized and, if diagnosed, remain undertreated (Lecrubier 2007; Merikangas et al 2007). Research on anxiety disorders has increased the understanding of their pathological basis and has resulted in proposed therapeutic approaches to their treatment. However, the complex central mechanisms underlying these disorders remain poorly understood. Besides, currently available drugs, although effective, were not specifically developed for treating anxiety disorders and possess unwanted side effects including sedation and dependence (Dias et al 2005).

Animal models are helpful to study the biological basis of anxiety and stress disorders, especially to evaluate the effectiveness of drug candidates. Since higher cognitive functions cannot be fully explored in animals, it is not without difficulty to use animals to investigate anxiety. In the development of anxiolytic drugs, it is not unusual for promising preclinical results to fail in the following clinical phase. The question is whether such expensive failures can only be attributed to the limited predictive clinical validity of the applied animal models, or whether methodological setup of these preclinical studies leads to a biased and unbalanced selection of the candidates to proceed to clinical trials

The extensive use of animal models has resulted in objective criteria for validity. In general, animal model validity can be assessed by three sets of criteria: whether the animal behavior is similar to the human condition (face validity), whether the animals respond to the drug treatments known to be effective in humans (predictive validity), and whether the model corresponds with the known disorder mechanisms (construct validity) (McKinney and Bunney 1969). A good animal-to-human translational model for the development of anxiolytic drugs would be able to provide early proof of concept of a drug's effectiveness before expensive clinical trials are initiated. Many different

approaches to modelling anxiety are available that focus on different aspects of the disorder (Cryan and Holmes 2005). The majority of animal models currently exploited in anxiety research use explorative approach-avoidance behavior under more or less aversive circumstances to assess anxiety states, among which the light-dark exploration test, the open field test and the elevated-plus maze. This approach has proven to be of great value, although it is difficult to differentiate anxiety-related avoidance from novelty-seeking or impulsivity-related approach behavior (Cryan and Holmes 2005), or to discriminate anxiolytic drug effects from sedation-induced locomotor changes. Furthermore, within-strain and between-strain locomotor variation can affect results (Carola et al 2002). The predictive value of these exploratory animal models for classes of anxiolytic drugs other than the classic benzodiazepines has been questioned (Borsini et al 2002). In addition, there are animal tests that use conditioned fear as another approach to model anxiety (Delgado et al 2006; Lissek et al 2005).

Taken as a whole, currently used models have limitations. As a result, we propose that an alternative approach to modeling anxiety in animals would be of valuable in anxiety research and drug development. In particular, we review the stress-induced hyperthermia (SIH) model, which uses the stress-induced autonomic nervous system activation by measuring the body temperature increase after stress exposure. This SIH response has proven to be a very consistent physiological stress reaction comparable across all species with less variability than the stress-induced heart rate reaction (Van Bogaert et al 2006a). In this review, we will further explore the background of the SIH response and present novel data that shows that the SIH paradigm can be used as a valuable approach in anxiety research with excellent animal-to-human translational properties.

## **2. The SIH paradigm**

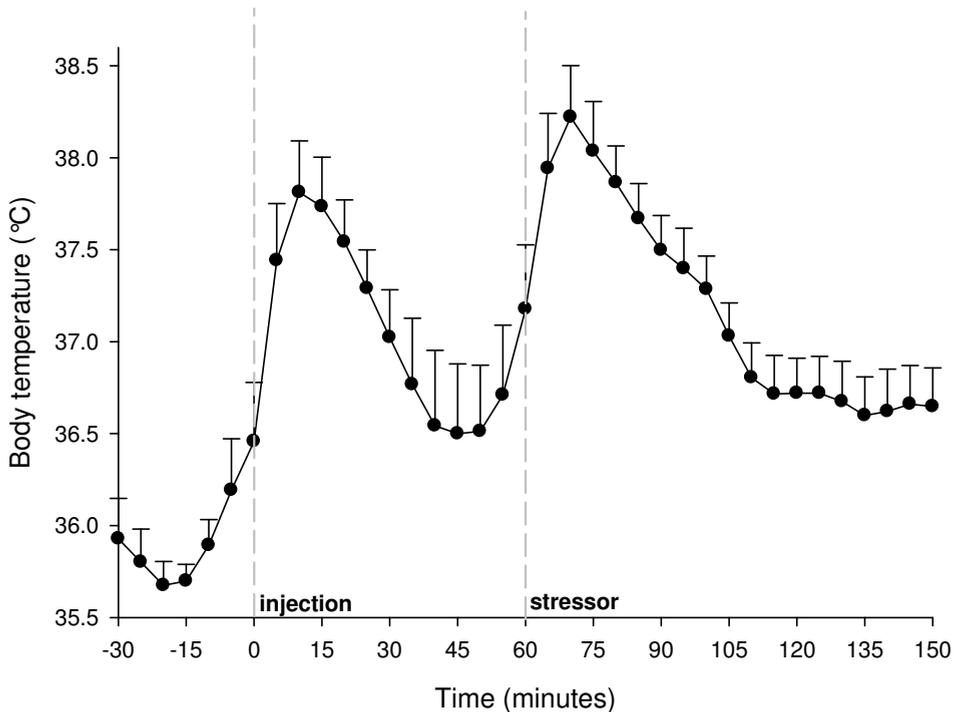
### **2.1 The SIH response**

Previous studies have shown that exposure to both physiological and psychological stress affects core body temperature. In humans, activities such as watching movies, attending boxing contests and taking a test all result in an increase in body temperature (Briese 1995; Kleitman and Jackson 1950; Marazziti et al 1992; Renbourn 1960), whereas in animals, exposure to noise, heat, novelty and pain have the same effect (Bouwknrecht et al 2007). This process is referred to as SIH, or emotional fever, and is a relative short lasting body temperature elevation in response to stress. Within 15 min, temperature rapidly rises  $\pm 1.0$ - $1.5$  °C. This elevation in temperature is considered important for survival as it is a preparation for fight-or-flight. In support of this, the SIH amplitude positively correlates with test performance in students (Briese 1995). In fact, the choice of fight or flight in lizards is influenced by the degree of temperature change after the stressor (Herrel et al 2007). However, the SIH response is not increased in mice with more anxious genotypes (Bouwknrecht et al 2007) and a higher SIH amplitude is not always accompanied with a more anxious phenotype in several behavioral paradigms. The SIH response is ubiquitous, being present in virtually any mammal that has been tested so far, among which humans, baboons, silver foxes, pigs, ground squirrels, rabbits, rats and mice (Bouwknrecht et al

2007). The SIH response is accompanied with an increased hypothalamic-pituitary-adrenal (HPA)-axis activity (Groenink et al 1994; Spooren et al 2002; Veening et al 2004), and habituation of a SIH response to stress is correlated with a similar blunting of corticosterone responses (Barnum et al 2007) (for more on habituation: 3.4). However, anxiolytic drugs that attenuate the SIH response do not influence basal and stress-induced levels of these stress-related endocrine parameters in mice (Groenink et al 1996b).

## **2.2 SIH and anxiety: development of a model**

The first use of SIH in anxiety research occurred after it was noted that removing mice one by one from a group-housed cage increased body temperature of the last mouse compared to the first. This response, putatively anticipatory anxiety, was utilized for the screening of anxiolytic drugs (Borsini et al 1989). Later on, this model was refined to a singly-housed version in which the rectal temperature was measured twice with an interval of 10 min (Van der Heyden et al 1997). This dramatically reduced the number of animals needed per experiment. In the singly-housed SIH experimental setup, the first rectal temperature measurement ( $T_1$ ) represents the basal unstressed core temperature, and functions as an adequate stressor as well. Since SIH peaks within 10-15 min, the second rectal temperature measurement ( $T_2$ ) represents the peak temperature after stress and the SIH response is calculated by subtracting  $T_1$  from  $T_2$ . The singly and group-housed versions correlate almost perfectly with each other, indicating that both approaches are valid (Spooren et al 2002). Telemetric systems measuring body temperature are increasingly employed and are more precise and accurate than labor-intensive manual rectal temperature measurements. Rectal measurements are fast, cheap and have been proven to be valid and easy to carry out.



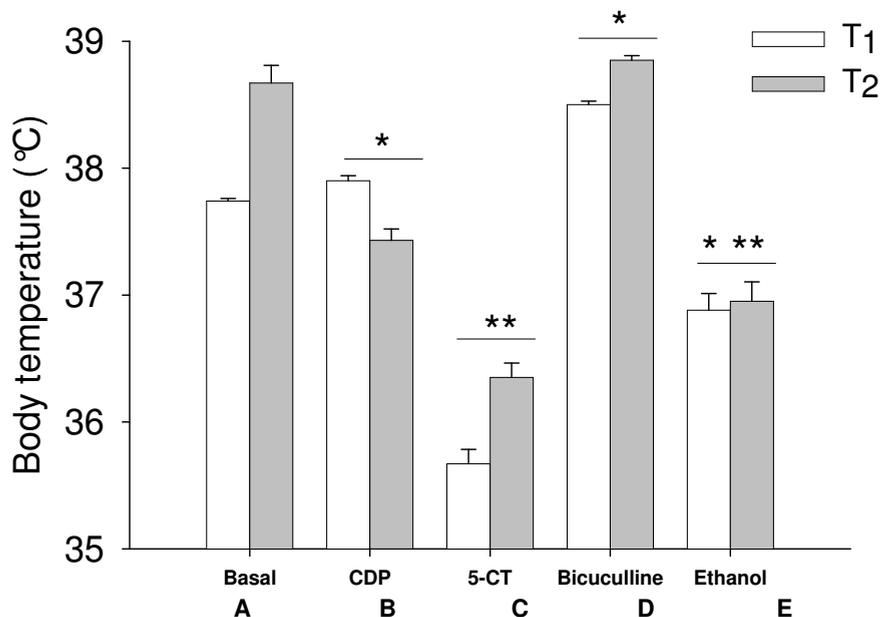
**Figure 1:** Telemetric measurement of the temperature course in a standard SIH test in 129Sv mice ( $n=7$ ). At  $t=0$  min, vehicle is injected, causing a transient SIH response. At  $t=60$ , a rectal temperature measurement is applied as a second stressor, leading to the basal SIH response used to assess anxiolytic action. In the classic SIH test, a second rectal measurement is carried out at  $t=70$  to assess the maximum temperature. Error bars represent the S.E.M..

Regardless, the use of telemetric equipment retains the general principle of increases in body temperature caused by a stressful situation. As illustrated in Fig. 1, SIH can easily be calculated by the difference of these temperatures (second peak in Fig. 1). Extensive experimenting has revealed that in mice, the SIH response remains intact when animals are repeatedly tested once a week (Bouwknicht and Paylor 2002; Bouwknicht et al 2004b; Van der Heyden et al 1997) (for more on habituation see section 3.4). The SIH response is very robust, but it displays a consistent ceiling effect (see below, Fig. 2 and 5). Applying more stress will not lead to higher stress-induced temperatures, which makes the SIH paradigm less appropriate for elucidating anxiogenic drug properties.

### 2.3 Drug testing in the SIH paradigm

The reproducible and robust SIH effects, combined with the ease of testing, make the SIH paradigm very suitable for drug screening. Administration of drugs with anxiolytic properties, such as GABA<sub>A</sub> and 5-HT receptor agonists results in a reduction or even ablation of the SIH response, whereas non-anxiolytic dopaminergic or noradrenergic drugs generally do not affect the SIH response (for more pharmacological data see section 5). However, many anxiolytic drugs also decrease basal body temperature even before

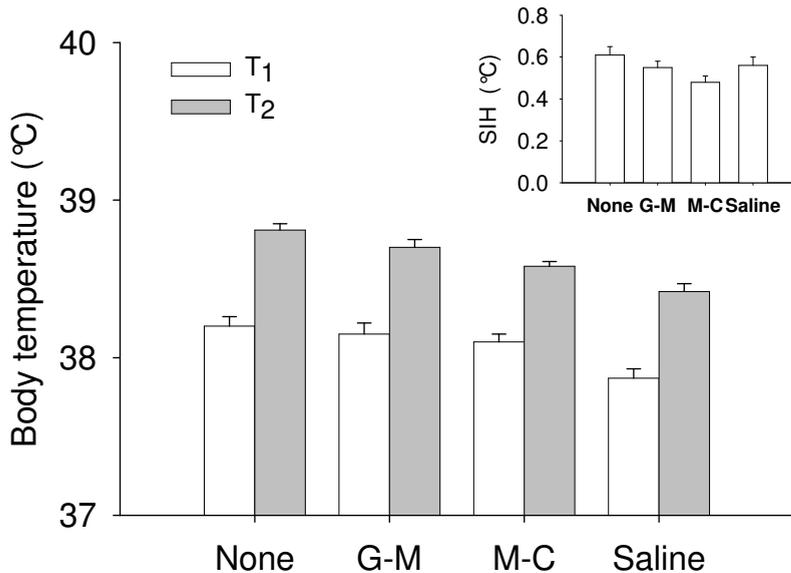
stress exposure occurs. Strong hypothermic effects of drugs reduce or even ablate the SIH amplitude without an anxiolytic mode of action (Olivier et al 2003). This is probably due to a disturbed homeostasis in which a SIH effect can no longer be present. It is therefore important not only to display temperature differences, but also to show the absolute temperature values to assess anxiolytic effects and effects on core body temperature.



**Figure. 2:** Pharmacological temperature reactions in C57BL/6J mice ( $n=10-12$ ) in the classical SIH test. Drugs are injected 60 min before measuring the body temperature (BT) rectally twice within 10 min ( $T_1$  and  $T_2$ , respectively). **(A)** basal SIH response (no drug) **(B)** SIH decrease, no effect on basal BT (chlordiazepoxide (CDP), 10 mg/kg IP) **(C)** No SIH reduction, hypothermic effect on basal BT (5-CT, 5 mg/kg IP) **(D)** Decreased SIH due to ceiling effect, increased basal BT (bicuculline, 10 mg/kg IP) **(E)** Decreased SIH, decreased basal BT (ethanol, 3 g/kg p.o.). Effects on SIH reduction (\*,  $p < 0.01$ ) and basal BT (\*\*:  $p < 0.01$ ). Error bars represent the S.E.M..

Unfortunately, the injection of a drug itself is a stressor that elevates body temperature. Therefore, drugs must be administered 60 min prior to the eventual stressor to assess SIH responses. This allows temperatures to return to approximately basal conditions. This period has been experimentally validated using various (genetically modified) strains and a wide variety of drugs (Olivier et al 2003). Because of a ceiling effect, it is not possible to superimpose two sequential stressors (injection plus rectal measurement). An interval of 60 min is sufficient to ensure body temperature has sufficiently declined to approximately pre-stress baseline levels. In mice, injections 60 min before assessing the SIH response lead to an identical SIH response as compared to the mice that had received no injection at all (Fig. 3), supporting a 60 min period between injection and SIH assessment.

Drugs tested in the SIH paradigm need to possess sufficiently long half lives to be present in effective blood concentrations at the time of stress application 60 min later. It is especially important, when comparing the intrinsic potencies of a series of candidate drugs, to make the comparisons with respect to peak concentrations of drug. In fact, several CNS active drugs have peak plasma concentrations well before 60 min. One way to address these comparisons would utilize stress-free administration via intravenous catheters connected to flexible injection lines, in which handling and even disturbing the animal would no longer be necessary.



**Figure 3:** Similar SIH response in C57BL/6J mice (n=12) regardless of the vehicle used 60 min earlier. A: no injection (None). B 0.5% gelatin/ 5% mannitol solution (G-M) C 0.5% methylcellulose solution (M-C) D 0.9% saline (Saline).

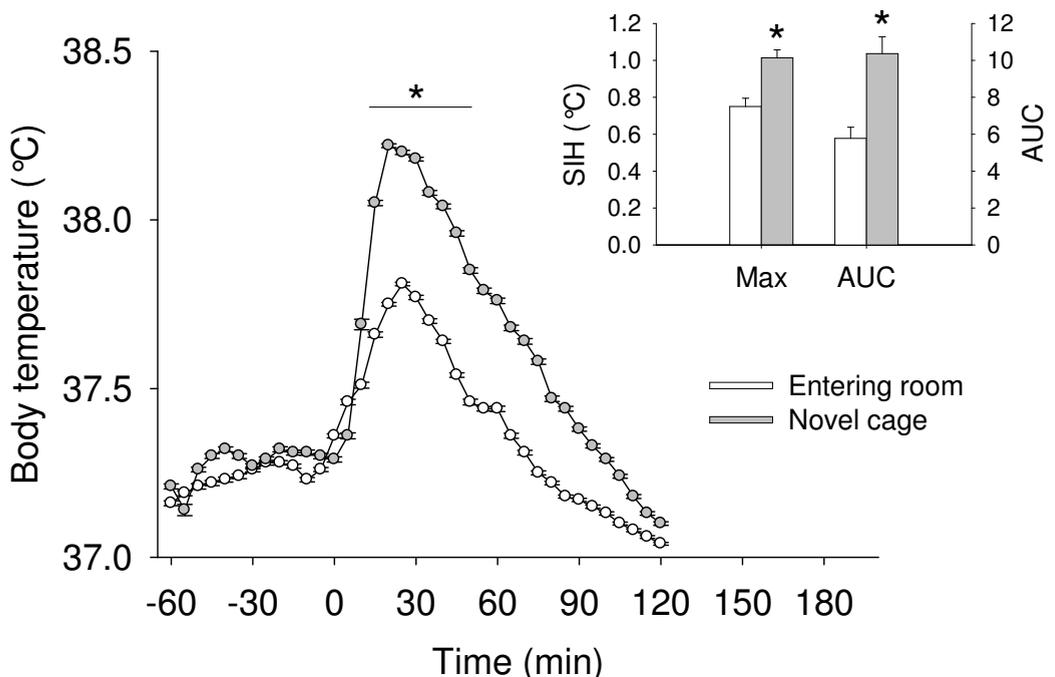
Extensive pharmacological testing has shown that the SIH paradigm is indeed suitable for predicting anxiolytic drug properties. Clinically effective anxiolytic drugs such as benzodiazepines and 5-HT<sub>1A</sub> receptor agonists such as buspirone all attenuate the SIH response (Olivier et al 2003). Most research has been carried out in rodents (for a review on the pharmacological SIH evidence (Bouwknicht et al 2007)), and a wide range of different drug classes has been tested so far. From this research, it has become evident that the GABA<sub>A</sub> and 5-HT systems are closely involved in the limbic stress response and the subsequent SIH reaction, whereas dopaminergic and noradrenergic drugs have little effect on the SIH response (Bouwknicht et al 2007). More importantly, acute effects of selective serotonin reuptake inhibitors (SSRIs) are absent (Olivier et al 2003). Chronic fluoxetine treatment is reported to have either no influence on SIH (Roche et al 2007) or to reduce SIH (Conley and Hutson 2007). All of these findings point to the fact that the SIH paradigm is sensitive to anxiolytic-like properties, contributing to its predictive validity. Before discussing a more detailed pharmacological background of drug classes and the

differences between and within animals, it is important to have a good understanding on the universality of the SIH response.

### 3. Factors influencing the SIH response

#### 3.1 Strain differences

The SIH response is robust, reproducible and reliable. However, the absolute temperature changes observed after a given stressor display significant variation, depending on various factors. First of all, animal strains differ in their ability to maintain homeostasis after stress in various anxiety animal models (Bouwknrecht and Paylor 2002; Rodgers et al 2002a; Van Bogaert et al 2006a), including the SIH paradigm. Testing revealed that 9 mouse strains displayed a variable SIH response between 0.6 and 1.9 °C (Bouwknrecht and Paylor 2002). Differences in locomotor activity and body weight alone do not account for the differences in basal or stress-induced levels of temperature and heart rate (Pardon et al 2004; Van Bogaert et al 2006a) (for more: see section 6.1). Therefore, it is vital to assess the stress responsiveness of animals of a given strain before any experiment is initiated.



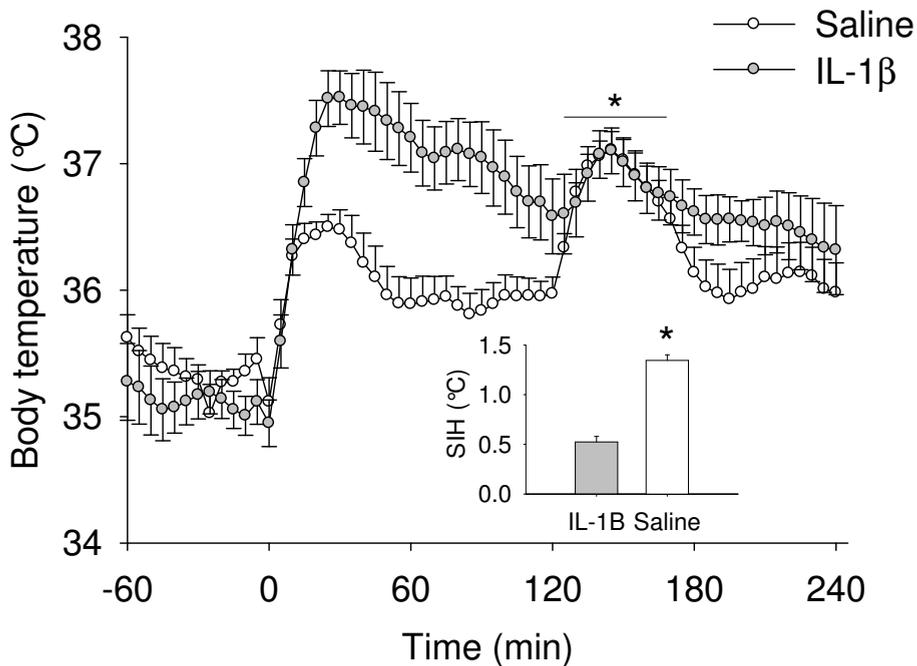
**Figure 4:** Entering the room of male Wistar rats (n=42) elicits a smaller SIH response than novel cage stress. At t=0 min, either the room was entered or rats were placed in a novel cage. Insert: Bars represent the maximum SIH response and the AUC (area under the curve) of both interventions. One-way ANOVA, treatment as within-subject factor: \*:  $p < 0.001$ . Error bars represent S.E.M..

### **3.2 Type of stressor**

Stressors apparently can increase body temperature only up to certain levels, above which no further temperature rise is possible. This ceiling effect limits the maximum SIH response, but nevertheless several types of stressors evoke reliable SIH responses from basal temperature (Van der Heyden et al 1997). Minor stressors, such as entering the room where the animal is housed induce a smaller SIH compared to intermediate stressors as putting animals into a novel cage (Fig. 4). Intense stressors such as repeated social defeat lead to consistently higher SIH responses than placing animals in a novel cage, restraint or cage confinement (Barnum et al 2007; Bhatnagar et al 2006; Pardon et al 2004). More subtle differences in stress intensity are generally not easily distinguished, although the duration of the stress effect – the time needed to return to baseline levels – correlates well with stressor intensity (Van Bogaert et al 2006a).

### **3.3 Fever state: high and low environment temperature**

Fever states do not eliminate the SIH response, but influence SIH amplitude. Even in interleukin-induced fever, mice display a small but significant SIH reaction to novel cage stress (Fig. 5). This small amplitude is due to the fact that SIH responses cannot further increase in a high temperature state. A high basal body temperature therefore interferes with the SIH response (Bouwknicht and Paylor 2002; Dymond and Fewell 1999), and housing mice at 35 °C rather than 23 °C increases body core temperature from around 37 °C to around 39.5 °C (Jiang et al 2000) and this likely reduces the SIH amplitude. On the contrary, housing animals at 11 °C instead of 24 °C does not interfere with SIH amplitude (Long et al 1990a). In general, environmental temperature has direct effects on body temperature homeostasis and resting body temperature (Jiang et al 2000), not only affecting SIH but also influencing lipopolysaccharide -induced fever (Buchanan et al 2006; Peloso et al 2003). In addition, body temperature displays circadian rhythmicity with a 1-2 °C temperature increase during the dark period. This influences SIH amplitude (Olivier 2005; Peloso et al 2002), although no consistent studies have been carried out to test whether this affects drug testing. Generally, SIH testing is performed during the light period, although one study showed a robust although smaller SIH response during the dark phase of the light-dark cycle (Caramaschi et al 2007).



**Figure 5:** The SIH response in C57BL/6J mice ( $n=8$ ) is present in IL-1 $\beta$ -induced fever states, although the SIH response is smaller due to a ceiling effect.  $t=0$ : IL-1 $\beta$  or saline injection,  $t=120$ : Novel cage stress. \*:  $p < 0.01$  (repeated measures ANOVA with IL-1 $\beta$ /saline as within subject factor,  $F_{1,7}=15.90$ ). Error bars represent S.E.M.

### 3.4 Habituation

Habituation of stress responses are confounding factors when studying anxiety in animal models (Holmes et al 2001; McIlwain et al 2001). Generally, repeated daily stress exposure results in stress response habituation in the light-dark box (Onaivi and Martin 1989), the open field test (Cook et al 2002) and the SIH paradigm (Van der Heyden et al 1997). Daily testing with moderate stressors decreases SIH amplitude, although the SIH response remains robust (Barnum et al 2007; Bhatnagar et al 2006; Thompson et al 2003; Van der Heyden et al 1997). Similarly, daily injection stress in rats reduces the amplitude of SIH response (unpublished data). However, if the interval between two tests is long enough as in the SIH paradigm, no habituation occurs. In the SIH paradigm, testing once a week with moderate stressors does not interfere with the SIH response for over a year (Bouwknicht and Paylor 2002; Bouwknicht et al 2004b; Olivier et al 2003; Van der Heyden et al 1997). Surprisingly, exposure to a more severe stressor like repeated social defeat does not lead to SIH habituation (Barnum et al 2007; Bhatnagar et al 2006; Pardon et al 2004). Generally, chronic stress needs to be unpredictable in order to maintain full SIH capacity (Van der Heyden et al 1997).

## 4. Underlying thermoregulatory pathways: is SIH a fever?

### 4.1 Relationship between stress and infectious fever

The construct validity of the SIH paradigm depends on the neuronal mechanisms subserved by this response. The debate whether SIH is an entirely passive response that is unrelated to the underlying neuronal correlates in brain circuits that are active during pathological states of anxiety is ongoing (Oka et al 2001) and focuses on how exactly stress exposure ultimately leads to changes in body temperature, and how this temperature increase compares to other changes in body temperature such as infectious fever.

Both stress and infectious fever result in the similar clinical signs: higher body temperature accompanied by shivering and cutaneous vasoconstriction (Briese and Cabanac 1991). Stress and infectious fever involve related systems. Stress activates the HPA axis and the sympathetic nervous system, and as a consequence acute and chronic stress affect the immune system (Connor and Leonard 1998; Croiset et al 1987; Leonard and Song 1999). Fever induces sickness behavior, reminiscent of depressive-like behavior (Frenois et al 2007), although this is only partially valid as an animal model (Dunn et al 2005). The central amygdala modulates HPA axis responses following systemic Interleukin-1 $\beta$  (IL-1 $\beta$ ) administration (Xu et al 1999). More specifically, the thermoregulatory preoptic area of the hypothalamus reacts to both aversive and rewarding stimuli, implying preoptic area involvement in stress responses (Hori et al 1986). Adrenalectomy and glucocorticoid antagonists result in both increased IL-6 levels and an increased stress response (McClellan et al 1994; Morrow et al 1993), and nitric oxide synthase inhibition suppresses both lipopolysaccharide induced-fever and SIH in rats indicating a common mediator in temperature effects (Soszynski 2001). Lipopolysaccharide induced fever can be inhibited by behavioral conditioning, making a limbic input to fever plausible (Bull et al 1994; Bull et al 1990), and the C5a receptor antagonist PMX53 produced a significant increase in the number of c-fos-positive neurons in the medial amygdala, possibly through the ventrolateral medulla (Crane and Buller 2007). The central amygdala is known to modulate HPA axis responses following systemic IL-1 $\beta$  (Weidenfeld et al 2005; Xu et al 1999), possibly via prostaglandin-mediated lipopolysaccharide-responsive ascending pathways from the nucleus tractus solitarius and the ventrolateral medulla (Gaykema et al 2007; Xu et al 1999). Furthermore, paroxetine treatment can prevent pro-inflammatory action of interferon-alpha in rats (Myint et al 2007).

Remarkable differences exist in the initiation, location and the neurotransmitters subserving the SIH response. While GABA<sub>A</sub> receptor agonists robustly abolish SIH (for review see (Bouwknicht et al 2007)), they have little effect on infectious fever (Olivier 2005). On the other hand, 5-HT<sub>1A</sub> receptor agonists reduce SIH, but since they also reduce infectious fever, their anxiolytic efficacies are not as specific as anxiolytic GABA<sub>A</sub> receptor agonists (Blessing 2004). On the other hand, prostaglandin blocking drugs (e.g. non-steroidal anti inflammatory drugs (NSAIDS)) have impressive effects on lipopolysaccharide-induced fever, whilst the effects of NSAIDS on SIH seem to be small or

even absent, although contradictory reports exist (Kluger et al 1987; Morimoto et al 1991; Singer et al 1986; Vellucci and Parrott 1995). Even when fever is present, SIH can be induced by stress exposure (Fig. 5). Also, SIH remains present in prostaglandin receptor null mutation (KO) mice (Oka et al 2003; Saha et al 2005) and in lipopolysaccharide tolerant animals (Soszynski et al 1998), in which fever states can no longer be evoked. Antiserum against IL-1 $\alpha$  and IL-1 $\beta$  does not affect the SIH response (Long et al 1990b), and direct corticosterone injection into the anterior hypothalamus attenuates lipopolysaccharide-induced fever but not the SIH response (Morrow et al 1996). The vagal nerve known to influence the lipopolysaccharide-induced fever response has no effect on the SIH response (Cabanac and Dardashti 1999).

Therefore, stress and infection are not independent, yet they display remarkable differences. In the next sections, we will present a short overview of the evidence for the central pathways subserving stress response vs. infectious fever. We will argue that both find their origins in anatomically distinct brain parts, but that both eventually connect to common pathways located in the dorsomedial hypothalamus to increase body temperature.

#### **4.2 Stress and the medial amygdala**

A typical flight-or-fight response is mediated by limbic brain areas, including various amygdala nuclei and central gray (Carrasco and Van de Kar 2003). SIH is initiated by extra-hypothalamic mechanisms that modulate stress and anxiety (Olivier et al 2002; Veening et al 2004). Since all central body temperature effects are eventually regulated in the hypothalamus, a connection between the anxiety-involved limbic system and the hypothalamic temperature execution areas is assumed.

The medial amygdala nucleus is involved in the stress reaction (Davis 1997), and has connections to various brain areas, including the striatum, bed nuclei of the stria terminalis and many parts of the hypothalamus (among which the paraventricular nucleus, medial preoptic area and anterior hypothalamus) (Canteras et al 1995) (Herman et al 2005). In addition, the medial amygdala displays *c-fos* activation after different kinds of acute stress (Cullinan et al 1995; Dayas et al 2001; Dayas et al 1999; Emmert and Herman 1999; Figueiredo et al 2003a; Kollack-Walker et al 1997; Pezzone et al 1992), but SIH has not been specifically evaluated for *c-fos* immunoreactivity in the medial amygdala (Veening et al 2004). Other treatments such as IL-1 $\beta$  injection, hypoxia or hemorrhage elicited no medial amygdala response (Figueiredo et al 2003b; Sawchenko et al 1996; Thirivikraman et al 1997). There is ample evidence for amygdaloid GABAergic projecting pathways (Swanson and Petrovich 1998), and medial amygdala-receptive regions like the hypothalamic paraventricular nucleus projecting neurons such as the dorsomedial hypothalamic nucleus and the preoptic area are largely GABAergic (Cullinan 2000; Cullinan et al 1996; Roland and Sawchenko 1993). The SIH response is known to be accompanied by a HPA response, and increases of adrenocorticotrophic hormone (ACTH) levels after stress through paraventricular nucleus activation (Dunn and Whitener 1986; Feldman et al 1994; Gray 1993). Lesioning the medial amygdala (but not the central amygdala) reduced restraint-induced paraventricular nucleus activation (Dayas et al 1999), and local administration of muscimol into the medial amygdala attenuated

restraint stress-induced responses (Kubo et al 2004). Amygdala-lesioned rats had less blood pressure increase after environmental stress (Folkow et al 1982), and social isolation influenced the medial amygdala-mediated activation HPA-axis response (Sanchez et al 1995). The medial amygdala proved critical for the activation of medullary noradrenergic cells after psychological stress, while the central amygdala had an inhibitory effect (Dayas and Day 2002). Noradrenergic release in the medial amygdala facilitated HPA-axis activation in response to acute stress (Ma and Morilak 2005). The posteromedial cortical amygdala shows increased c-fos expression in response to hot and cold environmental temperatures, although the medial amygdala was not assessed separately. The central amygdala, basolateral amygdala and lateral amygdala did not show a response (Bachtell et al 2003).

In contrast to medial amygdala involvement in acute responses to stress, there is less evidence for other limbic structures involved in stress. The central amygdala did not express c-fos after SIH (Veening et al 2004) or other stress-inducing procedures (Cullinan et al 1995; Herman et al 2005), but immune challenge does affect central amygdala c-fos expression (Buller and Day 2002; Dayas et al 2001; Ericsson et al 1994). The basolateral amygdala extensively innervates the central and medial amygdala (Davis 2006), and is thought to be involved in conditioned fear responses (e.g. fear potentiated startle) (Cardinal et al 2002; LeDoux 2000), but lesions of the basolateral amygdala do not modulate ACTH, corticosterone or CRH release (Feldman et al 1994; McGregor and Herbert 1992; Seggie 1987). In summary, these findings support the involvement of medial amygdala in the response to an acute stress, including the SIH procedure. Connections from the medial amygdala to hypothalamic neuroendocrine nuclei are thus involved in the stress-induced temperature effects.

### **4.3 Infection and the hypothalamic preoptic area**

The central area involved in infectious fever is the hypothalamic pre-optic area, containing warm and cold sensitive neurons (Blatteis and Sehic 1998; Boulant 2000). Exogenous pyrogens or parts thereof such as lipopolysaccharide lead to the activation of the immune system with subsequent release of pyrogenic cytokines, among which tumor necrosis Factor- $\alpha$ , IL-1 and IL-6 (Kakizaki et al 1999; Kluger 1991; Mastorakos and Ilias 2006). Through prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), these cytokines inhibit – directly or indirectly - the warm-sensitive neurons in the preoptic area, suppressing heat loss responses and activating cold-sensitive neurons to increase heat production. Together, these processes effectively elevate the set point for body temperature (Boulant 2000). This elevated set point persists as long as these pyrogen levels are elevated. PGE<sub>2</sub> is also reported to activate the bed nuclei of the stria terminalis and central amygdala (Lacroix et al 1996; Zhang and Rivest 2000).

There is evidence that cytokines activate the preoptic area through PGE<sub>2</sub> synthesis and release in the organum vasculosum laminae terminalis (the circumventricular organ system), surrounding the preoptic area but located outside of the blood-brain barrier (Rotondo et al 1988; Stitt 1986). In any case, PGE<sub>2</sub> mediated effects through prostanoid EP3 receptors in the hypothalamus play a pivotal role in fever (Nakamura et al 1999). The vagal nerve (Blatteis et al 2000; Romanovsky et al 2000) and the anteroventral third

ventricular region are also known to influence lipopolysaccharide-induced fever (Elmqvist et al 1996; Hunter 1997; Scammell et al 1996; Whyte and Johnson 2007), although their exact role remains to be elucidated. Ultimately, preoptic area activation by cytokines is necessary for an infectious fever to manifest itself.

#### **4.4 Thermoregulatory execution and the dorsomedial hypothalamus**

The medial amygdala and the preoptic area thus play a role in stress and infectious fever, respectively. Now, we focus on the assumption that a common neural pathway ultimately causes the actual temperature increases for both fever and SIH. From aforementioned literature, we hypothesize the dorsomedial hypothalamus to be the central projection area of both the medial amygdala and the preoptic area.

Generally, dorsomedial hypothalamus activation results in both vasoconstriction and shivering, via neurons in the dorsal dorsomedial hypothalamus that project directly to the rostral raphe pallidus. Neuronal disinhibition in the dorsomedial hypothalamus results in an increased body temperature through neuronal activity of premotor sympathetic neurons (vasoconstriction) in the rostral raphe pallidus and sympathetically mediated activation of brown adipose tissue (for reviews: DiMicco et al 2006; Dimicco and Zaretsky 2007). The rostral raphe pallidus directly controls sympathetic preganglionic neurons (SPNs) in the intermediolateral cell column of the thoracic spinal cord (Nakamura et al 2004; Nakamura et al 2005a). The dorsomedial hypothalamus also projects to the paraventricular nucleus, the principal location of neurons that contain corticotropin-releasing factor (Sawchenko et al 2000; ter Horst and Luiten 1986; Thompson et al 1996); disinhibition of the dorsomedial hypothalamus also results in increased c-fos expression in the paraventricular nucleus and elevated plasma ACTH (Zaretskaia et al 2002). Paraventricular nucleus neurons also project to the rostral ventrolateral medulla, and the intermediolateral cell column, and sacral preganglionic parasympathetic nuclei (Benarroch 2005), and thus the paraventricular nucleus could contribute to autonomic temperature effects.

It has been shown that lipopolysaccharide-induced fever increases c-fos expression in the dorsomedial hypothalamus (Elmqvist and Saper 1996; Lacroix and Rivest 1997; Rivest and Laflamme 1995; Zhang et al 2000) and that direct projections of EP3 receptor-expressing preoptic area neurons to the dorsomedial hypothalamus region exist, mediating temperature effects of infection (Nakamura et al 2005b). The preoptic area activates pathways that include neurons in the dorsomedial hypothalamus and the rostral raphe pallidus (Cerri and Morrison 2006). Muscimol microinjection (a GABA<sub>A</sub> receptor agonist) in the rostral raphe pallidus blocked increased body temperature, brown adipose tissue temperature, and sympathetic nerve activity caused by PGE<sub>2</sub> microinjection into the preoptic area (Madden and Morrison 2003; Madden and Morrison 2004; Nakamura et al 2002). Thus, infectious fever is the result of dorsomedial hypothalamus activation by the hypothalamic preoptic area. In the SIH response, sympathetic brown adipose tissue activation is involved (Shibata and Nagasaka 1982). Furthermore, the effector route from the dorsomedial hypothalamus to the rostral raphe pallidus seems to be likely in SIH as well, since saline microinjection into the dorsomedial hypothalamus or paraventricular nucleus results in an adequate SIH response, but muscimol microinjection in the

dorsomedial hypothalamus entirely ablates this temperature increase (Dimicco and Zaretsky 2007), making a role of the dorsomedial hypothalamus in the SIH response very likely.

#### **4.5 Is SIH a fever?**

All in all, a complete distinction between infectious fever and SIH cannot be made, yet both processes are quite different. The facts that SIH is similar in warm and cool environments and that SIH results in peripheral vasoconstriction (Briese and Cabanac 1991; Frank et al 2000; Long et al 1990a) indicates an active temperature increase and not a passive hyperthermia, and support the evidence that SIH (partially) uses the same thermoregulatory effector pathways as infectious fever. However, if fever is caused by continuous preoptic area activation of the dorsomedial hypothalamus, one could think of SIH as a short lasting medial amygdala-mediated activation of the dorsomedial hypothalamus. The SIH response generally does not last as long as fever since stress by nature does not last as long as an infection. Chronic unpredictable stress influences the 5-HT neurotransmitter system (Davis et al 1995), and also influences temperature stress responses (Matuszewich and Yamamoto 2003). Therefore, we hypothesize that chronic unpredictable stress would lead to persisting SIH responses. In contrast, repeating the same stressor for a longer period will lead to a smaller SIH response (see section 3.4).

## **5. SIH pharmacology**

### **5.1 The GABA<sub>A</sub> system and anxiety**

GABA<sub>A</sub> receptors likely modulate anxiety responses (Broocks et al 2003). These receptors are a family of pentameric ligand-gated chloride channels made up of five subunits (Barnard et al 1998), of which different isoforms exist. The most common subtype is a pentamer with 2  $\alpha$ , 2  $\beta$  and 1  $\gamma$  subunit (Sibille et al 2000), GABA<sub>A</sub> receptor subunit composition ultimately determines receptor properties and modulatory sites (Rudolph et al 1999). GABA<sub>A</sub> receptor subtypes have characteristic central distributions, indicative of distinct roles for each subtype (Pirker et al 2000; Sieghart and Sperk 2002). Pharmacological and genetic evidence from mice with diazepam insensitive point mutations has led to specific hypotheses regarding the contributions of each subtype to diazepam pharmacology (Rudolph and Mohler 2006).

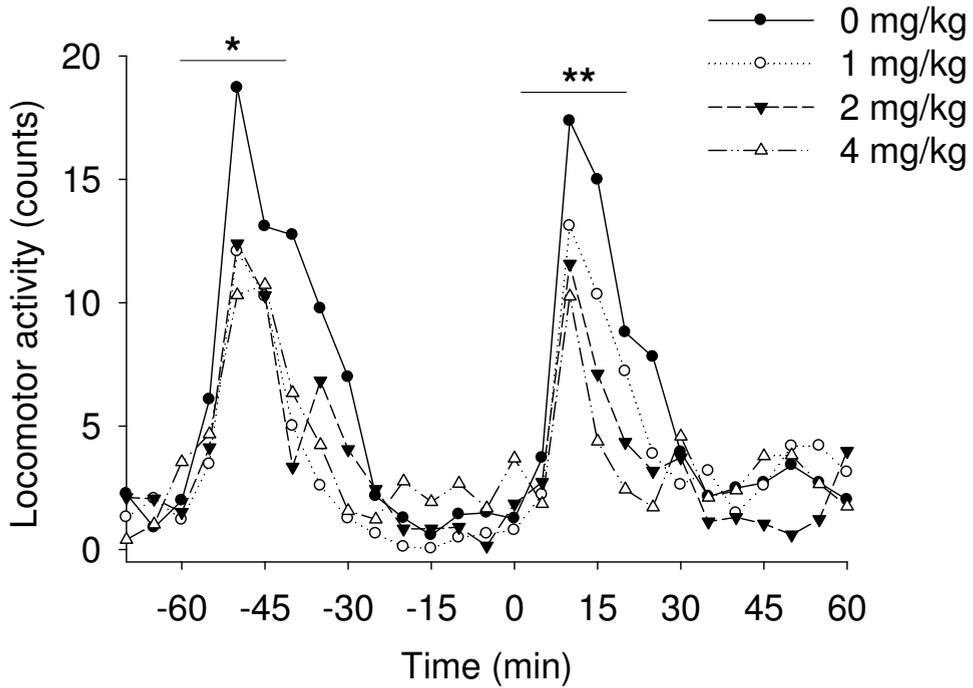
The benzodiazepine class of clinically efficacious anxiolytics such as diazepam allosterically potentiates GABA-induced currents. Of the  $\alpha$  subunits, only  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_5$  form a benzodiazepine binding site, representing around 75% of the total brain GABA<sub>A</sub> receptor population (McKernan and Whiting 1996). These  $\alpha$ -subunits thus mediate benzodiazepine effects such as anxiolysis, muscle-relaxant and anticonvulsant properties as well as unwanted benzodiazepine side effects as amnesia, tolerance, dependence, and alcohol potentiation. Since the advent of benzodiazepines, the search for novel anxiolytic drugs with reduced side-effects has a long history in drug development. In the 1980's this effort focused on compounds with reduced intrinsic efficacies (Haefely et al 1990), and more recently on compounds with selectivity away from  $\alpha_1$ -containing subtypes (Rudolph and Mohler 2006). It is remarkable that while many benzodiazepine anxiolytics

have been introduced to clinical practice until 1983, no non- benzodiazepine anxiolytics acting via GABA<sub>A</sub> receptors have been successfully developed and marketed since.

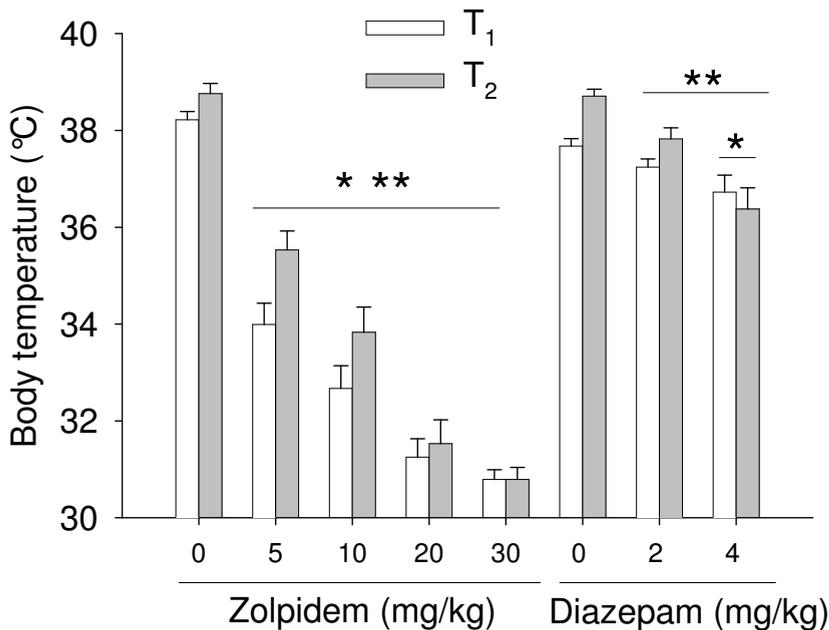
Positive modulation of  $\alpha_1$  subtypes appears to mediate sedative effects of benzodiazepines (Atack et al 2006; Rudolph et al 1999). In fact, zolpidem has ~10-fold selective affinity for  $\alpha_1$ -subunits and has indeed sedative effects in animals as well as humans (Griebel et al 2000a). The anxiolytic effect of benzodiazepines is ascribed to  $\alpha_2$  and/or  $\alpha_3$  subunit agonist activity (Atack et al 2006; Broocks et al 2003; Dias et al 2005). The  $\alpha_2$  and  $\alpha_3$  subunits are expressed in both the amygdala and the bed nuclei of the stria terminalis (Marowsky et al 2004; Pirker et al 2000) which are closely involved in anxiety responses. On a more detailed level, the medial amygdala expresses  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  GABA<sub>A</sub> subunits (Pirker et al 2000). Conflicting evidence exists on the presence of  $\alpha_1$  subunits in the central amygdala (Marowsky et al 2004; Pirker et al 2000). As a result, drug candidates selectively modulating  $\alpha_2$  and/or  $\alpha_3$  subtypes are sought after based on the premise of subtype selectivity potentially reducing the side effects of classical benzodiazepines (Atack et al 2006; Rowlett et al 2005).

## 5.2 SIH and GABA<sub>A</sub> receptor agonists

Since GABA<sub>A</sub> receptors play an important role in the autonomic stress and anxiety responses, it is not surprising that non-selective, as well as selective, GABA<sub>A</sub> receptor agonists are consistently effective in the SIH paradigm across species and strains (Bouwknicht et al 2007; Olivier et al 2002). A dose-dependent ablation of the SIH response is usually the result (Fig. 9), reflecting dose-dependent anxiolytic effects of classic benzodiazepines. Hypothermic effects on core body temperature are visualized by a reduced non stressed body temperature ( $T_1$ , Fig. 8 and 9-11). However, in rats, homeostasis is more tightly regulated, so that these effects are not always apparent. A way of measuring sedative effects of benzodiazepines in rodents is by looking at a dose-dependent reduction of locomotor activity (Fig. 6). Benzodiazepines are known to reduce locomotor activity (Gonzalez-Pardo et al 2006; McNamara and Skelton 1997; Siemiatkowski et al 2000). In general, GABA<sub>A</sub> drugs that modulate  $\alpha_1$ -containing subtypes affect basal body temperature as well as locomotor activity, whereas drugs selectively modulating  $\alpha_2$ - or  $\alpha_3$ -containing subtypes will reduce the SIH response with less effects on the basal body temperature or locomotor activity (Fig. 7). Such subunit-selective compounds are increasingly being developed and show promising results in the SIH paradigm. TP003, an  $\alpha_3$  subtype receptor agonist does not influence stress-induced activity levels, but reduces the SIH response (Dias et al 2005). Also, the effects of alcohol are at least partially mediated via GABA<sub>A</sub> receptors (Follesa et al 2006; Graham et al 1990; Kumar et al 2003). As such, alcohol reduces SIH but it is hypothermic at high doses (Fig. 11) (Olivier et al 2003). GABA<sub>A</sub> receptor modulators such as bretazenil are not effective in the SIH paradigm, and GABA<sub>A</sub> receptor antagonists such as flumazenil do not affect the SIH response, although they can block the SIH reducing effect of diazepam (Olivier et al 2002; Olivier et al 2003).



**Figure 6:** Dose-dependent reduction of locomotor activity with diazepam (0-1-2-4 mg/kg) in C57BL/6J mice (n=11), reflecting sedation. At t= -60 min, vehicle or diazepam is injected. At t= 0 min, a novel cage is applied as a stressor. Repeated measures ANOVA, treatment as within-subject factor: \* p<0.01 for injection stress at t=-60 ( $F_{3,30}=6.5$ ), \*\* p<0.05 for novel cage stress at t=0 ( $F_{3,30}=4.45$ ).



**Figure 7:** Effects of zolpidem (5-30 mg/kg, IP) and diazepam (2-4 mg/kg, IP) on the SIH response and basal body temperature in the classical SIH test in C57/BL6J mice ( $n=12$ ). All doses of zolpidem reduce core temperature (\*\*:  $p<0.001$ ) and reduce SIH (\*,  $p<0.001$ ). Diazepam reduces the SIH response at 2 mg/kg ( $p=0.07$ ) and 4 mg/kg (\*,  $p<0.001$ ), and also reduces basal body temperature at all doses (\*\*,  $p<0.001$ ). Error bars represent S.E.M..

### 5.3 Serotonergic system and anxiety

Serotonergic neurons are known to be extensively involved in anxiety responses (Millan 2003). Of the wide range of serotonin receptors known, the complex effects of 5-HT<sub>1A</sub> receptors in anxiety have been extensively studied (Olivier et al 2001). 5-HT<sub>1A</sub> receptors are mainly located in the raphe nuclei as somatodendritic autoreceptors and in limbic structures including the amygdala as postsynaptic receptors (Burnet et al 1995; Chalmers and Watson 1991; Cowen 2000; Hoyer et al 1986; Kung et al 1994; Pompeiano et al 1992; Verge et al 1986). Differential c-fos immunoreactivity after 5-HT<sub>1A</sub> receptor agonist flesinoxan administration has been described (Compaan et al 1996; Compaan et al 1997). 5-HT<sub>1A</sub> somatodendritic autoreceptors generally inhibit the amygdala through serotonergic projections from the dorsal raphe nucleus (Cervo et al 2000), which are known to be activated in acute stress states (Den Boer et al 2000). Generally, 5-HT<sub>1A</sub> receptor agonists like buspirone have been shown to exert consistent anxiolytic actions in rodents and humans (Bouwknicht et al 2004a; Millan 2003). SSRIs are clinically effective in treating anxiety disorders (Vaswani et al 2003), decreasing the inhibitory action of 5-HT<sub>1A</sub> autoreceptor in firing rates of the 5-HT neurons (Gartside et al 1995; Sharp et al 1997). Besides the pharmacological evidence indicating a role for the 5-HT<sub>1A</sub> receptors in anxiety, the use of gene knock-out models has led to helpful information on the role of these receptors in anxiety. 5-HT<sub>1A</sub> receptor KO mice (5-HT<sub>1A</sub>KO) display increased anxiety in various behavioral tests (Gingrich and Hen 2001; Holmes et al 2003c; Scarce-Levie et al

1999), including autonomic changes (Gingrich and Hen 2001; Groenink et al 2003b; Pattij et al 2002a).

The role of 5-HT<sub>1A</sub> receptors in anxiety is complex (Gingrich and Hen 2001; Pucadyil et al 2005). GABA can modulate 5-HT receptors (Forchetti and Meek 1981; Roberts et al 2004b), and vice versa, 5-HT<sub>1A</sub> receptors can modify GABAergic pathways (Fernandez-Guasti and Lopez-Rubalcava 1998; Sakaue et al 2001; Sibille et al 2000; Siemiatkowski et al 2000) and can also suppress the limbic release of GABA (Kishimoto et al 2000; Sakaue et al 2001). However, a complicating factor is that such interactions appear to be strain-dependent (Pattij et al 2002b).

Apart from 5-HT<sub>1A</sub> receptors, several other 5-HT receptors are implicated in anxiety. The role of 5-HT<sub>2</sub> receptors in anxiety states is less clear, and is postulated to be mediated via GABAergic neurons (Millan 2003). 5-HT<sub>7</sub> receptor antagonists could have an anxiolytic effect (Wesolowska et al 2006), but 5-HT<sub>7</sub> receptor KO mice do not display altered anxiety-like behavior (Guscott et al 2005; Roberts et al 2004a).

#### **5.4 Serotonergic receptors and basal body temperature**

Serotonin receptors influence basal body temperature. 5-HT<sub>1A</sub> receptor agonists are known to induce a hypothermic response in both animals (Bouwknicht et al 2000; Cryan et al 1999) and humans (Pitchot et al 2002; Pitchot et al 2004). This effect is induced through presynaptic 5-HT<sub>1A</sub> autoreceptors (Cowen 2000). Chronic treatment with SSRIs attenuates 5-HT<sub>1A</sub> agonist-induced hypothermia in healthy subjects (Lerer et al 1999; Sargent et al 1997) as well as in patients diagnosed with anxiety disorders and depression (Broocks et al 2003; Lesch et al 1991; Navines et al 2007), suggesting desensitization of the somatodendritic 5-HT<sub>1A</sub> receptor. The hypothermic effect of 5-HT<sub>1A</sub> receptor agonists originates in the medullary rostral raphe pallidus, leading to cutaneous vasodilatation and decreased brown adipose tissue thermogenesis (Ootsuka and Blessing 2003; Ootsuka and Blessing 2006a; Ootsuka and Blessing 2006b). A hypothermic effect of 5-HT<sub>1A</sub> receptor agonists in lipopolysaccharide-induced fever states can be explained by the common role of the rostral raphe pallidus in descending thermoregulatory pathways as we discussed in section 4.3 (Blessing 2004; Nalivaiko et al 2005).

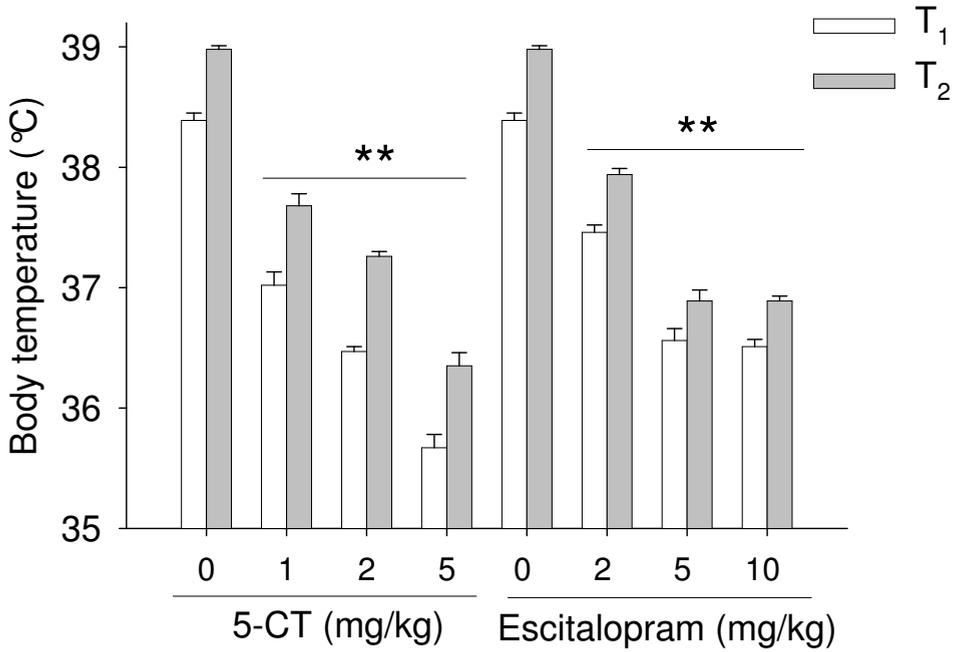
Stimulation of the 5-HT<sub>7</sub> receptors with the non-selective 5-HT<sub>7</sub> receptor agonist 5-carboxytryptamine (5-CT) results in hypothermia in mice (Fig. 8), an effect absent in 5-HT<sub>7</sub> receptor knockout mice (Guscott et al 2003). 5-HT<sub>2</sub> receptor agonists increase basal temperature, whereas 5-HT<sub>2</sub> receptor antagonists decrease basal temperature and can prevent the development of hyperthermia (Nisijima et al 2001; Yamada et al 2001; Zethof et al 1995). 5-HT<sub>2</sub> antagonistic effects are implicated in hypothermia during antipsychotic use (van Marum et al 2007). The effects of serotonin activation on basal body temperature makes interpretation of SIH results not always straightforward, even more since severe hypothermia can interfere with thermoregulatory homeostatic processes essential to SIH responses. The development of a more selective 5-HT<sub>1A</sub> receptor agonist with a postsynaptic preference could aid in distinguishing anxiolytic from hypothermic processes (Maurel et al 2007).

## 5.5 SIH and serotonergic drugs

The search for serotonergic anxiolytic drugs in the SIH paradigm has focused on 5-HT<sub>1A</sub> receptor agonists. Especially flesinoxan has received ample attention as an anxiolytic attenuating the SIH response (Bouwknicht et al 2004a). Flesinoxan reduces the SIH response at lower doses, but at higher doses, a severe hypothermia develops, making higher doses less readily interpretable (Fig. 10). Also, buspirone, which has been registered as an anxiolytic in humans, decreases the SIH response, an effect absent in 5-HT<sub>1A</sub> KO mice (Van Bogaert et al 2006a).

Radio telemetry testing in 5-HT<sub>1A</sub> KO mice has revealed an increased SIH response in KO mice after novel cage stress but not after rectally induced hyperthermia (Pattij et al 2002a), so that the classical SIH test using rectal temperature measurements did not reveal an increased SIH response in these KO mice (Pattij et al 2001). In addition, GABAergic drugs decrease the SIH response in 5-HT<sub>1A</sub> KO (Pattij et al 2001; Van Bogaert et al 2006a), suggesting that the 5-HT<sub>1A</sub> KO mice do not display changed GABA<sub>A</sub> receptor function. This effect however seems to be strain-dependent (Sibille et al 2000; Van Bogaert et al 2006a). Interestingly, effects of glutamate receptor antagonists on SIH can be antagonized with the 5-HT<sub>1A</sub> receptor antagonist WAY100,635 (Iijima et al 2007). All in all, the exact role of 5-HT<sub>1A</sub> receptors in anxiety including the SIH paradigm is complex.

Besides the consistent effects of 5-HT<sub>1A</sub> receptor agonists, other serotonergic drugs do not influence the SIH response, including 5-HT<sub>2A/C</sub> receptor agonists and antagonists, and 5-HT<sub>3</sub> receptor antagonists (Bouwknicht et al 2007). Also, 5-HT<sub>1B</sub> receptor agonists do not influence the SIH response, and 5HT<sub>1B</sub> KO mice do not display altered SIH response and drug sensitivity (Groenink et al 2003b). In addition, there are no acute effects of SSRI administration (Olivier et al 2003) (Fig. 8). Chronic SSRI treatment however, can decrease the SIH response in rats (Conley and Hutson 2007), although another study could not find such effects (Roche et al 2007).



**Figure 8:** No reduction of the SIH response with 5-HT<sub>7</sub> receptor agonist 5-CT (1-5 mg/kg, IP) and SSRI escitalopram (2-5 mg/kg, IP), but basal body temperature effects in the classical SIH test in C57BL/6J mice (n=12). All doses of 5-CT and escitalopram reduce core temperature (repeated measures ANOVA, \*\*, p<0.001) but do not influence the SIH response (Escitalopram: p=0.42, NS, 5CT: p=0.62, NS). Error bars represent S.E.M..

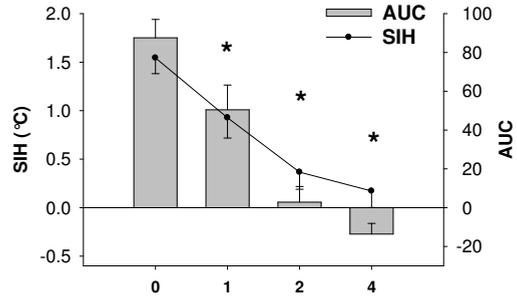
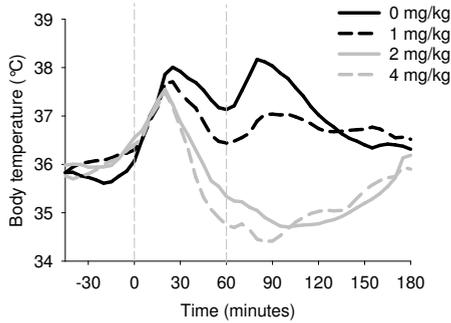
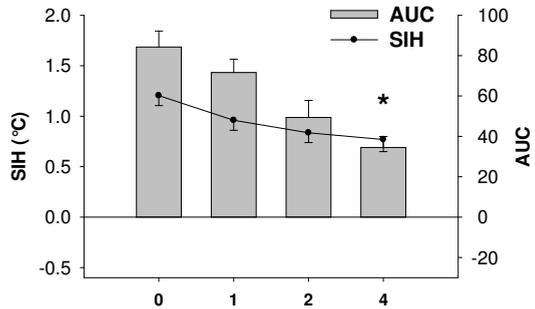
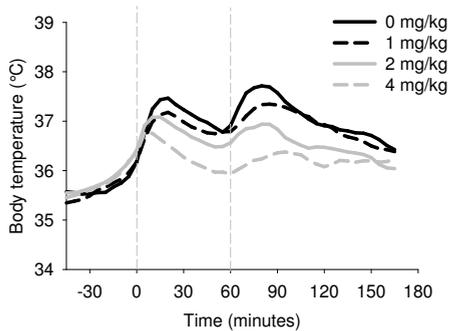
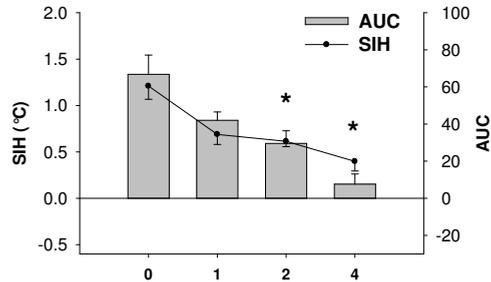
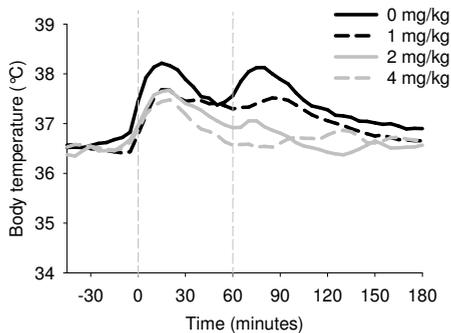
## 6. Variance in stress/SIH response

### 6.1 Species and strain differences

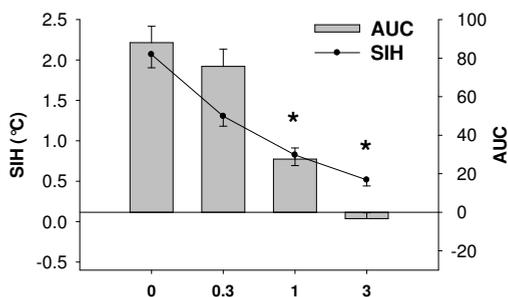
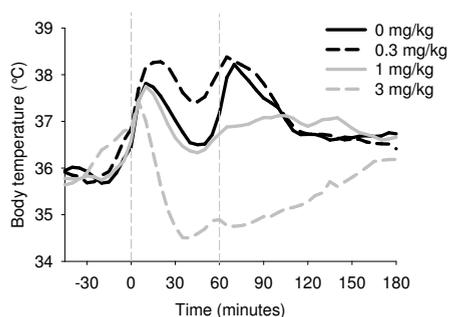
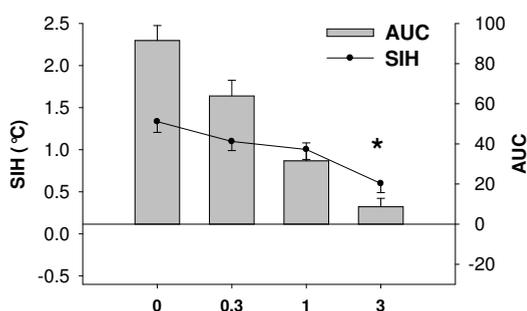
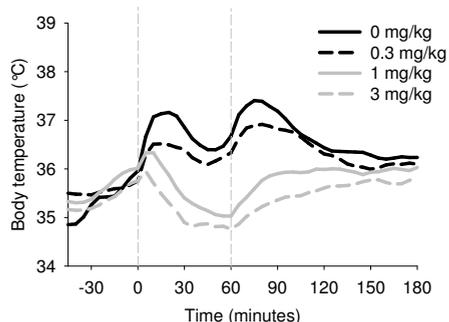
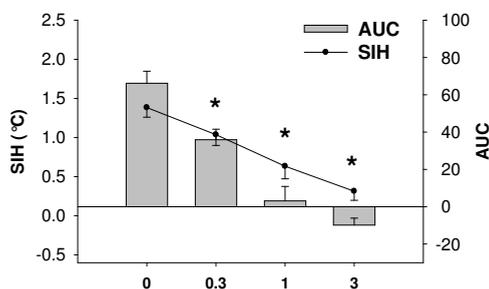
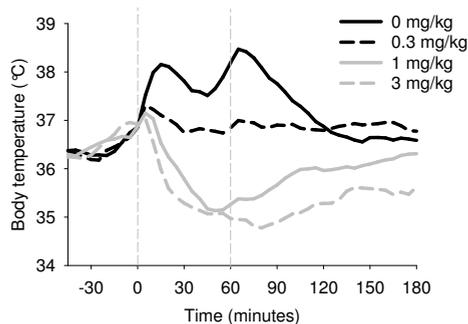
There are considerable differences in anxiety-like and depression-like behaviors between species and strains (Crabbe et al 1999; Cryan and Holmes 2005; Wahlsten et al 2003). These are attributable to genetic background (Turri et al 2001), but also epigenetic factors play a role (Francis et al 2003). Besides the variation in anxiety-like behavior, sensitivity to various anxiolytic drugs also depends on strain (Griebel et al 2000a; Lepicard et al 2000; Lucki et al 2001; Rodgers et al 2002b; Shanks and Anisman 1989; Tang et al 2005). 5-HT<sub>1A</sub> receptor responses are dependent on genetic background as well as the anxiety model used (Bouwknrecht et al 2004a). In the SIH paradigm, mouse strain differences exist in basal as well as stress-induced autonomic responses to stress. The C57BL/6J (B6) mice showed the largest autonomic response compared to Swiss-Webster (SW) and 129Sv/Ev (129Sv) mice (Bouwknrecht and Paylor 2002; van Bogaert et al 2006b). Between-strain SIH variance was however smaller than light-dark box test variance (Bouwknrecht and Paylor 2002).

We tested diazepam (0-4 mg/kg, IP, Fig. 9), the 5-HT<sub>1A</sub> receptor agonist flesinoxan (0-3 mg/kg, IP, Fig. 10) and ethanol (0-4 g/kg, PO, Fig. 11) in mice on three different genetic backgrounds (129Sv, B6 and SW) using radio-telemetry to determine whether anxiolytic drug effects are strain dependent in the SIH paradigm. The strains used are frequently used as genetic backgrounds for gene-targeting experiments and animal behavioral and pharmacological studies.

As expected, diazepam, flesinoxan and ethanol all dose-dependently reduced the SIH response. Even though the 129Sv strain shows highest SIH response after vehicle injection, none of the three strains was consistently more sensitive to anxiolytic-like (SIH) or intrinsic drug effects on basal body temperature. Depending on the specific receptor system investigated, a different strain was most sensitive and therefore no strain is more qualified to measure anxiolytic-like effects of drugs in the SIH paradigm compared to the others. Except for ethanol, all drugs were able to reduce T<sub>1</sub> with different sensitivities between the strains. After injection of diazepam, hypothermia was observed only in the 129Sv strain (Fig. 9). All mouse strains showed reductions after flesinoxan with the B6 and SW mice being most sensitive (Fig. 10). Ethanol decreased the basal body temperature (Fig. 11). Flesinoxan displayed anxiolytic-like effects in all strains, reducing SIH with the SW strain being most sensitive (Fig. 10). Interestingly, the B6 strain was only modestly sensitive to the effects of flesinoxan on SIH, but at the same time this strain was very sensitive to the effects of flesinoxan on the basal body temperature. These data clearly show that all drugs are robustly anxiolytic independent of strain, and this contributes to the predictive validity of the SIH paradigm. Furthermore, these data support that the SIH response can be determined independently from interfering drug effects on the basal body temperature.

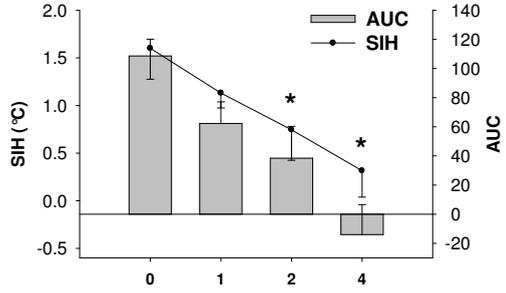
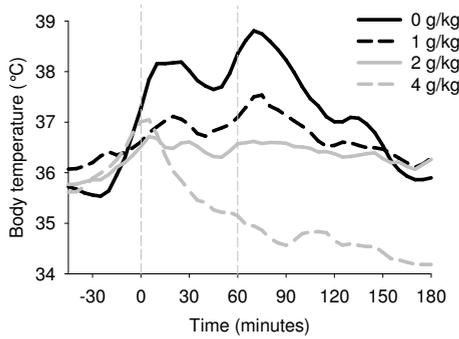
**A: 129Sv/Ev strain****B: C57BL/6 strain****C: SW strain**

**Figure 9:** Effects of non-selective GABA<sub>A</sub> agonist diazepam (0-4 mg/kg, IP) on the SIH response and basal body temperature) in three mouse strains (n=10-12): 129Sv/Ev (129Sv) (A), C57BL/6J (B6) (B), Swiss Webster (SW) (C). Diazepam reduced SIH in all strains ( $F_{3,30}=5.48$ ,  $p=0.004$ ). The 129Sv strain was most sensitive to the effects of diazepam, and reduced SIH was observed at all doses ( $F_{3,8}=11.73$ ,  $p=0.003$ ). In the SW strain SIH was reduced at 2 and 4 mg/kg ( $F_{3,8}=5.43$ ,  $p=0.026$ ), whereas the B6 strain was least sensitive and only showed reduced SIH after 4 mg/kg of diazepam ( $F_{3,8}=4.32$ ,  $p=0.038$ ). Also, diazepam reduced the basal body temperature in all strains ( $F_{3,30}=7.29$ ,  $p=0.001$ ). In the 129Sv strain, the basal body temperature was reduced at 2 and 4 mg/kg of diazepam ( $F_{3,8}=7.52$ ,  $p=0.001$ ). The SW strain showed a basal body temperature reduction at 4 mg/kg compared to the vehicle condition ( $F_{3,8}=8.71$ ,  $p=0.007$ ) and a trend on basal body temperature was observed in the B6 strain, although this was not significant ( $F_{3,9}=3.32$ ,  $p=0.07$ ).

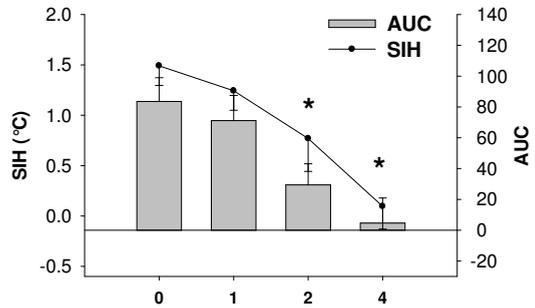
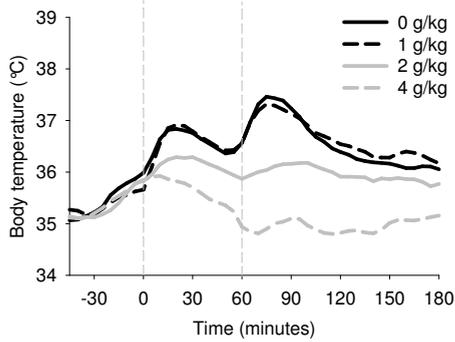
**A: 129Sv/Ev strain****B: C57BL/6 strain****C: SW strain**

**Figure 10:** Effects of 5-HT<sub>1A</sub> agonist flesinoxan (0-3 mg/kg, i.p.) on the SIH response and basal body temperature in three mouse strains (n=10-12): 129Sv/Ev (129) (A), C57BL/6J (B6) (B), Swiss Webster (SW) (C). Overall, flesinoxan reduced the SIH response ( $F_{3,27}=5.3$ ,  $p=0.006$ ). 129 mice showed anxiolytic-like effects after 1 and 3 mg/kg of flesinoxan, B6 mice only at 3 mg/kg flesinoxan and SW mice at all doses (129Sv:  $F_{3,7}=31.6$ ,  $p=0.003$ ; B6:  $F_{[3,8]}=5.4$ ,  $p=0.03$ ; SW:  $F_{3,8}=26.4$ ,  $p<0.001$ ). Flesinoxan also reduced the basal body temperature ( $F_{3,27}=5.0$ ,  $p=0.04$ ). Compared to vehicle, both B6 and SW mice showed a hypothermia after all doses of flesinoxan, whereas the 129Sv strain showed reduction only after 3 mg/kg of flesinoxan (129Sv:  $F_{3,7}=3.30$ , NS; B6:  $F_{3,8}=17.51$ ,  $p=0.001$ ; SW:  $F_{3,8}=17.72$ ,  $p=0.001$ ).

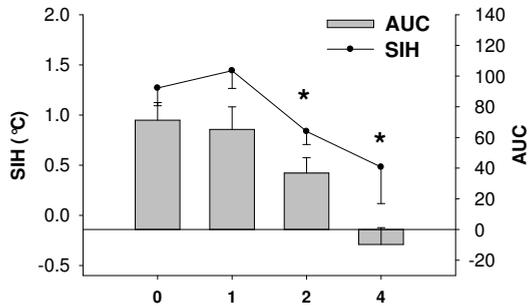
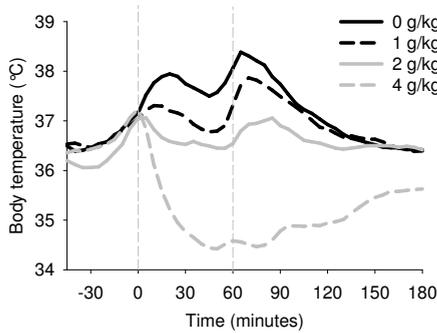
**A: 129Sv/Ev strain**



**B: C57BL/6 strain**



**C: SW strain**



**Figure 11:** Effects of ethanol (0-4 g/kg, p.o.) on the SIH response and basal body temperature in three mouse strains (n=10-12) 129Sv/Ev (129) **(A)**, C57BL/6J (B6) **(B)**, Swiss Webster (SW) **(C)**. All strains showed qualitatively comparable responses upon ethanol. Effects on both the SIH response as well as the basal body temperature were similar for all strains and were therefore analyzed together. In all strains SIH was reduced at 2 and 4 g/kg ( $F[3,27]=18.1, P<0.001$ ), reduction of T1 was found in all strains at 2 and 4 g/kg ( $F[3,27]=19.3, P<0.01$ ).

## 6.2 Sex differences

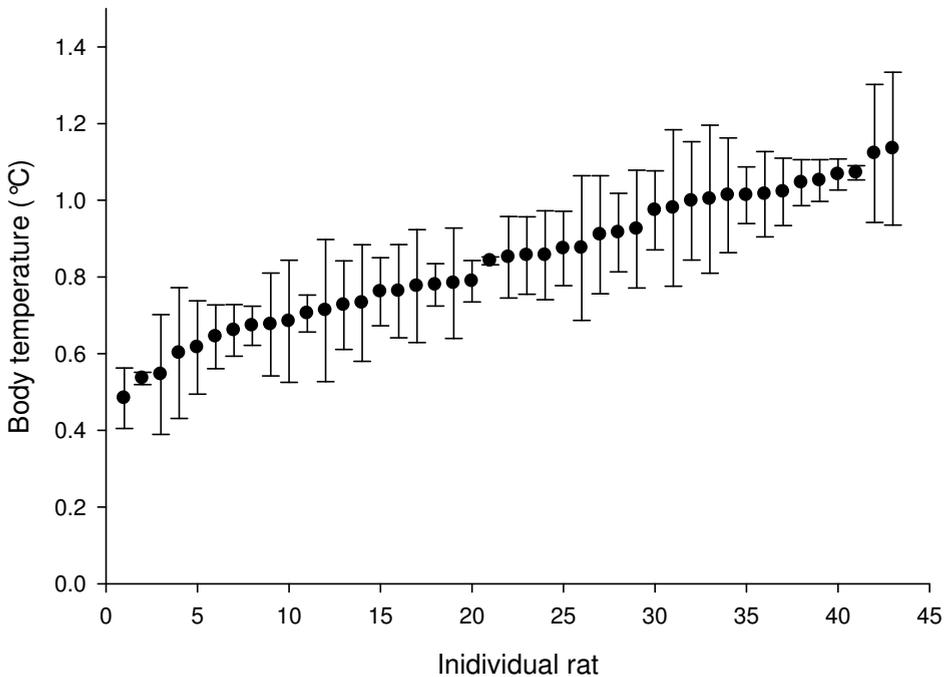
Women are more vulnerable to develop depression and anxiety disorders (Joffe and Cohen 1998), possibly due to fluctuating levels of estrogen. Females also exhibit larger stress-induced cortisol response as well as higher basal cortisol levels. However, estrogen has a complex role in modulating autonomic responsiveness after stress (Kajantie and Phillips 2006; Saleh et al 2000; Saleh and Connell 2003). In general, estrogen inhibits the sympathetic nervous system and enhances the parasympathetic system (Kajantie and Phillips 2006). Estradiol is known to decrease anxious behavior in ovariectomized rats (Petroski et al 2006), as well as attenuate HPA-axis reaction after stress (Puder et al 2001). Also in humans, estradiol can decrease stress-induced autonomic activity (Del Rio et al 1994; Komesaroff et al 1999; Lindheim et al 1992). Conflicting data on the effects of estrogen in tests of fear and anxiety exist (Walker et al 2003). In the SIH paradigm, female mice display a reliable but lower SIH response compared to male mice (Olivier et al 2003). Another study reported a higher SIH response of female rats compared to males (Thompson et al 2003). Also, effects of maternal deprivation on SIH are sex-dependent (Esposito 2006).

Hot flashes in the first menopausal years are ascribed to autonomic dysregulation which can be attenuated with SSRI treatment (Freedman 2005; Joffe et al 2007). Since stress can increase the number of hot flashes (Swartzman et al 1990), they can be regarded as a dysregulated SIH response. Postmenopausal women display altered levels of 5-HT (Duffy et al 2006). Stress responsiveness in females is also dependent on estrous cycle (Marcondes et al 2001; Mora et al 1996). In addition, estrous cyclicity alters benzodiazepine sensitivity (Fernandez-Guasti et al 2001; Fernandez-Guasti and Picazo 1990; Reddy and Kulkarni 1999), although 5-HT<sub>1A</sub> receptor agonist sensitivity was not altered (Fernandez-Guasti et al 2001; Fernandez-Guasti and Picazo 1990; Reddy and Kulkarni 1999). GABA<sub>A</sub> receptor subunit expression in the female medial amygdala depends on reproductive experience and state of estrous cyclicity, with increased proestrous  $\alpha_1$  and  $\alpha_2$  subunit expression in nulliparous females (Byrnes et al 2007). Sex steroid hormone levels also influence infectious fever responses (Avitsur et al 1995). After lipopolysaccharide administration, men showed a greater increase in core temperature than women (Coyle et al 2006).

How estradiol exactly modulates the stress response remains to be elucidated. There is evidence for a link between estradiol and the GABA<sub>A</sub> and 5-HT receptor systems. (Bethea et al 2002; McEwen and Alves 1999; Rybaczyk et al 2005). In rats, a high estrogen receptor density is present in the medial amygdala, bed nuclei of the stria terminalis and preoptic area (Canteras et al 1995; Rivest and Laflamme 1995; Shughrue et al 1997), as well as raphe pallidus (Hamson et al 2004). Furthermore, a GABA<sub>A</sub> receptor antagonist is able to block the estrogen-induced sympathetic effects, indicating that estrogen effects are at least partially mediated through the GABA<sub>A</sub> system (Saleh and Connell 2003). Estradiol also increases serotonin transporter mRNA levels (McQueen et al 1997).

### 6.3 Individual differences

Individuals differ in their susceptibility to develop an anxiety disorder due to genetic and environmental variation (Fyer et al 2006; Hettema et al 2001). A natural distribution in individual stress response is always present, and this also goes for the SIH response, where the SIH amplitude has a certain distribution as shown in male rats (Fig. 12). It is to be expected that the SIH amplitude correlates with perceived stress levels in humans, although this has never been investigated. In rats, maternal deprivation leads to longlasting behavioral influences, and this causes a higher SIH response to be present (Esposito 2006). Also, maternal behavior directly influences GABA<sub>A</sub> receptor expression (Caldji et al 2004). A history of stress induces a larger SIH response (Bhatnagar et al 2006). This supports the notion that individual life experiences directly affect the SIH amplitude.



**Figure 12:** Individual SIH distribution in Wistar rats ( $n=43$ ) undergoing 3 novel cage procedures inducing a robust SIH response. Data of one rat is the mean  $\pm$ S.E.M. of 3 novel cage responses over time.

## 7. Conclusion

The evidence supporting the SIH response as a valid paradigm to assess anxiety states has been consistent. All available anxiolytic drugs so far have been shown to reduce the SIH response in rodents. The possible potential of measuring body temperature to assess anxiety can be illustrated by the fact that peri-operative stress caused an increased temperature after surgery (Frank et al 2000). The relative ease to measure body temperature without extensive or intrusive procedures makes the SIH procedure a very attractive measurement to study stress and anxiety in humans. New applications like human telemetry make easy experimentation under non-intrusive circumstances possible. The fact that the SIH procedure is identical in humans as well as animals makes direct comparison possible, providing excellent animal-to-human translational possibilities. Surprisingly, little structural research has been carried out to characterize the SIH response in humans. Human pharmacological studies are needed to further validate the translational value of all the data gathered thus far in animals. We conclude that the SIH paradigm provides an enormous potential to study stress and anxiety in rodents as well as humans, and that it can be used to study the efficacy of new therapeutic anxiolytic agents



# Chapter 2

## The basic protocol to conduct SIH research

Christiaan H. Vinkers

Ruud van Oorschot

Berend Olivier

Lucianne Groenink

# 2

*Stress-Induced Hyperthermia in the Mouse. Mood and Anxiety Related Phenotypes in Mice: characterization using behavioral tests; Neuromethods (TD Gould, Ed) Vol. 42 (2009).*

## Abstract

The stress-induced hyperthermia (SIH) model studies the increase in body temperature in response to acute stress which is mediated by the autonomic nervous system. SIH is a simple and attractive paradigm to study putative anxiolytic drug properties as well as the effects of genetic or brain manipulations. In the SIH procedure, drugs are injected 60 min before the actual stressor, consisting of a manual rectal temperature measurement ( $T_1$ ). After 10 minutes, a second manual rectal temperature measurement is taken ( $T_2$ ), which represents the stress-induced body temperature. The SIH ( $\Delta T$ ) response is the difference between  $T_2$  and  $T_1$  ( $\Delta T = T_2 - T_1$ ). As drugs might exert intrinsic effects on the basal body temperature (measured via  $T_1$ ), the SIH response has to be interpreted in the context of absolute body temperatures. Mice can be repeatedly used (up to a year) if tested once a week because the SIH response remains very stable over such long periods. Animals can be socially housed except for the actual testing day (starting the day before until immediately after the procedure) when mice have to be singly housed.

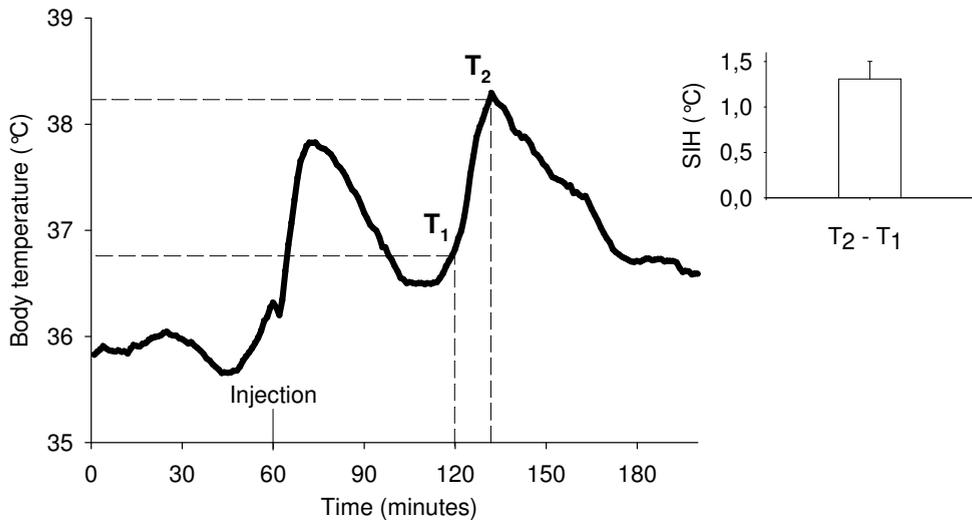
# 1. Background and historical overview

## 1.1 Introduction

The SIH paradigm uses the stress-induced activation of the autonomic nervous system by measuring the body temperature before and after stress exposure. The stress-induced increase in body temperature (SIH response, also called emotional or psychogenic fever) is a very consistent and stable physiological stress response with excellent translational properties. In mice, the SIH response remains intact for over a year when animals are used once a week (Bouwknrecht and Paylor 2002; Bouwknrecht et al 2004b; Van der Heyden et al 1997). Furthermore, anxiolytic drugs have been shown to selectively and dose-dependently reduce the SIH response (Bouwknrecht et al 2007). Besides, the SIH test is optimally suited to test the effects of various brain and genetic manipulations, e.g. null mutations or overexpression of genes (Groenink et al 2003b; Pattij et al 2001). Altogether, the SIH procedure is simple and robust, does not require time-consuming training, and drug effects on motor behavior, feeding, drinking, and nociception do not affect test outcome.

## 1.2 The SIH response

Exposure to both physiological and psychological stress increases body temperature in order to prepare for a fight-or-flight reaction. In humans, perceived stressful activities among which attending movies, boxing contests and taking an exam all increase body temperature (Briese 1995; Kleitman and Jackson 1950; Marazziti et al 1992; Renbourn 1960). In rats and mice, exposure to any stressor (including novelty, heat or pain) also induces an increase in body temperature (Figure 1). Within 15 minutes after stress exposure, body temperature rapidly rises up to 1.5 °C which usually returns to basal levels in two hours (Adriaan Bouwknrecht et al 2007). Besides humans and rodents, the SIH response has been found to be present in any mammal that has been tested so far, including impalas, silver foxes, sheep, pigs, rabbits and even cold-blooded animals as turtles (Adriaan Bouwknrecht et al 2007). The SIH curve parallels HPA-axis activity (Groenink et al 1994; Spooren et al 2002; Veening et al 2004). Moreover, the SIH response appears relatively independent of locomotor activity (Pardon et al 2004; Van Bogaert et al 2006a) which is a unique characteristic of an anxiety paradigm because it enables determination of anxiolytic activity of a drug independent from the often associated sedative properties that interfere with the primary parameter measured in the anxiety paradigm (e.g. movements on an elevated plus maze or in a light-dark box). Generally, the SIH procedure is not able to detect anxiogenic effects of drugs, probably due to a ceiling effect in the stress-induced temperature rise.



**Figure 1:** Telemetric measurement of the temperature course in a standard SIH test in 129Sv mice ( $n=7$ ). At  $t=60$  min, a vehicle is injected, causing a transient SIH response peaking at around 10-15 min post-injection and returning to baseline 45 minutes later. At  $t=120$  min, a rectal temperature measurement ( $T_1$ ) is applied as a stressor and measures basal body temperature, and leads to an SIH response. In the SIH procedure in singly-housed mice, a second rectal measurement is carried out at  $t=130$  min, to assess the stress-induced temperature ( $T_2$ ). The SIH response ( $T_2-T_1$ ) is around 1.4 °C. The error bar represents the SEM.

### 1.3 SIH and anxiety: development of a model

The first notice of a temperature effect in response to stress was when a gradual temperature increase was noted after removing mice one by one from a group-housed cage and measuring their rectal body temperature. This SIH response was thought to represent anticipatory anxiety (Borsini et al 1989). Later on, the SIH paradigm was refined to a singly-housed version in which the rectal temperature of a single mouse was measured twice with an interval of 10 minutes (Van der Heyden et al 1997). This reduced the number of animals needed per experiment. In this experimental setup, the first rectal temperature measurement ( $T_1$ ) represents the basal unstressed core temperature, but also functions as an adequate stressor. The second rectal temperature measurement ( $T_2$ ) measured 10 minutes later represents the peak temperature after stress. The SIH response is calculated by subtracting  $T_1$  from  $T_2$  ( $SIH=\Delta T=T_2-T_1$ ). The singly and group-housed versions correlate very well with each other, indicating that both approaches are measuring the same neural processes (Spooren et al 2002). Telemetric systems measuring body temperature and locomotor activity are increasingly used in the SIH paradigm, although the general principle of measuring an increase in body temperature in response to stress remains identical. However, because telemetric systems are able to measure locomotor activity, simultaneous application and comparison of body temperature and locomotor activity levels in response to stress is possible. As far as pharmacological studies have been performed in these SIH studies using telemetry equipment in rat and mice, results are very similar to those obtained in the standard SIH test in mice (van Bogaert et al 2006b; Vinkers et al 2008).

### 1.4 Drug testing in the SIH paradigm

The reproducible and robust SIH response combined with the ease of testing make the SIH paradigm ideal for detecting anxiolytic properties of drugs. The general principle is that anxiolytic drugs are able to reduce or even ablate the SIH response. Anxiolytic drugs including various GABA<sub>A</sub> receptor agonists, 5-HT receptor agonists and CRF receptor antagonists have been shown to indeed reduce the SIH response, whereas non-anxiolytic drugs including dopaminergic and noradrenergic compounds do not influence the SIH response (Bouwknicht et al 2007). However, anxiolytics can also exert hypothermic effects and decrease basal core body temperature even before stress exposure occurs (Olivier et al 2003). This is probably due to an activation of the GABA<sub>A</sub> receptor  $\alpha_1$  subunit, leading to a disturbed homeostatic regulation of the basal core body temperature (Vinkers et al 2009f). It is therefore important not only to regard temperature differences, but rather to study the absolute temperature values when assessing the effects of a compound (Figure 2).

The SIH paradigm possesses good predictive validity for anxiolytic drug properties. Clinically effective anxiolytic compounds such as benzodiazepines (including alprazolam, oxazepam, diazepam and chlordiazepoxide) and 5-HT<sub>1A</sub> receptor agonists such as buspirone decrease the SIH response (Olivier et al 2003). Most research has been carried out in rodents (for a review on the pharmacological SIH evidence (Adriaan Bouwknicht et al 2007)) and a wide range of different drug classes has been tested thus far (Vinkers et al 2008). Acute effects of SSRIs are absent in the SIH (Olivier et al 2003) whereas chronic SSRI treatment generates inconsistent findings: chronic fluoxetine treatment had either no influence (Roche et al 2007) or reduced the acute SIH response (Conley and Hutson 2007). Although there is a linear relationship between stressor intensity and magnitude of the SIH response (van Bogaert et al 2006b), so far no anxiogenic effects have been observed in the SIH test (Bouwknicht et al 2007; Vinkers et al 2008). Anxiogenic compounds such as FG7142 and mCPP did not alter basal body temperature or the SIH response, whereas a high dose of pentylenetetrazol significantly reduced basal body temperature but was also without effect on the SIH response (Olivier et al 2003).

In the standard manual SIH procedure, drugs are administered 60 minutes before stress is applied. This period has been experimentally validated (Olivier et al 2003), since drug administration in itself is a stressful event involving handling and manipulating the animal, causing an interfering SIH response. Because the maximum body temperature is limited up to certain levels, a newly applied stressor within the first 15 minutes does not cause any additional SIH response, although it prolongs the SIH response (Van der Heyden et al 1997). Thus, an injection-stressor interval of 60 minutes ensures that body temperature has sufficiently declined to approximately pre-stress baseline levels. In support, injections 60 minutes before assessing the SIH response in mice leads to an identical SIH response as compared to the mice that had received no injection at all (Figure 3C).

## **2. Factors influencing the SIH response**

### **2.1 Strain differences**

The SIH response has proven to be robust and reproducible between various labs. The absolute temperature increase after stress exposure depends on various factors. First of all, mouse strains generally differ in magnitude regarding their stress response which can be attributed to their genetic background as well as epigenetic factors (Bouwknicht and Paylor 2002; Rodgers et al 2002a; Van Bogaert et al 2006a). Nine different mouse strains (including Swiss, NMRI, C57BL6, 129Sv, FVB, and DBA/2) showed SIH responses between 0.6 and 1.9 °C (Bouwknicht and Paylor 2002). Differences in locomotor activity and body weight alone cannot account for the differences in SIH responses between strains, indicating the necessity to assess animal and strain stress responsiveness before any experiment is initiated (Van Bogaert et al 2006a). However, when comparing three different mouse strains (C57BL/6J, Swiss-Webster and 129Sv/Ev mice), none of the three strains was consistently more sensitive to anxiolytic-like (SIH) or intrinsic drug effects on basal body temperature (Vinkers et al 2008).

### **2.2 Type of stressor**

An increase in body temperature in response to stress can be induced up to certain levels above which no further temperature rise is possible. This ceiling effect limits the maximum SIH response, and any stressor seems to induce sufficient stress to reach a reliable SIH response (Van der Heyden et al 1997). However, minor stressors such as entering the room where the animal is housed induce smaller SIH amplitudes compared to placing animals in a novel cage, which is in turn exceeded by intense stressors such as repeated social defeat (Barnum et al 2007; Bhatnagar et al 2006; Pardon et al 2004). More subtle differences in stress intensity are generally not easily distinguishable, although the duration of the stress effect – the time needed to return to baseline level – correlates well with stress intensity (Van Bogaert et al 2006a).

### **2.3 Fever state, high and low environment temperature**

In infection-induced fever, the core body temperature is drastically enhanced by prostaglandins, lasting from hours to days long as prostaglandins activate the hypothalamic preoptic area. In contrast, the duration of a typical SIH response is maximally 2-3 hours. Furthermore, fever states do not eliminate the SIH response, although the SIH amplitude is decreased due to ceiling effects (Vinkers et al 2008). In addition, the SIH response is not sensitive to prostaglandin-blocking drugs, whereas infection-induced fever is hardly responsive to the effect of benzodiazepines (Vinkers et al 2009d). This indicates that, even though infection and stress both increase body temperature, they are mediated by different neurotransmitters and brain mechanisms and areas.

In general, environmental temperature has direct effects on body temperature homeostasis and resting body temperature (Jiang et al 2000), not only affecting SIH but also influencing infection-induced fever (Buchanan et al 2006; Peloso et al 2003). A high

basal body temperature interferes with the SIH response (Bouwknicht and Paylor 2002; Dymond and Fewell 1999), and housing mice at 35 °C rather than 23 °C increases body core temperature from around 37° C to around 39.5 °C, and thus likely decreases the SIH response (Jiang et al 2000). Oppositely, housing animals at 11° C instead of 24 °C does not interfere with SIH amplitude (Long et al 1990a). Body temperature displays circadian rhythmicity with a 1-2 °C temperature increase during the dark period. This can influence the SIH amplitude (Olivier 2005; Peloso et al 2002), although no consistent studies have been carried out to test whether this affects drug sensitivity. Generally, SIH testing is performed during the light period when basal body temperature is lower. In support, one study showed that the SIH amplitude was indeed smaller during the dark phase of the light-dark cycle (Caramaschi et al 2007).

## 2.4 Habituation

Repeated daily stress exposure results in habituation in many anxiety paradigms including the light-dark box (Onaivi and Martin 1989), the open field (Cook et al 2002) and to some - but very limited - degree in the SIH paradigm (Van der Heyden et al 1997). Because the SIH response partially depends on perceived stress intensity, repeated daily testing with moderate stressors results in a decreasing SIH amplitude, even though a robust SIH response is discernable (Barnum et al 2007; Bhatnagar et al 2006; Thompson et al 2003; Van der Heyden et al 1997). Similarly, daily injection stress for 6 consecutive days decreases the SIH response in rats (own unpublished data). Surprisingly, exposure to a very severe stressor like repeated social defeat does not lead to a habituated SIH response (Barnum et al 2007; Bhatnagar et al 2006; Pardon et al 2004). Generally, a one-week interval is sufficient to prevent any habituation in mice, and testing once weekly with moderate stressors has been shown not to interfere with the SIH response for over a year (Bouwknicht and Paylor 2002; Bouwknicht et al 2004b; Olivier et al 2003; Van der Heyden et al 1997).

## 3. Equipment, Materials & Setup

- Male mice, weighing 18-20 g upon arrival in the lab (e.g. NMRI mice, Charles River, strain and body weight are not critical). In general 10 to 12 animals per dose group are used. Mice are group-housed between experiments.
- Experimental cages for the animals: 27x16x12.5 cm cages with sawdust for group housing between experiments, and 12x18x13cm cages with sawdust, for individual housing.
- Balances with accuracy of 0.5 g (e.g., Mettler PG5000).
- Test compound solutions: prepare all suspensions and solutions fresh daily. For solutions, prepare compounds in physiological saline or in a 1% (w/v) methylcellulose/5% (w/v) mannitol mixture. For suspensions, prepare test compounds in 1% (w/v) tragacanth, 1% (w/v) methylcellulose or gelatin/mannitol. Other vehicles might also be used but have to be tested in advance to exclude intrinsic temperature effects or worse, act as a stressor (e.g. high concentrations of DMSO).

- 1-ml syringes, sterile, nontoxic, and pyrogen free; sterile 25-G x 5/8-in. (16-mm) needles.
- Silicon oil, peanut oil, or K-Y jelly, at room temperature.
- Digital thermometer, NiCr-NiAl thermocoupled with accuracy of 0.1 °C (e.g., Keithley 871A, Cleveland, OH, USA).
- Data analysis software for statistical analysis (e.g. SAS, SPSS).

## **4. Procedure**

### **4.1 Acclimatization of mice**

Mice should be ordered in time so that they can acclimatize to constant laboratory conditions at least one week before starting the experiment. Group-house the animals (5 per cage) at constant room temperature ( $21 \pm 2$  °C) and relative humidity ( $60 \pm 10\%$ ) under non-reversed 12 hr light/12 hr dark cycle (light on at 06:00). Provide cages with sawdust and free access to standard rodent diet and water.

### **4.2 Set up and run experiment**

In the afternoon before the test, house each mouse individually in an experimental cage in the experimental room. While isolating the mice, weigh and tail mark each animal and write down its weight on a run sheet. Make sure the housing conditions in the experimental room (day/night rhythm, temperature, and humidity) are identical to those in the acclimatization room, and that water and food are freely available. On the experimental day, prepare a balance, a digital thermometer, a tray with silicon oil (peanut oil or K-Y jelly), and sufficient cleaning tissues, 1-ml syringes, and 25-G x 5/8-inch needles. Prepare test compound solutions such that all substances are administered at 10 ml/kg. Use a coding system (A, B, C, etc) so that treatments are administered blindly. Ensure an even distribution of the different treatments over time (e.g. by using a Latin-square design).

### 4.3 Time Schedule

The time schedule below is used for testing 80 mice on one day, (40 mice in the morning, 40 mice in the afternoon, taking approximately 5 hours in total), with an injection test interval of 60 min. We assume starting in the morning at 9:00 AM, but adjustments can easily be made if starting at a different time.

- At 09:00, inject the first mouse (10 ml/kg body weight) with the test compound solution indicated by a previously prepared run sheet and return the mouse to its own cage. Then inject 9 other mice, using a 1-min interval between successive injections.
- Between 09:20 and 09:30 inject the second cohort of 10 mice, as described above.
- Between 09:40 and 9:55 inject the third and fourth cohort of 10 mice, as described above.
- Injections of the last 10 mice are performed a bit early so that they do not conflict with temperature measurements to be taken at 10:00.
- From 10:00 to 10:10 (i.e., 60 min after the injection), measure the rectal temperature ( $T_1$ ) of the first 10 mice using a 1-min interval between successive measurements.
- For temperature measurements fixate the mouse horizontally. While also fixating its tail at the base, dip the probe into silicon oil, insert it ~2 cm into the rectum, and hold until a stable rectal temperature is measured for approximately 20 sec. Write down the temperature read-out with 0.1 °C accuracy, and return the mouse to its individual cage.
- From 10:10-10:20 measure the rectal temperature ( $T_2$ ) of the first 10 mice again, using a 1-min interval between successive measurements.
- At 10:20, start rectal temperature measurement ( $T_1$ ) of the second cohort of 10 mice (injected between 09:20 and 09:30), with 1-min intervals as described before.
- From 10:30-10:40 measure the rectal temperature ( $T_2$ ) of the second cohort of 10 mice again using a 1-min interval between successive measurements as described previously.
- At 10:40, start rectal temperature measurement ( $T_1$ ) of the third and fourth cohort of 10 mice (injected between 09:40 and 09:55), with 1-min intervals.
- From 10:55-11:05 measure the rectal temperature ( $T_2$ ) of the third and fourth cohort of 10 mice again using a 1-min interval between successive measurements as described previously.
- Repeat injections and measurements for another 40 mice, with another compound in the afternoon.
- Return the mice to their group cages (keep always the same animals per cage) after completion of the whole test.

### 4.4 Perform statistical analysis

Use standard software packages to perform graphical presentations (e.g. Excel or Sigmaplot), and statistical analyses (e.g. SAS or SPSS). Enter the raw data (2 temperature measurements per mouse) in an empty raw data file. Calculate  $\Delta T$  (the SIH response) for each mouse as the difference between  $T_2$  and  $T_1$ . Then calculate mean  $T_1$ , mean  $T_2$ , and mean  $\Delta T$  for each treatment condition. Also calculate the standard error of the mean.

Check homogeneity of variance for  $T_1$ ,  $T_2$ , and  $\Delta T$  between treatments, and evaluate the effects of treatment by a two-way analysis of variance (ANOVA) with treatment (4 levels if vehicle plus three doses of a drug are tested) as between subject's factor and stress level ( $T_1$ ,  $T_2$ ) as within subject factor. For analyzing drug effects on  $\Delta T$  perform a one-way ANOVA with treatment condition as between subject's factor (4 levels). Subsequently, use a post-hoc analysis (Dunnett's multiple comparison test) to determine which treatment dose significantly alters SIH ( $\Delta T$ ) and/or basal core temperature ( $T_1$ ) relative to vehicle treatment.

#### **4.5 Experimental variables and troubleshooting**

This SIH procedure is based on measuring stress-induced changes in rectal temperature. Therefore it is important that the environmental noise in the experimental room is stable during the test day and over days (Bouwknicht et al 2007). Furthermore, room climate conditions should be kept constant to eliminate variation in results to prevent possibly interfering effects of environmental temperature on the SIH response (Long et al 1990a; Peloso et al 2003). Most SIH studies have been performed during the inactive light period (3-9 hr after lights on) when body temperature is 1-2 °C lower compared to the dark period, and relatively stable (Bouwknicht et al 2007).

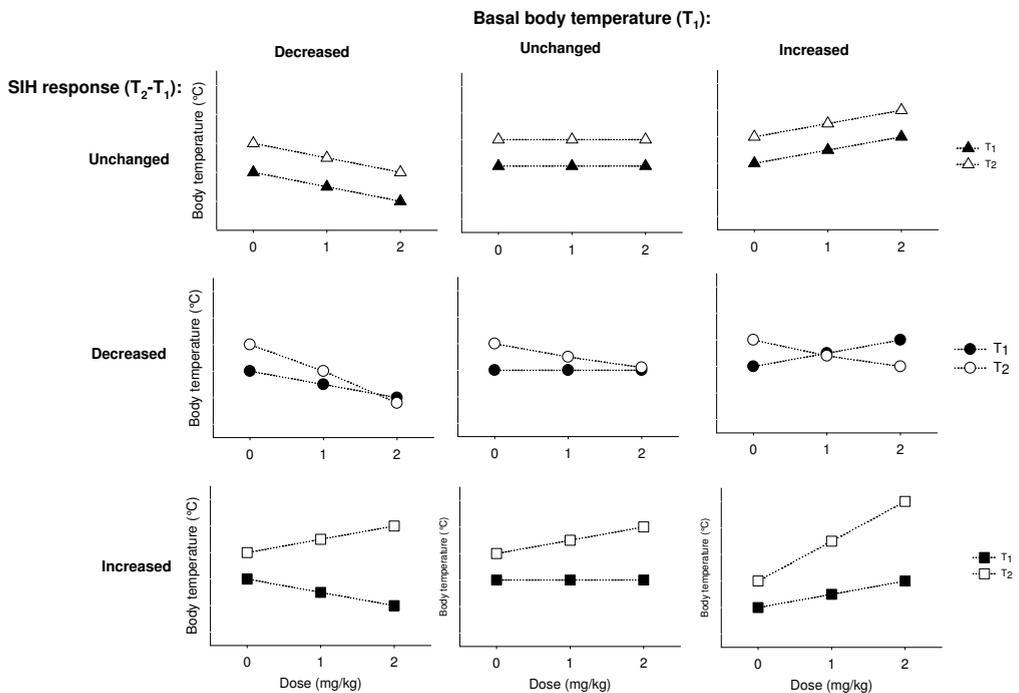
In the standard SIH set-up, mice are group-housed and isolated in the afternoon before the test day and returned to their original group cage after the test. It is not absolutely necessary however to group-house the mice first. Several studies have shown that also prolonged individual housing does not necessarily influence the SIH response or the pharmacological sensitivity (Van Bogaert et al 2006a). From an ethical point of view however, it is advised to group-house the mice, especially if they are tested several times in the SIH procedure.

Drugs may be administered orally, subcutaneously, or intraperitoneally. However, the injection-test interval should always be at least 45, but preferably 60 minutes. Drug administration injection itself induces a SIH response, and it takes approximately 60 min for the body temperature to return to basal levels (Van der Heyden et al 1997). Using a shorter injection-test interval, the resulting SIH response may be too small to detect significant drug effects in mice (Van der Heyden et al 1997). A drawback of this rather long injection-stress interval is that drugs need to possess sufficient long half-lives to be present in effective blood concentrations at the time of stress application 60 minutes later (e.g. nicotine:(Bouwknicht et al 2007)). Stress-free administration involving subcutaneous drug administration connected to flexible injection lines, in which handling and even disturbing the animal is no longer necessary are used in our laboratory to overcome the troubles associated with rapidly metabolized drugs with very short half-lives. In contrast to the results in mice, we have found that in rats, a 10 minute injection-test interval did not lead to an immediate increase in body temperature (Vinkers et al 2009c). This indicates that, at least in rats, a shorter injection-stressor interval may be applied.

It is also important to control for inter-experimenter effects on the SIH response. Different experimenters may obtain different mean temperature measurements, due to variations in the fixation techniques applied by investigators (e.g., head of the mouse up or down) (Zethof et al 1994). Although these inter-experimenter differences in mean temperature measurements not necessarily affect the SIH response (Zethof et al 1994), investigators should be trained in the technique holding the mice horizontally.

## 5. Anticipated results

Theoretically, a compound can either influence basal body temperature, the SIH response, both or none. Figure 2 graphically shows these possible drug effects on basal temperature ( $T_1$ ), the stress-induced temperature ( $T_2$ ) and the SIH response ( $\Delta T$ ). Statistically, an effect on basal body temperature is indicated by a main treatment drug effect in a two-way ANOVA, whereas an anxiolytic effect is indicated by a significant interaction of stress level ( $T_1/T_2$ ) x treatment in the two-way ANOVA.



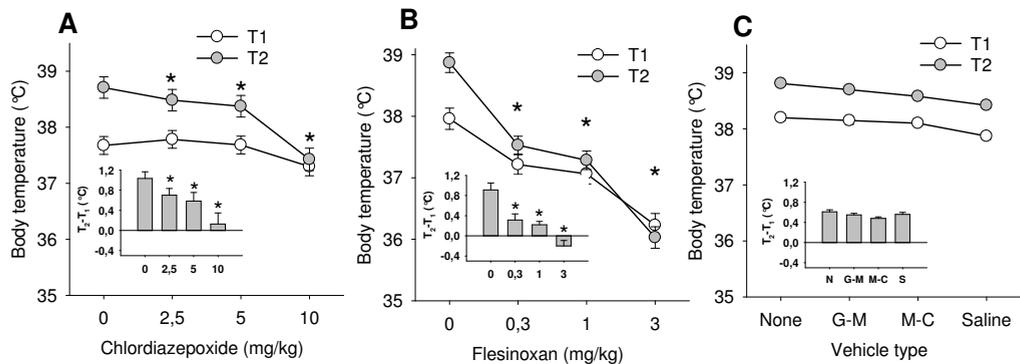
**Figure 2:** Theoretical outcome of the effects of a psychoactive drug (dose 0-1-2 on the x axis) in the SIH paradigm. Depending on whether a drug decreases (left column), has no effect on (middle column), or enhances (right column) the basal temperature ( $T_1$ ) the effects on SIH ( $\Delta T$ ) can be interpreted as either no effect (top row), anxiolytic effect (middle row), or angiogenic effect (bottom row). The difference between the rectal temperatures measured with a 10-min interval ( $T_2 - T_1$ ) is the SIH response ( $\Delta T$ ).

A compound that does not influence basal body temperature ( $T_1$ ) (middle column), has three theoretical effects: no influence on SIH (no anxiolytic activity), a decrease in SIH (anxiolytic effect), or an increase in SIH (anxiogenic effect).

A compound that decreases basal body temperature ( $T_1$ ) (left column) results in hypothermia. Subsequently, the hypothermia leads to no influence on SIH (no anxiolytic activity), a decreased SIH response (with a steeper decline in  $T_2$  compared to  $T_1$ , indicating an anxiolytic effect), or an increased SIH response (anxiogenic effect). Similar effects can be found with compounds that increase basal body temperature (right column).

Notes:

- Anxiogenic effects have not been reported in the SIH test because of an apparent ceiling effect of the stress-induced temperature.
- Increased basal body temperature with unaltered stress-induced temperature levels results in a decreased SIH response (and a significant stress level x treatment interaction). This is however not an anxiolytic drug effect. It is therefore vital to assess both the absolute temperature values as well as the SIH response.



**Figure 3:** Typical output from a stress-induced hyperthermia test in singly-housed male mice. The effects of a dose range of **(A)** the benzodiazepine receptor agonist chlordiazepoxide (a standard anxiolytic drug) and **(B)** the 5-HT<sub>1A</sub> receptor agonist flesinoxan and **(C)** different types of vehicles (none (N), gelatine-mannitol (G-M), methylcellulose (M-C) and saline (S)) on SIH ( $\Delta T$ ), basal temperature ( $T_1$ ) and stress-induced temperature ( $T_2$ ). Drugs (or vehicle) were administered 60 min before the first rectal temperature measurement ( $T_1$ ).  $T_2$  was measured 10 min later. The difference between  $T_2$  and  $T_1$ ,  $\Delta T$ , is indicated at each dose (insert; \*,  $\Delta T$  significantly different from vehicle treatment, indicating an anxiolytic effect). An asterisk (\*) indicates a significant difference ( $P < 0.05$ ) from the corresponding vehicle treatment (0 mg/kg).

Figure 3A shows the results of a typical SIH experiment using three doses of chlordiazepoxide, an anxiolytic GABA<sub>A</sub> receptor agonist. In drug-treated mice, the SIH response is significantly decreased compared to  $\Delta T$  in vehicle-treated animals (one-way ANOVA  $F_{3,46} = 4.96$ ,  $p=0.005$ , with Dunnett's multiple comparison as post-hoc test). Moreover, the stronger reduction in  $T_2$  than in  $T_1$  following treatment with

chlordiazepoxide is reflected in a significant stress x treatment interaction ( $F_{1,43}= 5.62$ ,  $p<0.01$ ). Together these data indicate an anxiolytic-like effect of chlordiazepoxide.

Figure 3B shows the effects of flesinoxan, a 5-HT<sub>1A</sub> receptor agonist in the standard SIH test. In drug-treated mice,  $\Delta T$  is significantly decreased compared to  $\Delta T$  in vehicle-treated animals (one-way ANOVA  $F_{3,43}= 15.32$ ,  $p<0.001$ , with Dunnett's multiple comparison as post-hoc test). Moreover, the stronger reduction in  $T_2$  than in  $T_1$  following treatment with flesinoxan is reflected in a significant stress x treatment interaction ( $F_{3,43}= 15.18$ ,  $p<0.001$ ). Together, these data indicate an anxiolytic-like effect of flesinoxan.

Figure 3C shows that different vehicle types (gelatin-mannitol, methylcellulose, saline) administered 60 minutes before measuring the rectal temperature leads to similar subsequent SIH responses, even compared when no vehicle at all is administered. Together these data indicate that a 60 minute injection-stressor interval is sufficiently long, ensuring that injection stress itself does not longer interfere with the stress procedure.

## 6. Conclusion

Altogether, the SIH procedure is relatively easy to perform without relying on the use of extensive or intrusive procedures. Moreover, the stress-induced increase in rectal temperature is a robust and reproducible parameter found in all mammals, including all strains of mice tested thus far (Bouwknicht and Paylor 2002; Rodgers et al 2002a). So far, many anxiolytic drugs have been shown to reduce the SIH response in rodents. The SIH response is therefore an autonomic stress response that can be successfully studied at the level of its physiology, pharmacology, neurobiology and genetics and possesses excellent animal-to-human translational properties.



## Chapter 3

# **Dissociating anxiolytic and sedative effects of GABA<sub>A</sub>ergic drugs using temperature and locomotor responses to acute stress**

Christiaan H. Vinkers

Marianne Klanker

Lucianne Groenink

S. Mechiel Korte

James M. Cook

Mike L. van Linn

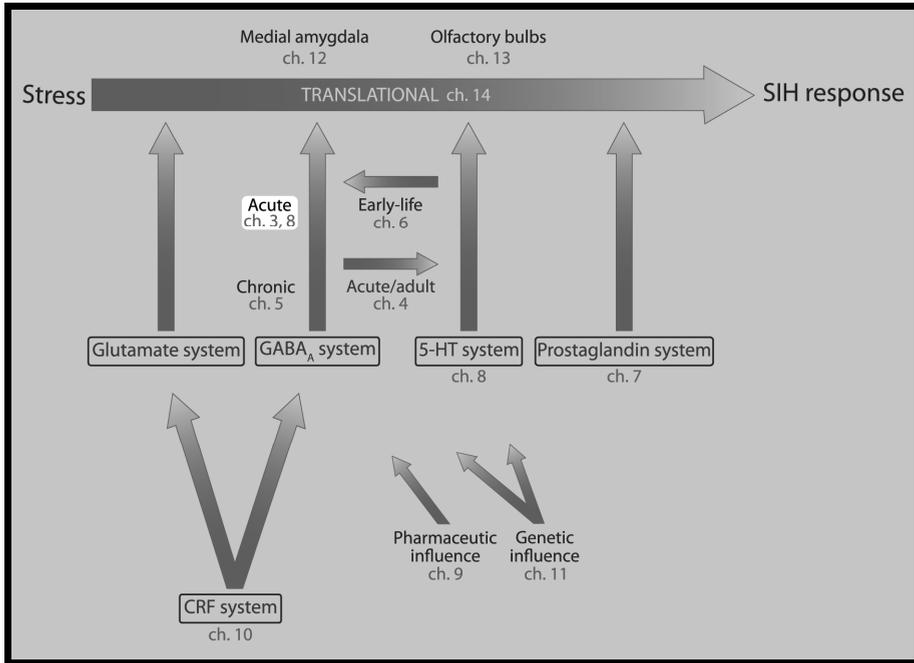
Seth C. Hopkins

Berend Olivier

3

*Psychopharmacology (Berl)* (2009) 204:299-311

## Abstract



**Background:** The stress-induced hyperthermia (SIH) model is an anxiety model that uses the transient rise in body temperature in response to acute stress. Benzodiazepines produce anxiolytic as well as sedative effects through non-selective binding to GABA<sub>A</sub> receptor subunits. The GABA<sub>A</sub> receptor  $\alpha_1$  subunit is associated with sedation, whereas the GABA<sub>A</sub> receptor  $\alpha_2$  and  $\alpha_3$  subunits are involved in anxiolytic effects.

**Aims:** We therefore examined the effects of (non-)subunit selective GABA<sub>A</sub> receptor agonists on temperature and locomotor responses to novel cage stress.

**Results:** Using telemetric monitoring of temperature and locomotor activity, we found that non-subunit selective GABA<sub>A</sub> receptor agonist diazepam as well as the  $\alpha_3$  subunit-selective receptor agonist TP003 dose-dependently attenuated SIH and locomotor responses. Administration of GABA<sub>A</sub> receptor  $\alpha_1$ -selective agonist zolpidem resulted in profound hypothermia and locomotor sedation. The GABA<sub>A</sub> receptor  $\alpha_1$ -selective antagonist  $\beta$ CCt antagonized the hypothermia, but did not attenuate the SIH response caused by diazepam and zolpidem. These results suggest an important regulating role for the  $\alpha_1$  subunit in thermoregulation and sedation. Ligands of extrasynaptic GABA<sub>A</sub> receptors such as alcohol and non-benzodiazepine THIP attenuated the SIH response only at high doses.

**Conclusions:** The present study confirms a putative role for the GABA<sub>A</sub> receptor  $\alpha_1$  subunit in hypothermia and sedation and supports a role for  $\alpha_{2/3}$  subunit GABA<sub>A</sub> receptor agonists in anxiety processes. In conclusion, we show that home cage temperature and locomotor responses to novel home cage stress provide an excellent tool to assess both anxiolytic and sedative effects of various (subunit selective) GABA<sub>A</sub>ergic compounds.

## 1. Introduction

The involvement of the GABA<sub>A</sub> receptor in anxiety has been extensively studied and confirmed (Nemeroff 2003). The pentameric GABA<sub>A</sub> receptor consists of five subunits ( $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\pi$ ), and the assembly of different combinations of subunits allows construction of different types of GABA<sub>A</sub> receptors, each having specific functional and pharmacological properties (Korpi et al 2002). The majority of GABA<sub>A</sub> receptors is composed of 2  $\alpha$ -, 2  $\beta$ -, and 1  $\gamma$ -subunit (Mihalek et al 1999). Classical benzodiazepines bind to GABA<sub>A</sub>-receptor containing  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and/or  $\alpha_5$  subunits, while binding affinity to  $\alpha_4$ - and  $\alpha_6$ -containing subunits is extremely weak (Rudolph and Mohler 2004). Besides the preferred anxiolytic action, the use of benzodiazepines is associated with dependence, anticonvulsant activity, sedation, amnesia and daytime drowsiness (Stewart and Westra 2002). These different benzodiazepine effects are thought to be mediated through different GABA<sub>A</sub> receptor subtypes. Therefore, the search for new anxiolytics focuses on subunit-selective GABA<sub>A</sub> receptor agonists. Both genetic and pharmacological studies suggest a major role of the  $\alpha_2$  and the  $\alpha_3$  GABA<sub>A</sub> receptor subunit in mediating anxiolysis (Atack et al 2005; Dias et al 2005; Low et al 2000; Rudolph and Mohler 2004). Consistent with this role, the  $\alpha_2$  and the  $\alpha_3$  GABA<sub>A</sub> receptor subunit are expressed in anxiety-involved areas like the amygdala and bed nucleus of the stria terminalis (Pirker et al 2000). The GABA<sub>A</sub> receptor  $\alpha_1$  subunit is associated with sedative and amnesic effects, while it is not involved in anxiolysis (McKernan et al 2000; Rowlett et al 2005; Rudolph et al 1999). Compounds lacking activity at the  $\alpha_1$ -containing GABA<sub>A</sub> receptor while modulating the  $\alpha_2$  and/or  $\alpha_3$  GABA<sub>A</sub> receptor subunit appear to be prime candidates for non-sedating anxiolytic drugs (de Haas et al 2007; Huang et al 2000).

The research for novel anxiolytics has focused on compounds with selective efficacy at different subunits (while binding to all subunits) rather than compounds with different affinities for the subunits (Atack 2005). We therefore aimed to characterize the effects of different GABA<sub>A</sub> ligands on temperature and locomotor responses to novel cage stress in rats, using home cage telemetry, in order to deduce the relative contributions of  $\alpha$  subunits of the GABA<sub>A</sub> receptor. Somatic stress symptoms are mediated by the autonomic nervous system and constitute a functional response in both humans and animals. The stress-induced hyperthermia (SIH) paradigm uses the physiological transient rise in body temperature in response to stress as a new and translational alternative in anxiety research (Bouwknicht et al 2007; Vinkers et al 2008). Using this paradigm, anxiolytic drugs including most benzodiazepines have been shown to dose-dependently attenuate the stress-induced hyperthermia response (Bouwknicht et al 2007; Olivier et al 2002; Van Bogaert et al 2006a). Using telemetry, stress-induced home cage temperature and locomotor activity responses can be simultaneously recorded, thus facilitating the comparison of effects on body temperature and locomotor activity caused by various GABA-ergic drugs. We hypothesized that anxiolytic effects would cause the SIH response to decrease without influencing basal body temperature and stress-induced locomotor activity responses. GABA-ergic sedative effects on the other hand would be characterized by a decrease in locomotor activity as well as general hypothermic state.

In the present study, we investigated the non-subunit selective GABA<sub>A</sub> receptor agonist diazepam (Pritchett et al 1989), the intermediate selective  $\alpha_1$  subunit GABA<sub>A</sub> receptor agonist zolpidem as well as the selective  $\alpha_3$  subunit GABA<sub>A</sub> receptor agonist TP003 (Dias et al 2005). Zolpidem is approximately 5- to 10-fold more selective for  $\alpha_1$  subunit-containing GABA<sub>A</sub> receptors than  $\alpha_2$  and  $\alpha_3$  subunit-containing receptors (Ebert et al 2006; Petroski et al 2006). However, zolpidem may demonstrate less selectivity *in vivo* compared to studies using recombinant receptors (Atack et al 1999). TP003 has lower efficacies at the different  $\alpha$  subtypes, with less than 15% potentiation at the  $\alpha_2$  and  $\alpha_5$  subunit compared to diazepam (Dias et al 2005). Exposure to higher drug doses may lead to loss of selectivity. We combined these compounds with the selective  $\alpha_1$  subunit GABA<sub>A</sub> receptor antagonist  $\beta$ CCt (Tietz et al 1999).

Less abundant populations of a  $\delta$ -subunit containing GABA<sub>A</sub> receptors are often located extra- and perisynaptically and are thought to be involved in a continuous active inhibitory tone instead of the phasic inhibitory tone caused by intrasynaptic agonists (Jia et al 2005; Nusser et al 1998). Alcohol is anxiolytic at low doses and has been shown to bind to extrasynaptic GABA<sub>A</sub> receptors containing  $\alpha_4$  or  $\alpha_6$  and  $\delta$  subunits (Wallner et al 2003). However, at higher doses, ethanol can modulate excitatory NMDA and non-NMDA glutamate receptors, serotonin and glycine receptors, as well as potassium and calcium channels (Crews et al 1996; Davies 2003; Harris 1999). Also, the fact that  $\delta$ -deficient mice demonstrated a normal anxiolytic and hypothermic response to ethanol and that the alcohol sensitivity of  $\alpha_4\beta_3\delta$  GABA<sub>A</sub> receptors could not be replicated (Borghese et al 2006), indicates that the discussion on the mechanism by which ethanol activates the GABA<sub>A</sub> receptor is ongoing (Mihalek et al 1999). Generally, the sedative and anxiolytic effects of alcohol were not altered after deletion of the  $\alpha_1$  subunit, suggesting that other yet unexplained factors may play a role (Kumar et al 2003). Moreover, we studied the non-benzodiazepine hypnotic drug THIP (gaboxadol) that also binds to extrasynaptic GABA<sub>A</sub> receptor  $\delta$ -subunits with putative anxiolytic effects (Elflin et al 2004; Wafford and Ebert 2006).

## 2. Materials and Methods

### 2.1 Animals

Male Wistar rats (Harlan Zeist, the Netherlands) were used in the current study. Rats were housed socially in a controlled environment with a non-reversed 12 hour light/dark cycle (white lights on from 7am-7pm). Animals had unlimited access to food (standard lab chow) and water. One week after arrival, telemetry transmitters were implanted. After recovery from surgery, rats were housed in groups of four in type IV macrolon<sup>®</sup> cages with a plastic tube as cage enrichment. Food (standard lab chow) and tap water were available *ad libitum*. Once a week, an experimental procedure was carried out. All experiments were carried out with approval of the ethical committee on animal experiments of the Faculties of Sciences, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki.

## 2.2 Surgery

A telemetric device (type ETA-F20, Data Sciences International, St Paul, MN, USA) was implanted in the abdominal cavity as described earlier (Pattij et al 2001). Prior to surgery, rats received a subcutaneous (s.c.) injection (2ml/kg) of the antibiotic Baytrill® (2.5% enrofloxacin). Rats were anaesthetized using O<sub>2</sub>/NO<sub>2</sub>/Isoflurane gas anesthesia. Carprofen (5 mg/kg, s.c.) was given as an analgetic immediately after surgery and twice daily for two days after surgery. After surgery, animals were housed individually for one week and recovery from surgery was monitored (weight, heart rate, temperature). Also, all rats had access to wet food and solid drinks (gel formula as a water replacement) for two days after surgery. Wound recovery was regularly checked. One animal repeatedly opened the abdomen wound and therefore had to be sacrificed and replaced. During the experiments, one rat was removed from the experiments due to an inflammation surrounding the telemetry device.

## 2.3 Experimental procedure

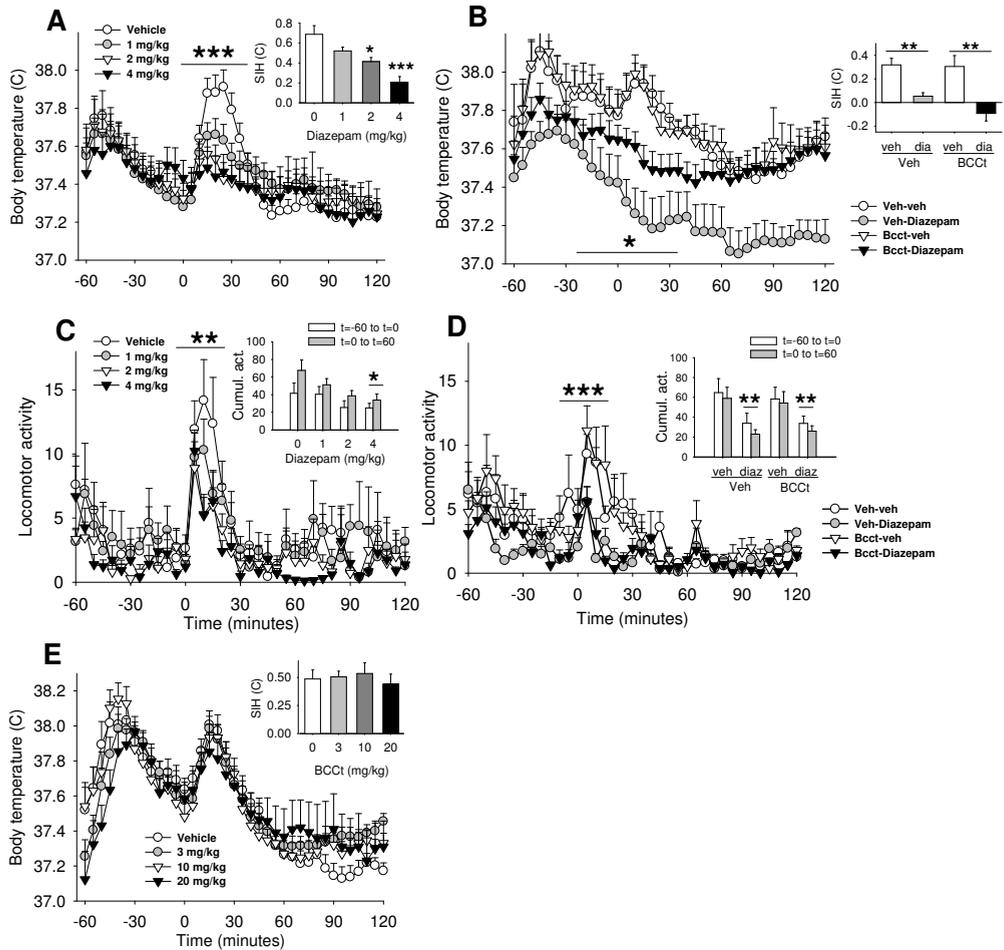
On the afternoon before an experimental day, rats were weighed and housed individually in a type III Macrolon® cage, located on a telemetric receiver. The telemetric transmitters were activated using a magnet. Data collection was subsequently started. The day after, the SIH procedure was initiated, consisting of an injection (intraperitoneal (i.p.) or oral (p.o.)) with vehicle or a certain drug dose. Immediately after injection, rats were placed back into their individual cage. Rats were placed in a novel cage (clean cage with fresh bedding and a paper tissue) 60 minutes later and left undisturbed for approximately two hours afterwards. At the end of the experimental day, rats were group-housed again and transmitters were turned off. To prevent habituation to the novel cage procedure, the interval between two experiments was set to be at least one week. A within subject design was used, and all animals received all (combined) doses of the drugs.

## 2.4 Drugs

Diazepam, zolpidem, alcohol and THIP HCl (gaboxadol) were obtained from Sigma Aldrich.  $\beta$ CCt (Beta-carboline-carboxy-tert-butyl ester) was synthesized by the laboratory of Dr. J.M.Cook, University of Wisconsin-Milwaukee. TP003 was synthesized according to published methods (Dias et al 2005; Humphries et al 2006). An injection volume of 2 ml/kg was used for intraperitoneal injections of all drugs, except THIP HCl (5 ml/kg, i.p.) and alcohol (5 ml/kg p.o.). Diazepam, zolpidem,  $\beta$ CCt, TP003 and gaboxadol were suspended in gelatin-mannitol 0.5% / 5%. When  $\beta$ CCt was combined with diazepam or zolpidem,  $\beta$ CCt at a dose of 10 mg/kg was injected 10 minutes prior to diazepam/zolpidem injection. Fresh solutions and suspensions were prepared each testing day.

## 2.5 Data analysis

All data were collected in 5-minute blocks and are displayed  $\pm$  SEM. All experiments were carried out with a within-subject design. Body temperature and locomotor activity were analyzed during the first hour after novel cage using a univariate repeated measures analysis of variance (ANOVA) with manipulations time and treatment as within-subject factors. Simple contrast tests were used to compare drug with vehicle conditions whenever a significant main effect for drug (indicating an effect on the basal body temperature) or a significant drug  $\times$  time interaction effect (indicating an effect on the SIH response) was observed. Also, the SIH response was calculated from the telemetry data for each individual rat by subtracting the body temperature at  $t=0$  from the maximum temperature reached within the first 30 minutes after the novel cage procedure and compared using a repeated measures ANOVA with drug as within subject factor and simple contrasts to compare drug with vehicle conditions. In addition, cumulative locomotor activity after the first 60 minutes after injection and cumulative locomotor activity after the first 60 minutes after the novel cage procedure were calculated and compared using repeated measures ANOVA. A probability level of  $p<0.05$  was set as statistically significant, probability levels between  $p=0.05$  and  $p=0.1$  were regarded as trends.



**Figure 1:** The effects of diazepam with and without  $\beta$ CCt on the novel cage-induced temperature and locomotor responses ( $t=-60$  injection,  $t=0$  novel cage stress). \*:  $p<0.05$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ . Inset: Calculated SIH response from the telemetry data. **A:** Diazepam (0-4 mg/kg) dose-dependently reduced the SIH response. Inset: Calculated SIH response from the telemetry data. **B:** Diazepam at a dose of 4 mg/kg reduced core body temperature and the SIH response. Prior injection with  $\beta$ CCt (10 mg/kg) prevented basal core body temperature reduction without affecting the diazepam-induced reduction of the SIH response \*:  $p<0.05$ : diazepam effect and diazepam x  $\beta$ CCt interaction. Inset: calculated SIH response from the telemetry data. **C:** Diazepam (4 mg/kg) reduced stress-induced locomotor activity responses. **D:** Diazepam (4 mg/kg) reduced stress-induced locomotor activity responses with no effect of  $\beta$ CCt. **E:**  $\beta$ CCt (0-20 mg/kg) did not affect stress-induced hyperthermia responses. Inset: calculated SIH response from the telemetry data. **Inset 1C-D:** white bar: cumulative locomotor activity  $t=-60$  to  $t=0$  (after injection); grey bar: cumulative locomotor activity  $t=0$  to  $t=60$  (after novel cage).

## 3. Results

### 3.1 Diazepam (Figure 1A-C, n=11)

*Summary:* Diazepam dose-dependently attenuated the SIH response to novel cage stress without affecting basal body temperature, and reduced locomotor activity levels before and after the novel cage procedure only at higher doses.

*Body temperature:* The novel cage stress-induced hyperthermia response ( $F(12,120)=22.0$ ,  $p<0.001$ ) was reduced by diazepam (diazepam x time interaction:  $F(36,360)=4.31$ ,  $p<0.001$ ). Diazepam did not influence basal body temperature (diazepam effect:  $F(3,30)=1.04$ ,  $p=0.39$ , NS) (figure 1A). The calculated SIH response revealed a diazepam effect on the SIH response ( $F_{3,30}=12.74$ ,  $p<0.001$ ). Simple contrasts revealed SIH attenuation at higher doses (1mg/kg-veh:  $F_{1,10}=3.85$ ,  $p=0.08$ , trend; 2 mg/kg-veh  $F_{1,10}=6.03$ ,  $p<0.05$ ; 4 mg/kg-veh  $F_{1,10}=33.51$ ,  $p<0.001$ ) (Figure 1A, inset).

*Locomotor activity:* The novel cage stress-induced locomotor activity response ( $F(12,120)=20.55$ ,  $p<0.001$ ) was overall diminished by diazepam (diazepam effect  $F(3,30)=4.98$ ,  $p<0.01$ ), although not dependent upon time (diazepam x time interaction  $F(3,30)=1.33$ ,  $p=0.11$ , NS). Only the higher doses of diazepam influenced locomotor activity (planned comparisons: vehicle-1 mg/kg ( $F(1,10)=1.68$ ,  $p=0.22$ , NS), vehicle-2 mg/kg ( $F(1,10)=8.18$ ,  $p<0.05$ ) and vehicle-4 mg/kg ( $F(1,10)=7.03$ ,  $p<0.05$ ). When cumulating locomotor activity levels after injection and after stress (figure 1C, inset), diazepam reduced locomotor activity levels at higher doses (main diazepam effect  $F(3,30)=3.03$ ,  $p<0.05$ ; Simple contrasts: 2 mg/kg vs vehicle  $F(1,10)=3.85$ ,  $p=0.08$ , NS; 4 mg/kg vs vehicle  $F(1,10)=5.19$ ,  $p<0.05$ ). Activity levels were larger after the novel cage procedure than after injection stress (Stress effect  $F(1,10)=8.55$ ,  $p<0.05$ ).

### 3.2 $\beta$ CCt (Figure 1E, n=11)

*Body temperature:*  $\beta$ CCt alone did not affect the SIH response (time effect  $F(12,120)=53.00$ ,  $p<0.001$ ) ( $\beta$ CCt effect  $F(3,30)=0.70$ ,  $p=0.56$ , NS;  $\beta$ CCt x time interaction  $F(36,360)=1.17$ ,  $p=0.24$ , NS) (Figure 1E). The calculated SIH response revealed no  $\beta$ CCt effect on the SIH response ( $F_{3,30}=0.33$ ,  $p=0.80$ , NS). Simple contrasts revealed that there was no attenuation of the SIH response at any dose (3mg/kg-veh:  $F_{1,10}=0.06$ ,  $p=0.83$ , NS; 10 mg/kg-veh  $F_{1,10}=0.29$ ,  $p=0.61$ , NS; 20 mg/kg-veh  $F_{1,10}=0.13$ ,  $p=0.72$ , NS) (Figure 1E, inset).

*Locomotor activity:*  $\beta$ CCt did not influence the stress-induced locomotor activity responses (time effect  $F(12,120)=13.72$ ,  $p<0.001$ ;  $\beta$ CCt effect  $F(3,30)=0.23$ ,  $p=0.88$ , NS;  $\beta$ CCt x time interaction  $F(36,360)=1.02$ ,  $p=0.45$ , NS) (data not shown).

### 3.3 Diazepam and $\beta$ CCt (Figure 1B and D, n=8)

*Summary:*  $\beta$ CCt was able to partially reverse the diazepam-induced hypothermia without affecting diazepam's ability to reduce the SIH response.  $\beta$ CCt was not able to reverse the diazepam-induced locomotor reduction.

*Body temperature:* When combined with  $\beta$ CCt, the SIH response (time effect ( $F(12,84)=9.85$ ,  $p<0.001$ ) was overall reduced by diazepam (diazepam x time interaction  $F(12,84)=5.17$ ,  $p<0.001$ ).  $\beta$ CCt did not influence the SIH response ( $\beta$ CCt x time interaction  $F(12,84)=1.38$ ,  $p=0.19$ , NS). Diazepam reduced basal body temperature (diazepam effect  $F(1,7)=6.96$ ,  $p<0.05$ ), and  $\beta$ CCt influenced the diazepam-induced hypothermia (diazepam

x  $\beta$ CCt interaction  $F(1,7)=6.18$ ,  $p<0.05$ ) without altering the body temperature itself ( $\beta$ CCt effect  $F(1,7)=1.24$ ,  $p=0.30$ , NS). The calculated SIH response revealed that diazepam reduced the SIH response (diazepam effect  $F_{1,7}=16.94$ ,  $p<0.01$ ).  $\beta$ CCt itself did not affect the SIH response ( $\beta$ CCt effect  $F_{1,7}=1.86$ ,  $p=0.22$ , NS), nor did  $\beta$ CCt affect the attenuation of the SIH response by diazepam (diazepam x  $\beta$ CCt interaction  $F_{1,7}=0.68$ ,  $p=0.44$ , NS). (Figure 1B, inset).

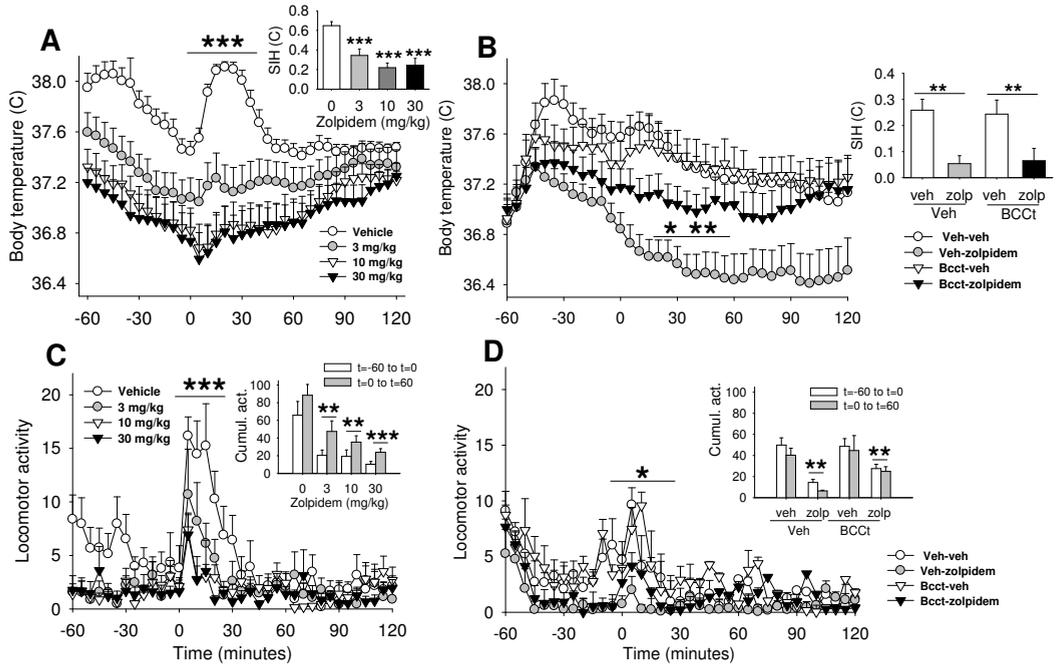
*Locomotor activity*; When diazepam was injected after  $\beta$ CCt, the stress-induced locomotor activity response (time effect  $F(12,84)=7.65$ ,  $p<0.001$ ) was generally and time-dependently reduced by diazepam (diazepam effect  $F(1,7)=9.48$ ,  $p<0.05$ ; diazepam x time interaction  $F(12,84)=3.45$ ,  $p<0.001$ ) with no effect of  $\beta$ CCt ( $\beta$ CCt effect  $F(1,7)=0.20$ ,  $p=0.68$ , NS;  $\beta$ CCt x time interaction  $F(12,84)=0.32$ ,  $p=0.98$ , NS;  $\beta$ CCt x diazepam interaction  $F(1,7)=0.02$ ,  $p=0.88$ , NS). When cumulating locomotor activity levels after injection and after stress (figure 1D, inset), diazepam generally reduced locomotor activity levels (main diazepam effect  $F(1,7)=10.86$ ,  $p=0.01$ ; stress effect  $F(1,7)=2.47$ ,  $p=0.16$ , NS) without effect of  $\beta$ CCt ( $\beta$ CCt effect  $F(1,7)=0.05$ ,  $p=0.82$ , NS;  $\beta$ CCt x diazepam interaction  $F(1,7)=0.21$ ,  $p=0.66$ , NS).

### 3.4 Zolpidem (Figure 2A and C, n=12)

*Summary*: Zolpidem dose-dependently reduced basal body temperature and the SIH response, and attenuated stress-induced and basal locomotor activity levels.

*Body temperature*: Zolpidem reduced basal body temperature (main zolpidem effect:  $F(3,33)=9.85$ ,  $p<0.001$ ). Basal body temperature was found to be reduced in all three dosages (planned comparisons: vehicle-3 mg/kg ( $F(1,11)=8.89$ ,  $p<0.05$ ), vehicle-10 mg/kg ( $F(1,11)=27.98$ ,  $p<0.001$ ) and vehicle-30 mg/kg ( $F(1,11)=26.73$ ,  $p<0.001$ ). The SIH response (time effect:  $F(12,132)=15.70$ ,  $p<0.001$ ) was reduced by zolpidem (zolpidem x time interaction:  $F(36,396)=10.12$ ,  $p<0.001$ ). The calculated SIH response revealed that zolpidem reduced the SIH response ( $F_{3,33}=12.71$ ,  $p<0.001$ ). Simple contrasts revealed SIH attenuation at all doses (3 mg/kg-veh:  $F_{1,11}=18.17$ ,  $p<0.001$ ; 10 mg/kg-veh  $F_{1,11}=100.61$ ,  $p<0.001$ ; 30 mg/kg-veh  $F_{1,11}=24.10$ ,  $p<0.001$ ) (Figure 2A, inset).

*Locomotor activity*: The stress-induced locomotor response (main time effect:  $F(12,132)=12.48$ ,  $p<0.001$ ) was reduced by zolpidem (main zolpidem effect:  $F(3,33)=7.41$ ,  $p<0.001$ ; zolpidem x time interaction  $F(36,396)=1.98$ ,  $p<0.001$ ). Locomotor activity was found to be reduced in all three dosages (planned comparisons: vehicle-3 mg/kg ( $F(1,11)=8.27$ ,  $p<0.05$ ), vehicle-10 mg/kg ( $F(1,11)=12.25$ ,  $p<0.01$ ) and vehicle-30 mg/kg ( $F(1,11)=13.34$ ,  $p<0.01$ )). When cumulating locomotor activity levels after injection and after novel cage stress (figure 2C, inset), zolpidem was found to reduce overall locomotor activity (main zolpidem effect  $F(3,33)=12.21$ ,  $p<0.001$ ; zolpidem x stress interaction  $F(3,33)=0.31$ ,  $p=0.69$ , NS). Simple contrasts showed that all doses of zolpidem reduced cumulative locomotor activity (veh-3mg/kg:  $F(1,11)=12.28$ ,  $p<0.01$ ; veh-10mg/kg:  $F(1,11)=12.37$ ,  $p<0.01$ ; veh-30 mg/kg:  $F(1,11)=25.11$ ,  $p<0.001$ ). Novel cage-induced locomotor levels were larger than injection-induced locomotor levels (stress effect  $F(3,33)=17.18$ ,  $p<0.01$ ).



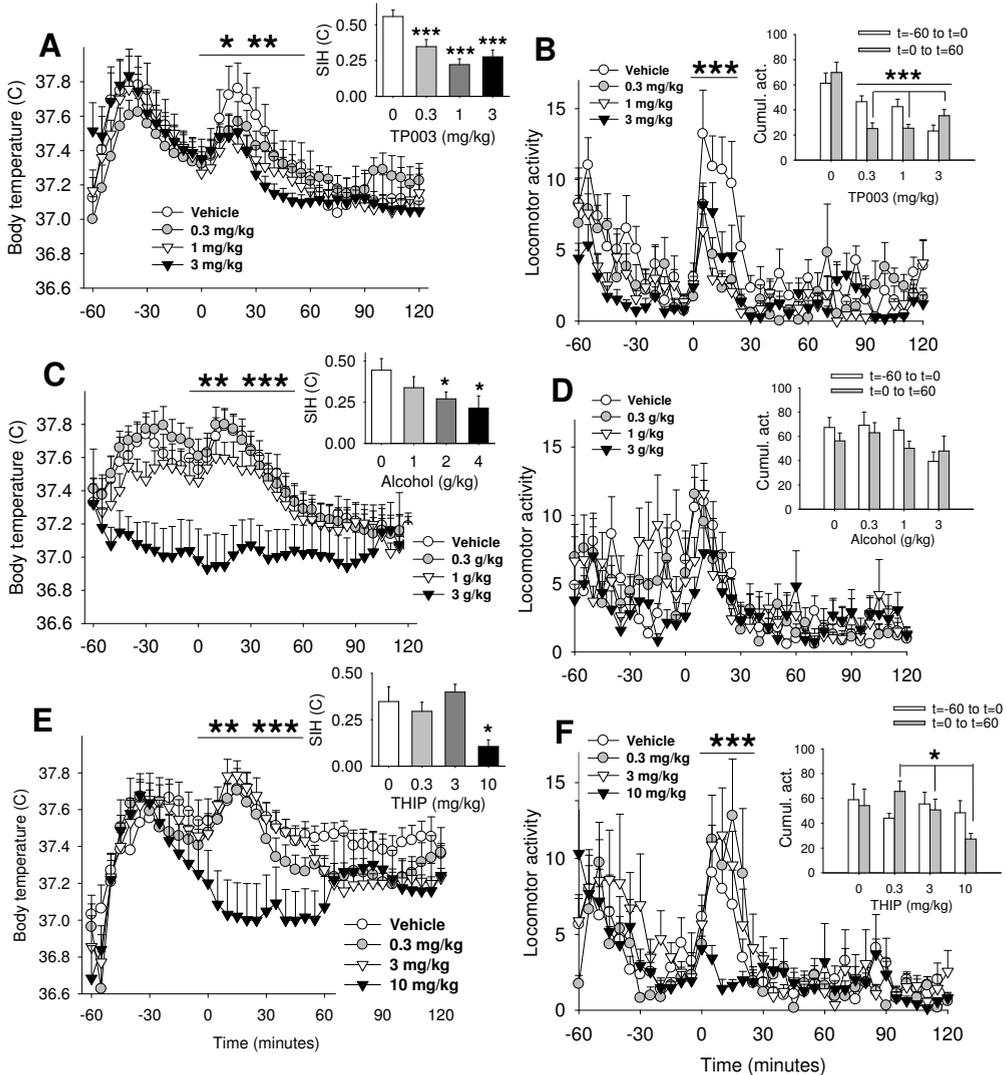
**Figure 2:** The effects of zolpidem with and without  $\beta$ CCt on the novel cage-induced temperature and locomotor responses ( $t=-60$  injection,  $t=0$  novel cage stress). \*:  $p<0.05$ ; \*\*:  $p<0.01$ ; \*\*\*:  $p<0.001$ . **A:** Zolpidem (0-30 mg/kg) dose-dependently reduced the SIH response and basal body temperature. Inset: calculated SIH response from the telemetry data. **B:** Zolpidem at a dose of 10 mg/kg reduced core body temperature and the SIH response. Prior injection with  $\beta$ CCt (10 mg/kg) reversed basal core body temperature reduction without affecting the zolpidem-induced reduction of the SIH response \*\*:  $p<0.01$ , zolpidem effect; \*:  $p<0.05$ , zolpidem x  $\beta$ CCt interaction. Inset: calculated SIH response from the telemetry data. **C:** Zolpidem (0-30 mg/kg) dose-dependently reduced stress-induced locomotor activity responses. **D:** Zolpidem (10 mg/kg) reduced stress-induced locomotor activity responses.  $\beta$ CCt partially reversed zolpidem-induced locomotor sedation. (\*:  $p<0.05$ : zolpidem\* $\beta$ CCt interaction). **Inset 2C-D:** white bar: cumulative locomotor activity  $t=-60$  to  $t=0$  (after injection); grey bar: cumulative locomotor activity  $t=0$  to  $t=60$  (after novel cage).

### 3.5 Zolpidem and $\beta$ CCt (Figure 2B and D, n=8)

*Summary:*  $\beta$ CCt was able to partially reverse the overall zolpidem-induced hypothermia as well as time-dependently partially reverse the zolpidem-induced locomotor sedation.

*Body temperature:* When combined with  $\beta$ CCt, zolpidem did not significantly reduce the SIH response (main time effect  $F(12,84)=9.75$ ,  $p<0.001$ ; zolpidem x time interaction  $F(12,84)=0.93$ ,  $p=0.54$ , NS). Also,  $\beta$ CCt did not influence the SIH response ( $\beta$ CCt x time interaction  $F(12,84)=0.92$ ,  $p=0.53$ , NS). Zolpidem reduced basal body temperature (main zolpidem effect  $F(1,7)=11.12$ ,  $p<0.01$ ), and  $\beta$ CCt influenced the zolpidem-induced hypothermia (zolpidem x  $\beta$ CCt interaction  $F(1,7)=6.31$ ,  $p<0.05$ ) without altering the body temperature itself ( $\beta$ CCt effect  $F(1,7)=2.54$ ,  $p=0.16$ , NS). The calculated SIH response revealed that zolpidem reduced the SIH response (zolpidem effect  $F_{1,7}=11.31$ ,  $p=0.01$ ).  $\beta$ CCt itself did not affect the SIH response ( $\beta$ CCt effect  $F_{1,7}=0.01$ ,  $p=0.97$ , NS), nor did  $\beta$ CCt affect the attenuation of the SIH response by zolpidem (zolpidem x  $\beta$ CCt interaction  $F_{1,7}=0.25$ ,  $p=0.63$ , NS) (Figure 2B, inset).

*Locomotor activity:* When combined with  $\beta$ CCt, zolpidem overall reduced locomotor responses (main zolpidem effect  $F(1,7)=7.80$ ,  $p<0.05$ ; zolpidem x time interaction  $F(1,7)=1.99$ ,  $p<0.05$ ).  $\beta$ CCt had no overall effect on locomotor responses ( $\beta$ CCt effect  $F(1,7)=1.94$ ,  $p=0.21$ , NS). However,  $\beta$ CCt reversed locomotor activity in the zolpidem group dependent upon time (zolpidem x  $\beta$ CCt x time interaction  $F(12,84)=1.91$ ,  $p<0.05$ ; zolpidem x  $\beta$ CCt interaction  $F(1,7)=0.18$ ,  $p=0.69$ , NS). When cumulating locomotor activity levels after injection and after novel cage stress (figure 2D, inset), zolpidem reduced basal and stress-induced locomotor activity (zolpidem effect  $F(1,7)=16.73$ ,  $p<0.01$ ; stress effect  $F(1,7)=1.61$ ,  $p=0.25$ , NS) without overall effect of  $\beta$ CCt ( $\beta$ CCt effect  $F(1,7)=2.09$ ,  $p=0.19$ , NS;  $\beta$ CCt x zolpidem interaction  $F(1,7)=1.47$ ,  $p=0.26$ , NS).



**Figure 3:** The effects of TP003 (0-3 mg/kg, figures 3A-B), alcohol (0-3 g/kg, figures 3C-D) and THIP (0-10 mg/kg, figure 3E-F) on the novel cage-induced temperature and locomotor responses (t=-60 injection, t=0 novel cage stress). \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

**A:** TP003 reduced the SIH response at higher doses. **Inset:** calculated SIH response from the telemetry data. **B:** TP003 dose-dependently reduced stress-induced locomotor responses (\*\*\*). **Inset:** calculated SIH response. **C:** Alcohol at the highest dose reduced the SIH response (\*\*\*) and basal body temperature (\*\*). **Inset:** calculated SIH response from the telemetry data. **D:** Alcohol did not affect stress-induced locomotor activity responses. **E:** THIP at the highest dose reduced the SIH response (\*\*\*) and basal body temperature (\*\*). **F:** THIP at the highest dose reduced stress-induced locomotor activity responses. **Inset graphs:** white bar: cumulative locomotor activity t=-60 to t=0 (after injection stress); grey bar: cumulative locomotor activity t=0 to t=60 (after novel cage stress). **Inset 3B:** TP003 reduced overall locomotor activity (TP003 effect: \*: p<0.001), but more so after the novel cage procedure (TP003\*stress effect, \*: p<0.001). **Inset 3D:** Alcohol did not influence locomotor activity responses. **Inset 3F:** THIP did not reduce overall locomotor activity, but did reduce locomotor activity after the novel cage procedure (gaboxadol\*stress effect, \*\*\*: p<0.05).

### 3.6 TP003 (Figure 3A-B, n=10)

*Summary:* TP003 reduced the SIH response at higher doses as well as reduced basal body temperature, and attenuated novel cage-induced activity more than injection-induced activity.

*Body temperature:* The SIH response (main time effect:  $F(12,108)=19.27$ ,  $p<0.001$ ) was attenuated by TP003 (TP003 x time interaction:  $F(36,324)=1.93$ ,  $p<0.01$ ). TP003 did influence basal core body temperature ( $F(3,27)=2.96$ ,  $p=0.050$ ). Planned comparisons revealed a significant difference between the vehicle and 1 mg/kg condition ( $F(1,9)=6.26$ ,  $p<0.05$ ), a trend for a difference between vehicle and 3 mg/kg ( $F(1,9)=3.65$ ,  $p=0.09$ , NS), and no difference between vehicle and the 0.3 mg/kg condition ( $F(1,9)=0.27$ ,  $p=0.62$ , NS). The calculated SIH response revealed TP003 reduced the SIH response ( $F_{3,27}=12.57$ ,  $p<0.001$ ). Simple contrasts revealed SIH attenuation at all doses (0.3 mg/kg-veh:  $F_{1,9}=22.25$ ,  $p<0.001$ ; 1 mg/kg-veh  $F_{1,9}=25.50$ ,  $p<0.001$ ; 3 mg/kg-veh  $F_{1,9}=27.79$ ,  $p<0.001$ ) (Figure 3A, inset).

*Locomotor activity:* Stress-induced locomotor responses (main time effect ( $F(12,108)=22.43$ ,  $p<0.001$ ) were reduced by TP003 (TP003 effect  $F(3,27)=14.43$ ,  $p<0.001$ ; TP003 x time interaction:  $F(36,324)=1.42$ ,  $p=0.06$ ). All three TP003 doses resulted in significant activity reduction after novel cage stress as compared to the vehicle group ( $F(1,9)=23.94$ ,  $p<0.001$  for vehicle–0.3 mg/kg,  $F(1,9)=25.56$ ,  $p<0.001$  for vehicle–1 mg/kg and  $F(1,9)=14.09$ ,  $p<0.01$  for vehicle–3 mg/kg, simple contrasts). When cumulating locomotor activity levels after injection and after novel cage stress (figure 3B, inset), TP003 reduced locomotor activity levels after novel cage stress more than after injection (TP003 effect  $F(3,27)=9.34$ ,  $p<0.001$ ; TP003 x prepost interaction  $F(1,9)=9.81$ ,  $p<0.001$ ), although activity levels were comparable after both injection and novel cage (stress effect  $F(1,9)=1.63$ ,  $p=0.23$ , NS).

### 3.7 Alcohol (Figure 3C-D, n=11)

*Summary:* Alcohol reduced the SIH response and basal body temperature only at higher doses. Alcohol did not affect locomotor activity levels after injection and after novel cage stress.

*Body temperature:* Novel cage stress led to a significant increase in temperature (time effect  $F(12,120)=15.68$ ,  $p<0.001$ ) and alcohol reduced the SIH response (time x alcohol interaction  $F(36,360)=2.56$ ,  $p<0.001$ ). Alcohol decreased basal body temperature (main alcohol effect  $F(3,30)=4.82$ ,  $p<0.01$ ). Simple contrasts revealed differences only between vehicle–3 g/kg regarding basal body temperature (dose contrasts: veh–0.3 g/kg:  $F(1,10)=0.79$ , NS; veh–1g/kg:  $F(1,10)=0.01$ ,  $p=0.98$ , NS; veh–3g/kg:  $F(1,10)=5.71$ ,  $p<0.05$ ). The calculated SIH response showed a trend for alcohol to reduce the SIH response ( $F_{3,30}=2.30$ ,  $p=0.09$ , NS). Simple contrasts revealed that SIH attenuation at higher doses (1 g/kg-veh:  $F_{1,10}=1.32$ ,  $p=0.28$ , NS; 2 g/kg-veh  $F_{1,10}=5.19$ ,  $p<0.05$ ; 4 g/kg-veh  $F_{1,10}=5.12$ ,  $p<0.05$ ) (Figure 3C, inset).

*Locomotor activity:* The locomotor reaction in response to novel cage stress (time effect  $F(12,120)=16.87$ ,  $p<0.001$ ) was not affected by alcohol (main alcohol effect  $F(3,30)=0.62$ ,  $p=0.61$ , NS; alcohol x time interaction  $F(36,360)=1.23$ ,  $p=0.18$ , NS). When cumulating locomotor activity levels after injection and after novel cage stress (figure 3D, inset), alcohol did not affect locomotor activity levels after injection and after novel cage stress (alcohol effect  $F(3,30)=1.67$ ,  $p=0.20$ , NS; alcohol x stress  $F(3,30)=0.90$ ,  $p=0.45$ , NS).

### 3.8 THIP (Figure 3E-F, n=10)

*Summary:* THIP reduced the SIH response and basal body temperature at its highest dose. THIP reduced locomotor activity after novel cage stress.

*Body temperature:* Basal body temperature was overall reduced by THIP (THIP effect  $F(3,27)=5.44, p<0.01$ ). Also, the SIH response (time effect:  $F(12,108)=12.59, p<0.001$ ) was reduced by THIP (THIP x time interaction:  $F(36,324)=4.25, p<0.001$ ). Simple contrasts revealed a significant difference in basal body temperature between the vehicle and 10 mg/kg condition ( $F(1,9)=15.40, p<0.01$ ), whereas the other doses did not affect basal body temperature (vehicle-0.3 mg/kg condition  $F(1,9)=1.11, p=0.32, NS$ ; vehicle-3 mg/kg condition  $F(1,9)=0.11, p=0.75, NS$ ). The calculated SIH response revealed that THIP reduced the SIH response ( $F_{3,27}=4.64, p=0.01$ ). Simple contrasts revealed that only the highest dose reduced the SIH response (0.3 mg/kg-veh:  $F_{1,9}=0.31, p=0.59, NS$ ; 3 mg/kg-veh  $F_{1,9}=0.25, p=0.63$ ; 10 mg/kg-veh  $F_{1,10}=6.15, p<0.05$ ) (Figure 3E, inset).

*Locomotor activity:* The stress-induced locomotor response (time effect:  $F(12,108)=12.35, p<0.001$ ) was reduced by THIP (THIP x time interaction  $F(36,324)=1.96, p<0.001$ ; THIP effect  $F(3,27)=2.43, p=0.09, \text{trend}$ ). When cumulating locomotor activity levels after injection and after novel cage stress (figure 3F, inset), THIP reduced locomotor activity levels only after the novel cage procedure (THIP x stress interaction  $F(1,9)=3.37, p<0.05$ ), although overall locomotor activity was not reduced (THIP effect  $F(3,27)=1.18, p=0.34, NS$ ) and overall locomotor activity levels after injection and novel cage stress were not different (stress effect  $F(1,9)=0.18, p=0.68, NS$ ).

## 4. Discussion

In the present study, we examined the effects of various GABA<sub>A</sub>ergic compounds on temperature and locomotor responses to acute stress. The stress-induced hyperthermia model uses the rise in body temperature in response to stress to assess anxiolytic drug effects, and provides a translational approach to anxiety research (Vinkers et al 2008). We found that the administration of the non-selective GABA<sub>A</sub> receptor agonist diazepam resulted in a dose-dependent attenuation of the stress-induced hyperthermia and basal and stress-induced locomotor activity responses, indicating that diazepam induces both anxiolytic and sedative effects. These findings support and extend previous studies on diazepam in the stress-induced hyperthermia paradigm in mice (Olivier et al 2002). Zolpidem, an intermediate selective GABA<sub>A</sub> receptor  $\alpha_1$  subunit agonist, decreased basal core body temperature and attenuated basal and stress-induced locomotor and temperature responses in a dose-dependent fashion. The sedative effect of zolpidem *in vivo* is mediated by the  $\alpha_1$  subunit (Crestani et al 2000), and zolpidem does not possess any anxiolytic properties (Kumar et al 2003; Mirza et al 2008). However, we cannot exclude that the results of the higher doses of zolpidem may be the result of non-specific GABA<sub>A</sub> receptor activation. Also, the reduction of the SIH response by zolpidem is most likely the result of strong hypothermic effects on basal body temperature, disturbing physiological homeostatic mechanisms (Olivier et al 2003).

$\beta$ CCt shows a high affinity for the GABA<sub>A</sub> receptor  $\alpha_1$  subunit with considerably lower affinity for GABA<sub>A</sub> receptor  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_4$  subunits, and has comparable low efficacy at all  $\alpha$  subunits. (Huang et al 2000; Popik et al 2006). Administration of  $\beta$ CCt alone had no effect on either basal body temperature or novel cage-induced temperature and locomotor activity responses. However, prior injection with  $\beta$ CCt antagonized hypothermic effects of both diazepam and zolpidem, and reversed zolpidem-induced locomotor sedation (figures 1 and 2). In contrast,  $\beta$ CCt did not antagonize the diazepam-induced locomotor sedation, which may be attributed to the fact that diazepam exerts a broader pharmacological GABA<sub>A</sub> agonistic profile while zolpidem action is restricted to the GABA<sub>A</sub> receptor  $\alpha_1$  subunit. Basal body temperature reduction after diazepam administration was only observed in the combination  $\beta$ CCt/diazepam experiment and not when solely diazepam was injected. We do not have an explanation for this puzzling observation; the only experimental difference was the presence of a double injection within 10 minutes. Mice do consistently show a reduction in basal body temperature after administration of diazepam (Olivier et al 2002; Van Bogaert et al 2006a), and better regulated homeostasis in the rat may account for an absent hypothermia when diazepam was administered. Our results suggest a role for the GABA<sub>A</sub> receptor  $\alpha_1$  subunit in hypothermic and locomotor sedative actions of GABA<sub>A</sub>ergic drugs. Some studies have suggested that anxiolytic effects of benzodiazepines can be reversed with  $\beta$ CCt (Griebel et al 2000a; Griebel et al 1999). However, this may be the result of decreased sedation rather than a reversal of anxiolytic effects, since sedation caused by diazepam and zolpidem has been shown to be reversible with high doses of  $\beta$ CCt (Griebel et al 1999; Popik et al 2006). The hypothermic effects after activation of the GABA<sub>A</sub> receptor  $\alpha_1$  subunit has been extensively studied in mice (Van Bogaert et al 2006a). The  $\alpha_1$  subunit is abundantly expressed throughout the brain, and a higher expression of the  $\alpha_1$  subunit is present in the hypothalamic preoptic area and dorsomedial hypothalamus as compared to the  $\alpha_2$  and  $\alpha_3$  subunits (Pirker et al 2000). These areas are thought to play a major role in thermoregulation (Boulant 2000; Dimicco and Zaretsky 2007; Nagashima et al 2000) and may account for the  $\alpha_1$  involvement in the regulation of basal body temperature.

A putative role for the GABA<sub>A</sub> receptor  $\alpha_3$  subunit in anxiety was confirmed with GABA<sub>A</sub> receptor  $\alpha_3$  subunit agonist TP003 that attenuated the SIH response without affecting basal body temperature levels (figure 3A). Also, when combined with GABA<sub>A</sub> receptor  $\alpha_1$  subunit antagonist  $\beta$ CCt, diazepam reduced the SIH response, possibly through activation of the  $\alpha_{2/3}$  subunit. Although transgenic mice lacking benzodiazepine sensitivity in the  $\alpha_3$  subunit did not show altered anxiolytic actions of diazepam (Low et al 2000; Rudolph and Mohler 2004), pharmacological studies have pointed to a role for this subunit in anxiolysis (Atack et al 2005; Carling et al 2005; Dias et al 2005) as might be expected from high  $\alpha_3$  subunit expression in brain areas involved in acute stress responses (Pirker et al 2000). Anxiolytic effects of TP003 were found in the elevated plus maze (rats) and in a conditioned emotional response test (squirrel monkeys) (Dias et al 2005). Our findings support and extend previous experiments suggesting GABA<sub>A</sub> receptor  $\alpha_2$  and  $\alpha_3$  subunits as the main regulatory subunits mediating anxiolytic effects (Atack et al 2005; Dias et al 2005). Stress-induced locomotor responses after novel cage stress were reduced at all TP003 doses, but only after novel cage stress and not immediately after injection like in the case of diazepam and zolpidem (figure 3B). This contrasts with another study in mice

that did not show any sedative locomotor effects of TP003 (Dias et al 2005). Although being  $\alpha_3$  subunit selective, TP003 also has low modulation via  $\alpha_{1-}$ ,  $\alpha_{2-}$ , and  $\alpha_{5-}$ -containing subtypes (Dias et al 2005). Also, differences in metabolizing enzymes exist between animal species, resulting in different clearance rates which are frequently thought to be responsible for differences in behavioral responses. For GABA<sub>A</sub> receptor agonists, one study of nitrazepam found much higher plasma levels in rats than in mice after a dose nitrazepam (Takeno et al 1993), whereas another study found that the oral bioavailability of L-838417, a  $\alpha_1$  antagonist and  $\alpha_2/\alpha_3$  partial agonist, in mice was very poor compared to bioavailability in rats (Scott-Stevens et al 2005). Therefore, a lack of sedative action of TP003 in mice compared to rats can possibly be ascribed to lower plasma levels of TP003 caused by a more rapid metabolism of TP003 in mice.

Locomotor activity responses to stress are used as an output parameter in various anxiety paradigms such as the elevated plus maze, the open field test and the light-dark test. Open-arm entries, a lit box or centre of a field all putatively lead to an anxiety state, but also cage exchange as used in the current experiments leads to similar increases in distances travelled and velocities (de Visser et al 2006). In general, stress-induced behavior in rodents consists of exploration on one hand, and anxiety-driven avoidance behavior on the other hand, and there is no easy way of establishing the relationship between exploration and anxiety. Anxiolytic drugs increase explorative behavior and locomotor activity (Belzung and Berton 1997), but in higher doses cause general locomotor sedation, interfering with a good test interpretation (Dawson et al 1995). Therefore, sedative effects of both diazepam and zolpidem cause a decrease in locomotor activity (Davies et al 1994; Elliot and White 2001). However, the sedative effects of benzodiazepines in the elevated plus maze are no longer present after a point mutation of the  $\alpha_1$  subunit (McKernan et al 2000; Rudolph et al 1999), indicating that the  $\alpha_1$  subunit is closely involved in benzodiazepine-induced locomotor activity reduction. McKernan et al. showed that diazepam (3 mg/kg) even increased locomotor activity in  $\alpha_1$  point mutated mice compared to wild-type controls. In contrast, myorelaxant effects of diazepam in the rotarod assay remain present in the  $\alpha_1$  subunit KO mice, suggesting that the locomotor activity attenuation is not the mere result of muscle relaxation. Other studies showed that  $\beta$ CCt antagonized the locomotor depressant effects of zolpidem and diazepam on open field locomotor activity in mice (Griebel et al 1999) as well as the elevated plus maze (Savic et al 2004). All in all, there is ample evidence that locomotor depressant actions of zolpidem and the benzodiazepines are mediated via the  $\alpha_1$  subunit of the GABA<sub>A</sub> receptor. Anxiolytic drugs completely devoid of sedative side effects would therefore either increase or not affect locomotor activity parameters after novelty-induced stress.

Ethanol reduced basal body temperature at higher doses without affecting stress-induced locomotor responses (figure 3C). Only the highest dose reduced the SIH response, an effect that was already earlier observed in mice (Olivier et al 2003). Although acute administration of alcohol is known to possess an anxiolytic profile, the effects are known to be different from benzodiazepines (Langen et al 2002) as alcohol binds to extrasynaptic GABA<sub>A</sub> receptors containing  $\alpha_4$  or  $\alpha_6$  and  $\delta$  subunits (Wallner et al 2003). However, we used higher doses that could have lost extrasynaptic binding selectivity. Also, alcohol at higher doses may act on NMDA, serotonin and glycine receptors, (Crews et al 1996; Davies

2003; Harris 1999). THIP reduced basal body temperature and stress-induced hyperthermia and locomotor activity responses only at the highest dose tested (10 mg/kg), whereas lower doses did not have any effect (figure 3E). THIP has been shown to enhance sleep episodes (Lancel and Langebartels 2000) with little affinity for benzodiazepine receptors. Rather, THIP binds to extrasynaptic GABA<sub>A</sub> receptors containing a  $\delta$ -subunit (Wafford and Ebert 2006), and a role for the GABA<sub>A</sub> receptor  $\delta$  subunit in neurosteroid-mediated anxiolytic effects has been proposed (Mihalek et al 1999). However, it seems more likely that the strong hypothermic effects of the highest dose of THIP are due to interference with physiological thermoregulation (Olivier et al 2003). Interaction between effects on sleep and thermoregulation are possible because of common neural pathways within the preoptic area and anterior hypothalamus (Frosini et al 2004). Indeed, THIP synchronized hypothermic and EEG effects in rabbits (Frosini et al 2004). Interestingly, alcohol did not affect locomotor activity at all doses and THIP affected locomotor activity only at high doses (figures 3D and 3F), whereas the other synaptic compounds all reduced locomotor activity to some extent. Other studies have found that alcohol impaired rotarod performance at lower doses (Zaleski et al 2001). Although our high doses may have lost extrasynaptic selectivity, this indicates that locomotor activity may be differentially controlled by extrasynaptic and synaptic receptor populations. This is supported by a lack of cross-tolerance in the rotarod test between zolpidem and THIP (Voss et al 2003).

The SIH amplitude decreased over the course of the experiments from 0.7 °C at the start of the experiments to 0.2-0.3 °C in the final experiments, as did locomotor activity levels after novel cage stress. Habituation to the experimental procedure may account for a decreased SIH response, although previous methodological testing has not revealed any habituation using a one week interval, even when testing occurred for over a year (Bouwknicht et al 2007; Olivier et al 2003; Van der Heyden et al 1997). Also, the manually calculated SIH response from the time graphs is generally in complete agreement with the time graphs. Only when drugs are tested at doses that markedly decrease body temperature, there appears to be a small difference between the calculated SIH response and the time graphs. This difference is attributable to the fact that the calculated SIH response is based on the maximum temperature during the first thirty minutes after stress. In those cases in which body temperature is decreasing after stress induction, the maximum is likely to be close to the start of that 30-minute period. In this way, the calculated SIH response in these cases is likely to yield a result close to 0 °C, whereas a decreasing basal body temperature seems to indicate a negative SIH response. The differences however are small and do not change the interpretation of our data.

The most important finding in the present study is that the GABA<sub>A</sub> receptor modulates temperature and locomotor stress responses as well as basal body temperature processes through different GABA<sub>A</sub> receptor subunits. More specifically, the GABA<sub>A</sub>  $\alpha_1$  receptor subunit was found to be essential for basal body temperature regulation and for inducing locomotor sedation, whereas the GABA<sub>A</sub> receptor  $\alpha_2$  and  $\alpha_3$  subunit exerted anxiolytic effects by attenuating the SIH response. Non-benzodiazepine GABA<sub>A</sub> activity is less involved in thermoregulation and locomotor sedation, as suggested by the effects of alcohol and THIP. In conclusion, we show that the use of home cage temperature and

locomotor stress responses provides a successful approach to anxiety research and possesses an enormous potential to pharmacologically study the effects of GABA<sub>A</sub>-ergic drugs. The stress-induced hyperthermia model uses a simultaneously collected independent parameter and may possess additional value over locomotor activity parameters only.

### **Acknowledgements**

We would like to thank Ruud van Oorschot and Koen Westphal for their excellent technical assistance.

# Chapter 4

## **5-HT<sub>1A</sub> receptor blockade reverses anxiolytic effects mediated by the GABA<sub>A</sub> receptor $\alpha_3$ subunit**

Christiaan H. Vinkers

Ruud van Oorschot

S. Mechiel Korte

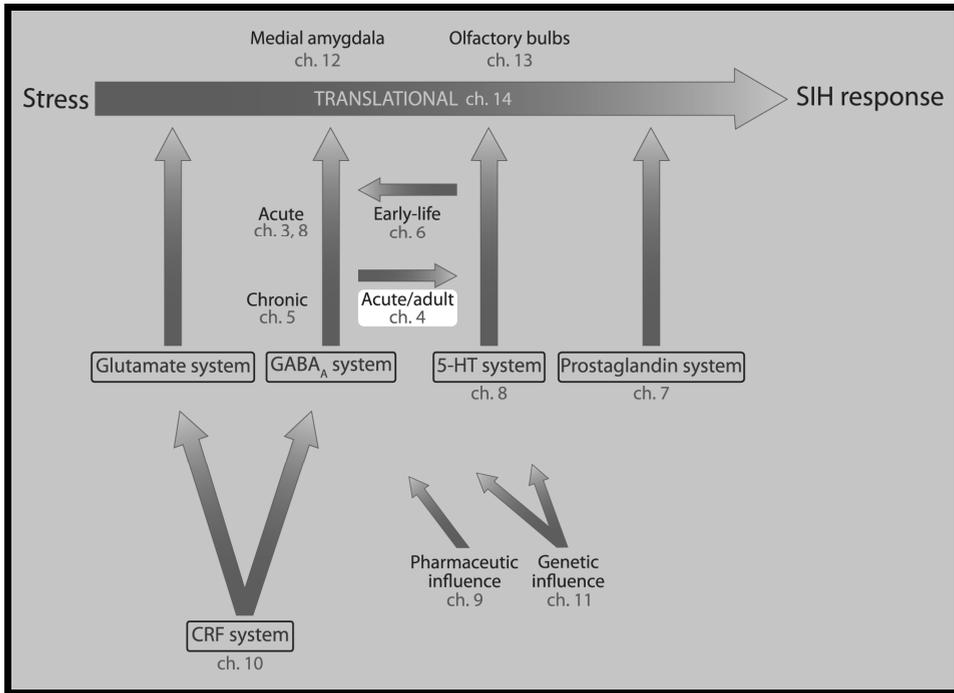
Berend Olivier

Lucianne Groenink

# 4

*Submitted*

## Abstract



**Rationale:** Stress-related disorders are associated with dysfunction of both serotonergic and GABAergic pathways and clinically effective anxiolytics act via both neurotransmitter systems. As there is evidence that the GABA<sub>A</sub> and the serotonin receptor system directly interact, a serotonergic component in the anxiolytic actions of benzodiazepines could be present.

**Objectives:** The aim of the present study was to investigate whether the anxiolytic effects of (non-)selective  $\alpha$  subunit GABA<sub>A</sub> receptor agonists could be reversed with 5-HT<sub>1A</sub> receptor blockade using the stress-induced hyperthermia (SIH) paradigm.

**Results:** The 5-HT<sub>1A</sub> receptor antagonist WAY-100635 (0.1-1 mg/kg) reversed the SIH-reducing effects of the non  $\alpha$ -subunit selective GABA<sub>A</sub> receptor agonist diazepam (1-4 mg/kg) and the GABA<sub>A</sub> receptor  $\alpha_3$ -subunit selective agonist TP003 (1 mg/kg), whereas WAY-100635 alone was without effect on SIH or basal body temperature. At the same time, co-administration of WAY-100635 with diazepam or TP003 reduced basal body temperature. WAY-100635 did not affect the SIH response when combined with the preferential  $\alpha_1$  subunit GABA<sub>A</sub> receptor agonist zolpidem (10 mg/kg). Zolpidem did not affect the SIH response, but markedly reduced basal body temperature when administered alone.

**Conclusions:** The present study suggests a direct interaction between GABA<sub>A</sub> receptor  $\alpha_3$  subunits and 5-HT<sub>1A</sub> receptors on stress-induced hyperthermia. Specifically, our data indicate that benzodiazepines affect serotonergic signalling via GABA<sub>A</sub>-ergic  $\alpha_3$  subunits. Further understanding of the interactions between the GABA<sub>A</sub> and serotonin system in reaction to stress may be valuable in the search for novel anxiolytic drugs.

## 1. Introduction

Stress-related disorders are associated with dysfunction of both serotonergic and GABAergic pathways (Akimova et al 2009; Kalueff and Nutt 2007; Nemeroff 2003). The clinical anxiolytic effects of selective serotonin reuptake inhibitors, 5-HT<sub>1A</sub> receptor agonists and GABA<sub>A</sub>R agonists indicate that both the GABA<sub>A</sub>ergic as well as the serotonergic system may be involved in the pathological basis underlying anxiety disorders (Nutt 2005; Zohar and Westenberg 2000). There is evidence that the GABA and the serotonergic system directly interact (Fernandez-Guasti and Lopez-Rubalcava 1998; Gao et al 1993; Lista et al 1989), although the evidence is inconsistent (Shephard et al 1982; Thiebot 1986). Specifically, a serotonergic component in the anxiolytic actions of benzodiazepines has been suggested (Harandi et al 1987; Stein et al 1977; Thiebot et al 1984). Hence, studying the interactions of the GABA<sub>A</sub> and serotonin system in stress and anxiety could be valuable in the search for novel anxiolytic drugs.

Here, we investigate whether the anxiolytic effects of GABA<sub>A</sub> receptor agonists are dependent on 5-HT<sub>1A</sub> receptor activation using the stress-induced hyperthermia (SIH) paradigm. The SIH response is the transient rise in body temperature in response to acute stress that is mediated by the autonomic nervous system (Bouwknrecht et al 2007; Vinkers et al 2008). Both classical benzodiazepines and 5-HT<sub>1A</sub> receptor agonists consistently reduce the SIH response (as well as basal body temperature at higher doses), whereas dopaminergic and noradrenergic systems are generally ineffective (Olivier et al 2003). Classical (non-subunit selective) benzodiazepines bind to GABA<sub>A</sub> receptor  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  subunits, and the various benzodiazepine effects are thought to be mediated through different GABA<sub>A</sub> receptor subtypes (Rudolph and Mohler 2006). Interactions with the serotonergic system may thus depend on the GABA<sub>A</sub> receptor composition. In the present study, we investigated whether the silent 5-HT<sub>1A</sub> receptor antagonist WAY-100635 (WAY) could reverse the anxiolytic effects of the non-subunit selective GABA<sub>A</sub> receptor agonist diazepam, the selective GABA<sub>A</sub> receptor  $\alpha_3$  subunit agonist TP003 (Dias et al 2005) and the preferential GABA<sub>A</sub> receptor  $\alpha_1$  subunit agonist zolpidem.

## 2. Materials and methods

### 2.1 Animals

Male NMRI mice (Charles River, The Netherlands) were housed in Macrolon type 3 cages enriched with bedding and nesting material under a 12-h light/12-h dark cycle (lights on from 0600 to 1800 h) at controlled temperature (22±2 °C) and relative humidity (40–60%) with free access to standard food pellets and tap water. Experiments were carried out with approval of the ethical committee on animal experiments of the Faculties of Sciences, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki.

## 2.2 The stress-induced hyperthermia (SIH) procedure

The SIH tests were carried out according to standard procedures (Groenink et al 2009). A between-subject design was used. Cages were randomly and evenly allocated over daytimes (morning–afternoon). The temperature of mice was measured by rectally inserting a thermistor probe by a length of 2 cm. Digital temperature recordings were obtained with an accuracy of 0.1 °C using a Keithley 871A digital thermometer (NiCr– NiAl thermocouple). The probe, dipped into silicon oil before inserting, was held in the rectum until a stable rectal temperature had been obtained for 20 s. Animals were injected intraperitoneally with vehicle or WAY-100635 on the left flank and with vehicle, diazepam, zolpidem or TP003 on the right flank. All drugs were injected 60 min before the first temperature measurement ( $T_1$ ). The temperature was again measured 10 min later ( $T_2$ ), representing the stress-induced body temperature. The stress-induced hyperthermia response was calculated by subtracting  $T_1$  from  $T_2$ .

## 2.3 Drugs

Diazepam (base), zolpidem (tartaric acid) and WAY-100635 (maleate) (N-2-[4-(2-methoxy)-1-piperazinyl]ethyl]-N-(2-pyridinyl) cyclohexanecarboxamide tri-chloride) were obtained from Sigma Aldrich. TP003 was synthesized according to published methods (Dias et al 2005; Humphries et al 2006). An injection volume of 10 ml/kg was used for intraperitoneal injections of all drugs. WAY-100635 was dissolved in saline. Diazepam, zolpidem and TP003 were suspended in gelatin-mannitol 0.5% / 5%. Fresh solutions and suspensions were prepared each testing day.

## 2.4 Data analysis

For each individual mouse, a basal temperature ( $T_1$ ), an end temperature ( $T_2$ ) and the difference (SIH response =  $T_2 - T_1$ ) was determined. Treatment effects were evaluated using a two-way analysis of variance with explanatory factors drug<sub>1</sub> (WAY-100635 or vehicle) and drug<sub>2</sub> (diazepam/zolpidem/TP003 or vehicle). A probability level of  $p < 0.05$  was set as statistically significant, probability levels between  $p = 0.05$  and  $p = 0.1$  were regarded as trends.

### 3. Results

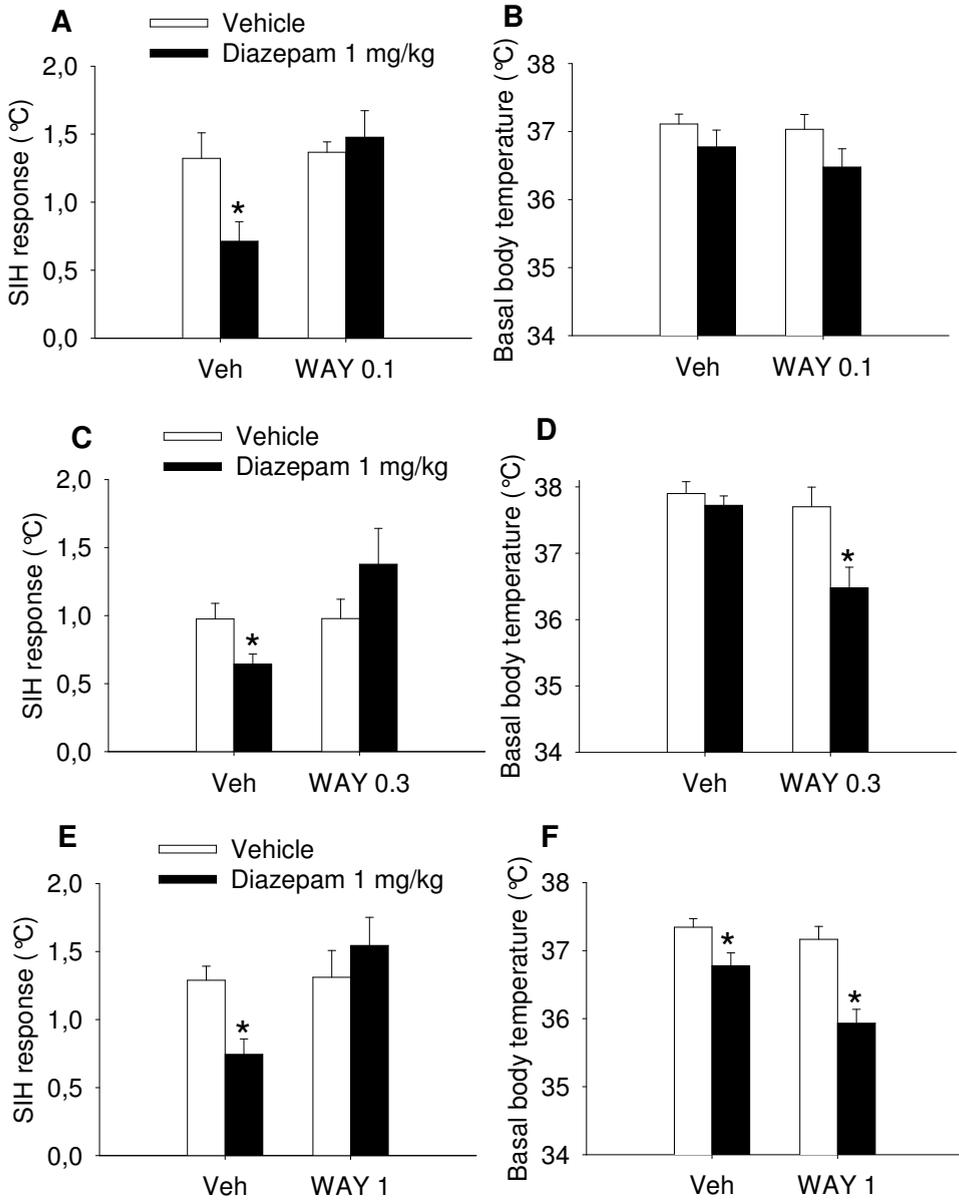
#### 3.1 Diazepam 1 mg/kg and WAY-100635

##### SIH response (Figure 1A, C, E)

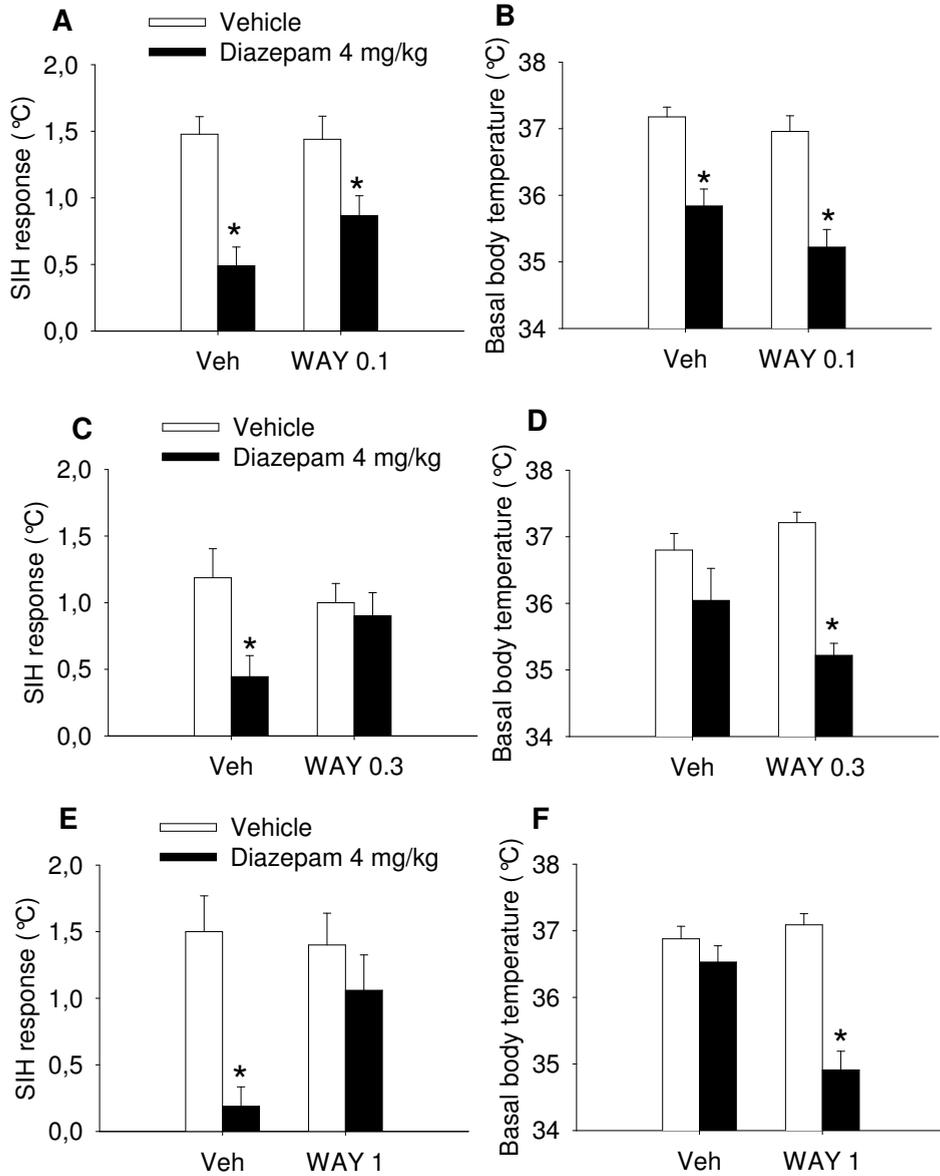
As shown in figure 1, WAY significantly altered the diazepam effect at all three doses tested (*WAY 0.1 mg/kg*: WAY x diazepam interaction  $F_{1,31}=5.02$ ,  $p<0.05$ ; *WAY 0.3 mg/kg*: WAY x diazepam interaction  $F_{1,31}=4.71$ ,  $p<0.05$ ; *WAY 1.0 mg/kg*: WAY x diazepam interaction  $F_{1,32}=5.76$ ,  $p<0.05$ ). Post-hoc analysis showed that in vehicle-treated mice, diazepam at a dose of 1 mg/kg reduced the SIH response relative to the vehicle-vehicle treated animals in all three experiments (*WAY 0.1 mg/kg*: diazepam effect:  $F_{1,16}=6.14$ ,  $p<0.05$ ; *WAY 0.3 mg/kg*: diazepam effect:  $F_{1,16}=5.70$ ,  $p<0.05$ ; *WAY 1 mg/kg*: diazepam effect:  $F_{1,17}=6.91$ ,  $p<0.05$ ). In contrast, diazepam did not reduce the SIH response in WAY-treated animals in any of the three experiments (*WAY 0.1 mg/kg*: diazepam effect:  $F_{1,17}=0.10$ ,  $p=0.76$ , NS; *WAY 0.3 mg/kg*: diazepam effect:  $F_{1,17}=1.79$ ,  $p=0.20$ , NS; *WAY 1 mg/kg*: diazepam effect:  $F_{1,17}=0.18$ ,  $p=0.68$ , NS).

##### Basal body temperature (Figure 1B, D, F)

In the experiment with the 0.1 mg/kg WAY dose, WAY did not alter the effect of diazepam (WAY x diazepam interaction,  $F_{1,31}=0.23$ ,  $p=0.63$ , NS), but diazepam tended to reduce basal body temperature (diazepam effect,  $F_{1,31}=3.86$ ,  $p=0.06$ , trend). In the experiments with the higher WAY doses, WAY appeared to enhance the temperature reducing effects of diazepam (*WAY 0.3 mg/kg*: WAY x diazepam interaction,  $F_{1,31}=4.40$ ,  $p<0.05$ ; *WAY 1.0 mg/kg*: WAY x diazepam interaction,  $F_{1,32}=3.43$ ,  $p=0.07$ , trend). Post-hoc analysis showed that combined administration of diazepam with the two higher WAY doses resulted in hypothermic effects relative to the WAY-vehicle treated animals (*WAY 0.3 mg/kg*: diazepam effect  $F_{1,17}=8.07$ ,  $p=0.01$ ; *WAY 1 mg/kg*: diazepam effect:  $F_{1,17}=14.57$ ,  $p<0.01$ ).



**Figure 1:** Effects of WAY-100635 (0.1-1 mg/kg, IP) on stress-induced hyperthermia and basal body temperature response after vehicle or diazepam (1 mg/kg, IP) administration. \*: p<0.05.



**Figure 2:** Effects of WAY-100635 (0.1-1 mg/kg, IP) on stress-induced hyperthermia and basal body temperature response after vehicle or diazepam (4 mg/kg, IP) administration. \*: p<0.05.

### 3.2 Diazepam 4 mg/kg and WAY-100635

#### SIH response (Figure 2A,C,E)

At the lowest dose, WAY did not alter the effect of diazepam (*WAY 0.1 mg/kg*: WAY x diazepam interaction  $F_{1,35}=1.20$ ,  $p=0.28$ , NS), nor did it have an intrinsic effect on the SIH response (*WAY effect*,  $F_{1,35}=2.04$ ,  $p=0.16$ , NS). Overall, diazepam (4 mg/kg) reduced the SIH response (*WAY 0.1 mg/kg*: diazepam effect,  $F_{1,35}=24.37$ ,  $p<0.001$ ). At higher doses, WAY significantly reversed the diazepam effects on the SIH response (*WAY 0.3 mg/kg*: WAY x diazepam interaction  $F_{1,32}=3.88$ ,  $p=0.05$ ; *WAY 1.0 mg/kg*: WAY x diazepam interaction  $F_{1,36}=4.74$ ,  $p<0.05$ ). Post-hoc analysis showed that in vehicle-treated mice, diazepam reduced the SIH response relative to the vehicle-vehicle treated animals in all experiments (*WAY 0.3 mg/kg*: diazepam effect:  $F_{1,17}=9.07$ ,  $p<0.01$ ; *WAY 1 mg/kg*: diazepam effect:  $F_{1,19}=20.40$ ,  $p<0.001$ ). In contrast, in WAY-treated animals (0.3 and 1.0 mg/kg) diazepam did not reduce the SIH response (*WAY 0.3 mg/kg*: diazepam effect:  $F_{1,17}=0.19$ ,  $p=0.67$ , NS; *WAY 1 mg/kg*: diazepam effect:  $F_{1,19}=1.01$ ,  $p=0.33$ , NS).]

#### Basal body temperature (Figure 2B,D,F)

The lowest dose of WAY-100635 did not alter the effect of diazepam (*WAY 0.1 mg/kg*: WAY x diazepam interaction,  $F_{1,35}=0.91$ ,  $p=0.35$ , NS and WAY effect,  $F_{1,35}=3.50$ ,  $p=0.07$ , trend), whereas diazepam (4 mg/kg) reduced basal body temperature (*WAY 0.1 mg/kg*: diazepam effect,  $F_{1,35}=48.87$ ,  $p<0.001$ ). The higher WAY doses enhanced the effect of diazepam on basal body temperature (*WAY 0.3 mg/kg*: WAY x diazepam interaction,  $F_{1,32}=4.86$ ,  $p<0.05$ ; *WAY 1 mg/kg*: WAY x diazepam interaction,  $F_{1,36}=18.47$ ,  $p<0.001$ ). Post-hoc analysis showed that diazepam combined with the higher WAY doses resulted in hypothermic effects in combination relative to the WAY-vehicle treated animals (*WAY 0.3 mg/kg*: diazepam effect  $F_{1,17}=67.79$ ,  $p<0.001$ ; *WAY 1 mg/kg*: diazepam effect:  $F_{1,19}=49.40$ ,  $p<0.001$ ). In contrast, diazepam alone did not reduce the basal body temperature compared to vehicle-vehicle treated animals (*WAY 0.3 mg/kg*: diazepam effect:  $F_{1,17}=1.64$ ,  $p=0.22$ , NS; *WAY 1 mg/kg*: diazepam effect:  $F_{1,19}=1.44$ ,  $p=0.25$ , NS).

### 3.3 TP003 1 mg/kg and WAY-100635

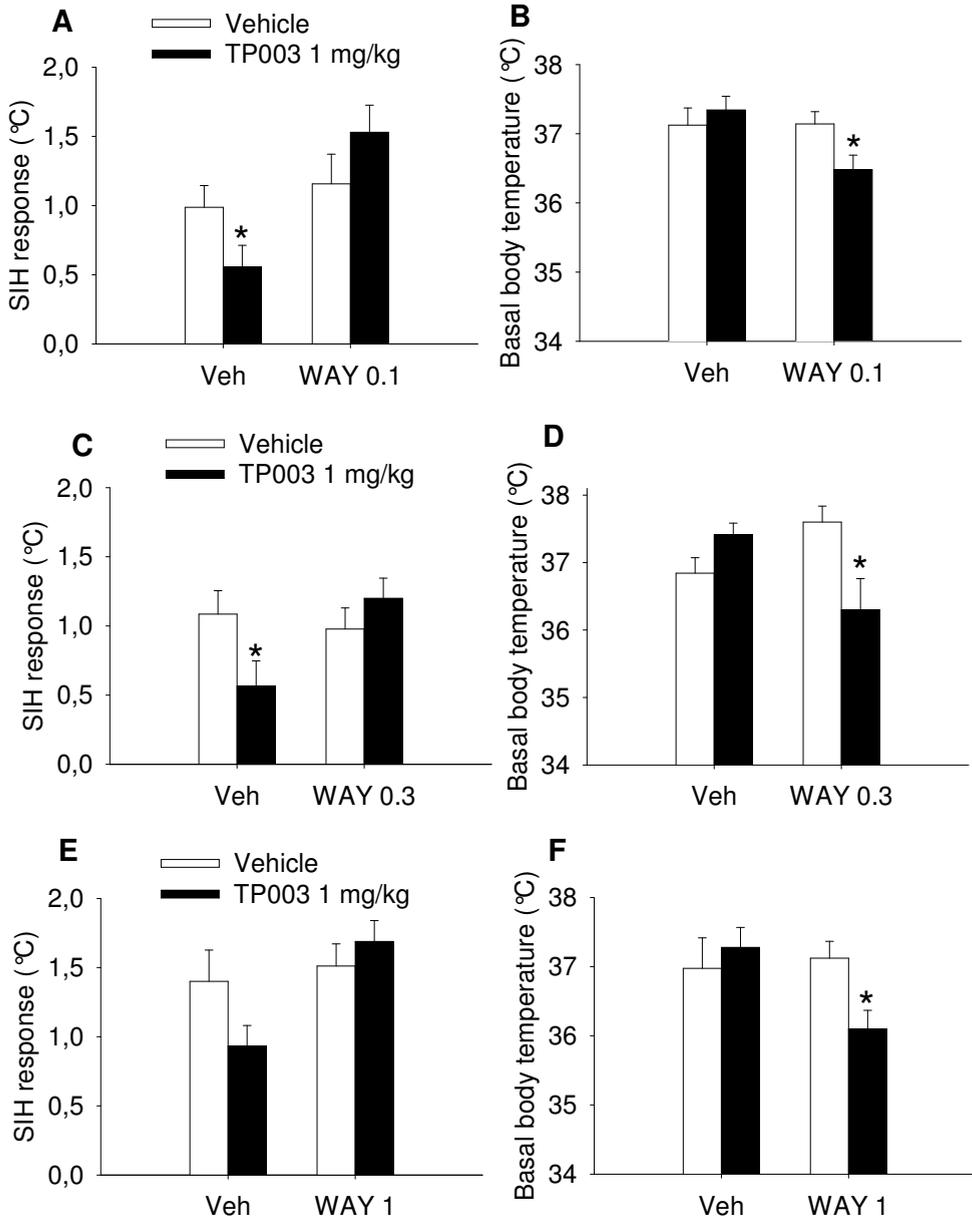
#### SIH response (Figure 3A,C,E)

As shown in figure 3A, C and E, WAY influenced the TP003 effect at all three WAY doses tested (*WAY 0.1 mg/kg*: WAY x TP003 interaction,  $F_{1,30}=4.56$ ,  $p<0.05$ ; *WAY 0.3 mg/kg*: WAY x TP003 interaction,  $F_{1,30}=4.87$ ,  $p<0.05$ ; *WAY 1.0 mg/kg*: WAY x TP003 interaction,  $F_{1,31}=3.38$ ,  $p=0.06$ , trend; WAY effect  $F_{1,31}=6.11$ ,  $p<0.05$ ). Post-hoc analysis showed that in vehicle-treated mice, TP003 reduced the SIH response compared to vehicle-vehicle treated animals in two of the three experiments (*WAY 0.1 mg/kg*: TP003 effect:  $F_{1,16}=4.56$ ,  $p<0.05$ ; *WAY 0.3 mg/kg*: TP003 effect:  $F_{1,15}=4.94$ ,  $p<0.05$ ; *WAY 1 mg/kg*: TP003 effect:  $F_{1,16}=2.87$ ,  $p=0.11$ , NS). In contrast, TP003 did not reduce the SIH response in WAY-treated animals compared to vehicle-WAY treated animals in any experiment (*WAY 0.1 mg/kg*: TP003

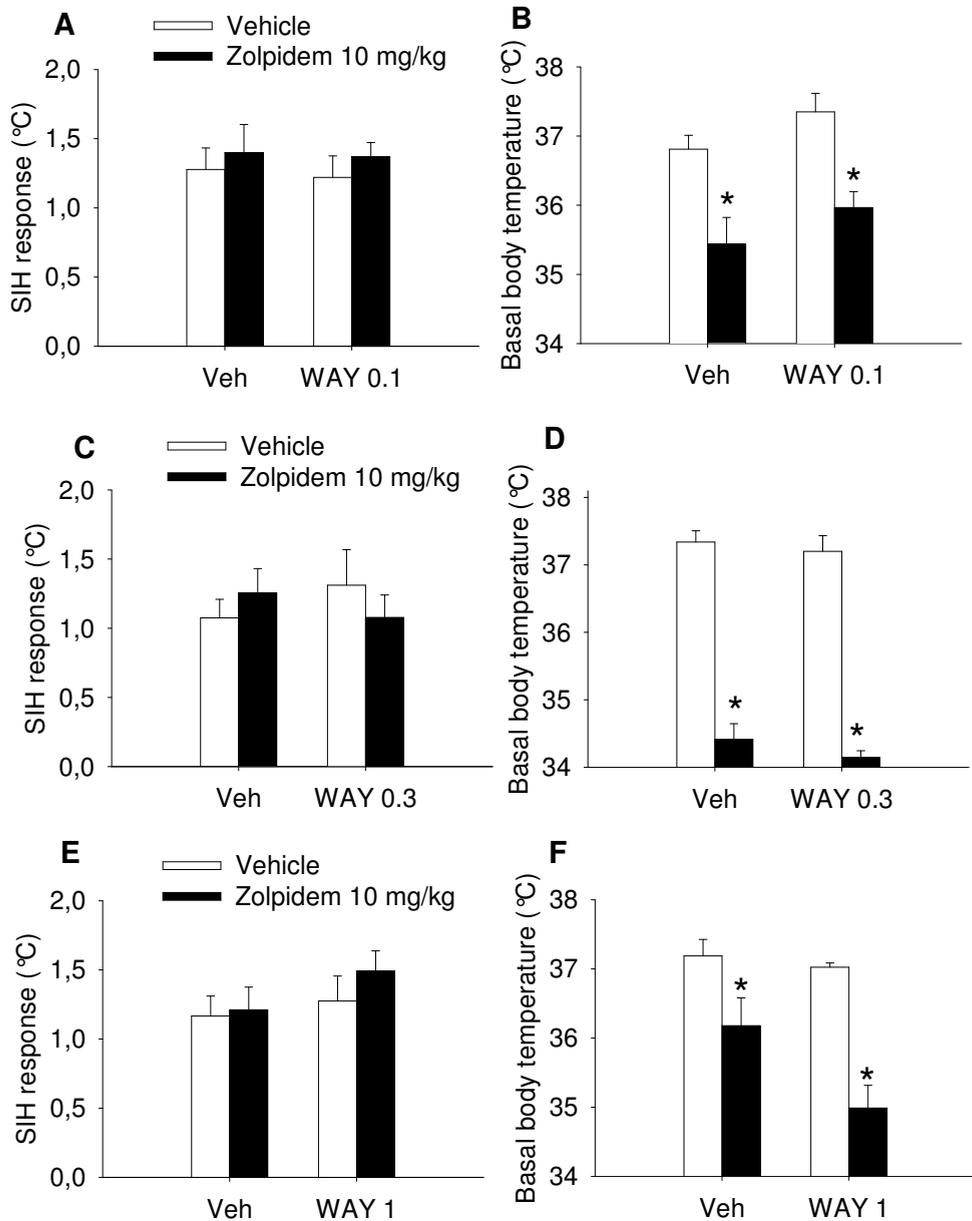
effect:  $F_{1,16}=1.54$ ,  $p=0.23$ , NS; *WAY 0.3 mg/kg*: TP003 effect:  $F_{1,17}=1.11$ ,  $p=0.31$ , NS; *WAY 1 mg/kg*: TP003 effect:  $F_{1,17}=0.65$ ,  $p=0.43$ , NS).

#### Basal body temperature (Figure 3B,D,F)

The effect of TP003 on basal body temperature was influenced by WAY treatment (0.1, 0.3 and 1 mg/kg) (*WAY 0.1 mg/kg*: WAY x TP003 interaction,  $F_{1,30}=4.54$ ,  $p<0.05$ ; *WAY 0.3 mg/kg*: WAY x TP003 interaction,  $F_{1,30}=9.01$ ,  $p<0.01$ ; *WAY 1 mg/kg*: WAY x TP003 interaction,  $F_{1,31}=4.28$ ,  $p<0.05$ ). Post-hoc analysis showed that combined treatment of TP003 with WAY (0.1, 0.3 and 1 mg/kg) resulted in significant hypothermic effects relative to the WAY-vehicle treated animals (*WAY 0.1 mg/kg*: TP003 effect  $F_{1,16}=5.55$ ,  $p<0.05$ ; *WAY 0.3 mg/kg*: TP003 effect  $F_{1,17}=6.27$ ,  $p<0.05$ ; *WAY 1 mg/kg*: TP003 effect:  $F_{1,17}=7.96$ ,  $p=0.01$ ). In contrast, TP003 alone did not reduce the basal body temperature compared to vehicle-vehicle treated animals (*WAY 0.1 mg/kg*: TP003 effect  $F_{1,16}=0.52$ ,  $p=0.48$ , NS; *WAY 0.3 mg/kg*: TP003 effect  $F_{1,15}=3.56$ ,  $p=0.08$ , trend; *WAY 1 mg/kg*: TP003 effect:  $F_{1,16}=0.32$ ,  $p=0.58$ , NS).



**Figure 3:** Effects of WAY-100635 (0.1-1 mg/kg, IP) on stress-induced hyperthermia and basal body temperature response after vehicle or TP003 (1 mg/kg, IP) administration. \*: p<0.05.



**Figure 4:** Effects of WAY-100635 (0.1-1 mg/kg, IP) on stress-induced hyperthermia and basal body temperature response after vehicle or zolpidem (10 mg/kg, IP) administration. \*: p<0.05.

### 3.4 Zolpidem 10 mg/kg and WAY-100635

#### SIH response (Figure 4A,C,E)

Zolpidem did not affect the SIH response in all three experiments (*WAY 0.1 mg/kg*: zolpidem effect,  $F_{1,32}=0.77$ ,  $p=0.39$ , NS; *WAY 0.3 mg/kg*: zolpidem effect,  $F_{1,31}=0.30$ ,  $p=0.87$ , NS; *WAY 1 mg/kg*: zolpidem effect,  $F_{1,32}=0.72$ ,  $p=0.40$ , NS). WAY did not have any effect on the SIH response nor changed the zolpidem effects (*WAY 0.1 mg/kg*: WAY effect,  $F_{1,32}=0.09$ ,  $p=0.77$ , NS; WAY x zolpidem interaction  $F_{1,32}=0.001$ ,  $p=0.94$ , NS; *WAY 0.3 mg/kg*: WAY effect,  $F_{1,31}=0.32$ ,  $p=0.86$ , NS; WAY x zolpidem interaction  $F_{1,31}=1.63$ ,  $p=0.21$ , NS; *WAY 1 mg/kg*: WAY effect,  $F_{1,32}=1.93$ ,  $p=0.17$ , NS; WAY x zolpidem interaction  $F_{1,32}=0.29$ ,  $p=0.59$ , NS).

#### Basal body temperature (Figure 4B,D,F)

Zolpidem reduced basal body temperature in all three experiments (*WAY 0.1 mg/kg*: zolpidem effect,  $F_{1,32}=25.07$ ,  $p<0.001$ ; *WAY 0.3 mg/kg*: zolpidem effect,  $F_{1,31}=136.20$ ,  $p<0.001$ ; *WAY 1 mg/kg*: zolpidem effect,  $F_{1,32}=41.39$ ,  $p<0.001$ ). WAY did not alter zolpidem-induced hypothermia (*WAY 0.1 mg/kg*: WAY x zolpidem interaction,  $F_{1,32}=0.01$ ,  $p=0.98$ , NS; *WAY 0.3 mg/kg*: WAY x zolpidem interaction,  $F_{1,31}=0.38$ ,  $p=0.54$ , NS; *WAY 1 mg/kg*: WAY x zolpidem interaction,  $F_{1,32}=2.28$ ,  $p=0.14$ , NS). WAY had no significant effect on basal body temperature, except for the highest dose tested (*WAY 0.1 mg/kg*: WAY effect,  $F_{1,32}=3.76$ ,  $p=0.11$ , NS; *WAY 0.3 mg/kg*: WAY effect,  $F_{1,31}=1.37$ ,  $p=0.25$ , NS; *WAY 1 mg/kg*: WAY effect,  $F_{1,32}=4.48$ ,  $p<0.05$ ).

## 4. Discussion

The present study investigated the putative GABA-serotonin interactions using the stress-induced hyperthermia (SIH) paradigm. Using a pharmacological approach, we show that the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 reversed the SIH-reducing effects of the non-selective GABA<sub>A</sub> receptor agonist diazepam and the GABA<sub>A</sub> receptor  $\alpha_3$ -selective agonist TP003. In contrast, WAY-100635 did not affect the SIH response when it was combined with the preferential  $\alpha_1$  subunit GABA<sub>A</sub> receptor agonist zolpidem, which was ineffective itself in reducing the SIH response. Moreover, co-administration of WAY-100635 with diazepam or TP003 resulted in augmented hypothermia. As WAY-100635 has no affinity for GABA<sub>A</sub> receptors (Fletcher et al 1996), our data indicate that in the SIH paradigm, anxiolytic effects of GABA<sub>A</sub> receptor agonists may be mediated via the serotonin system. The 5-HT<sub>1A</sub> receptor antagonist WAY-100635 is generally assumed to act as silent antagonist but has also been reported to exert anxiolytic or even anxiogenic effects (Cao and Rodgers 1997; Fletcher et al 1996; Forster et al 1995; Griebel et al 2000b; Groenink et al 1996a; Stanhope and Dourish 1996). In the present study, WAY-100635 never affected the SIH response when it was administered alone, which is in line with earlier studies (Olivier et al 2003; Olivier et al 2008a). Moreover, WAY-100635 has been shown to reverse the SIH-reducing effects of 5-HT<sub>1A</sub> receptor agonists such as buspirone and flesinoxan, confirming that WAY-100635 targets 5-HT<sub>1A</sub> receptors (Iijima et al 2007; Olivier et al 1998).

The main finding of the present study is that the non-selective GABA<sub>A</sub> receptor agonist diazepam and the  $\alpha_3$  subunit selective GABA<sub>A</sub> receptor agonist TP003 no longer reduced the SIH response in the presence of the 5-HT<sub>1A</sub> receptor antagonist WAY-100635, suggesting a direct interaction between GABA<sub>A</sub> receptor  $\alpha_3$  subunits and 5-HT<sub>1A</sub> receptors. Some studies have found decreased serotonin activity and turnover after benzodiazepine administration (Chase et al 1970; Pratt et al 1979; Stein et al 1977; Trulson et al 1982; Wright et al 1992), although others have not found such effects (Shephard et al 1982; Thiebot 1986; Thiebot et al 1984). Also, a reduction in serotonin turnover has been linked to the anxiolytic effects of the classical benzodiazepine chlordiazepoxide on social interaction (Vellucci and File 1979). Our data may indicate that benzodiazepines affect serotonergic signaling via  $\alpha_3$  subunits on a distinct and selective group of serotonergic neurons. In support, the vast majority of serotonergic neurons express GABA<sub>A</sub> receptor  $\alpha_3$ -subunit immunoreactivity but not GABA<sub>A</sub> receptor  $\alpha_1$ -subunit staining (Gao et al 1993). This is remarkable as the  $\alpha_1$  subunit is highly prevalent in the central nervous system. Thus, benzodiazepines could at least partially produce their anxiolytic effects by activating  $\alpha_3$  subunits located on serotonergic neurons. Presynaptically, 5-HT<sub>1A</sub> receptors are located as somatodendritic receptors on serotonergic neurons in the dorsal and median raphe nuclei with ascending projecting throughout the brain. Postsynaptic 5-HT<sub>1A</sub> receptors are present at high density in central cortical and limbic areas. Activation of both receptor types inhibits the firing rate of serotonergic neurons. Blockade of presynaptic 5-HT<sub>1A</sub> receptors using WAY-100635 may disinhibit serotonin release and turnover at synaptic levels (Wesolowska et al 2003), which may then activate postsynaptic 5-HT receptors. This way, WAY-100635 could reverse or block the  $\alpha_3$ -induced inhibitory effects on serotonergic neurons. Electrophysiological studies also show that WAY-100635 increases serotonergic neuronal activity probably by blocking 5-HT<sub>1A</sub> autoreceptors (Corradetti et al 1996; Fornal et al 1996; Mundey et al 1996). Thus, a presynaptic location of  $\alpha_3$  subunits on serotonergic cell with 5-HT autoreceptors seems to be plausible. In support, serotonergic raphe nuclei receive a prominent GABAergic input via distant sources as well as interneurons (Bagdy et al 2000; Gervasoni et al 2000; Harandi et al 1987; Varga et al 2001). However, this can only provide a partial explanation as WAY-100635 also augmented the benzodiazepine-induced hypothermia, putatively via an activation of  $\alpha_1$  subunits (Vinkers et al 2009f). Furthermore, it is striking that WAY-100635 reverses the SIH response while it augments the hypothermia. It may be hypothesized that specialized thermoregulatory GABA<sub>A</sub>-serotonin interactions exist in the hypothalamus, or, alternatively, that dorsal and median raphe projections differentially affect basal and stress-induced body temperature levels. Also, we cannot exclude the possibility that downstream activation of hypothalamic 5-HT<sub>1A</sub> receptors, subsequent to the direct activation of GABA<sub>A</sub> receptors, is needed to maintain the core body temperature. Thus, detailed hypotheses on the potential interactive sites of GABA<sub>A</sub> receptors and 5-HT<sub>1A</sub> receptors and their functional relevance must await further experimental analysis.

The effects of diazepam, TP003 and zolpidem on the SIH response and basal body temperature from our experiments are in line with earlier studies from our group (Olivier et al 2002; Vinkers et al 2009f; Vinkers et al 2008). Classical (non-subunit selective) benzodiazepines all reduce the SIH response and may induce hypothermia (at higher

doses). Classical (non-selective) benzodiazepines allosterically enhance the inhibitory actions of GABA by binding to GABA<sub>A</sub> receptors that contain  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  subunits in combination with a  $\beta$  and a  $\gamma_2$  subunit (Rudolph and Mohler 2006). Recently, genetic and pharmacological evidence has indicated that  $\alpha$  subunits may differentially contribute to the various classical benzodiazepine induced effects such as anxiolysis, dependence, anticonvulsant activity, sedation and amnesia (Crestani et al 2001; Rudolph et al 1999). More specifically, we have shown a putative role for the GABA<sub>A</sub> receptor  $\alpha_1$  subunit in hypothermia and a role for GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonists in anxiety processes (Vinkers et al 2009f). Zolpidem is approximately five- to tenfold more selective for  $\alpha_1$  subunit-containing GABA<sub>A</sub> receptors than  $\alpha_2/\alpha_3$  subunit-containing receptors (Petroski et al 2006), whereas TP003 is  $\alpha_3$  subunit selective with low modulation via  $\alpha_1$ -,  $\alpha_2$ -, and  $\alpha_5$ -containing subtypes (Dias et al 2005). In the present study, we confirm and extend the earlier findings that the GABA<sub>A</sub> receptor  $\alpha_1$  subunit leads to hypothermia, whereas GABA<sub>A</sub> receptor  $\alpha_3$  subunits attenuated the SIH response without affecting basal body temperature levels.

Interestingly, WAY-100635 has also been able to reverse the SIH reduction caused by the mGluR<sub>2/3</sub> receptor antagonist MGS0039, suggesting that the 5-HT<sub>1A</sub> receptors may also be involved in the effects of glutamate receptor antagonists (Iijima et al 2007). In support, MGS0039 increased serotonin release in the medial prefrontal cortex (Kawashima et al 2005). Moreover, 5-HT<sub>1A</sub> receptor antagonists WAY-100635 and NAD-299 attenuated the passive avoidance impairment caused by the muscarinic receptor antagonist scopolamine and the noncompetitive *N*-methyl-D-aspartate receptor antagonist MK-801 in rats, indicating that 5-HT<sub>1A</sub> receptor-muscarinic receptor and 5-HT<sub>1A</sub> receptor-NMDA receptor interactions may also exist, possibly on the level of hippocampal pyramidal cells (Carli et al 1997; Madjid et al 2006; Misane and Ogren 2003). Altogether, these results suggest that functional interactions with the serotonin system are not limited to the GABA<sub>A</sub> system, but may also include glutamatergic, muscarinergic and NMDAergic receptor systems.

In conclusion, the present study shows that 5-HT<sub>1A</sub> receptor blockade reversed the anxiolytic effects of the non-selective GABA<sub>A</sub> receptor agonist diazepam and the  $\alpha_3$ -selective GABA<sub>A</sub> receptor agonist TP003 on the autonomic SIH response and enhanced TP003- and diazepam-induced hypothermia. In contrast, these effects were not present in combination with the selective GABA<sub>A</sub> receptor  $\alpha_1$  subunit agonist zolpidem. Our data suggest that the GABA<sub>A</sub>ergic system functionally interacts with the serotonergic system to cause its anxiolytic effects.

# Chapter 5

## **Differential tolerance after chronic treatment with GABA<sub>A</sub> receptor $\alpha$ -subunit selective compounds**

Christiaan H. Vinkers

Naheed R. Mirza

Henrik H. Hansen

Ruud van Oorschot

James M. Cook

Shengming Huang

Terry Clayton Sr.

Sundari Rallapalli

Lucianne Groenink

S. Mechiel Korte

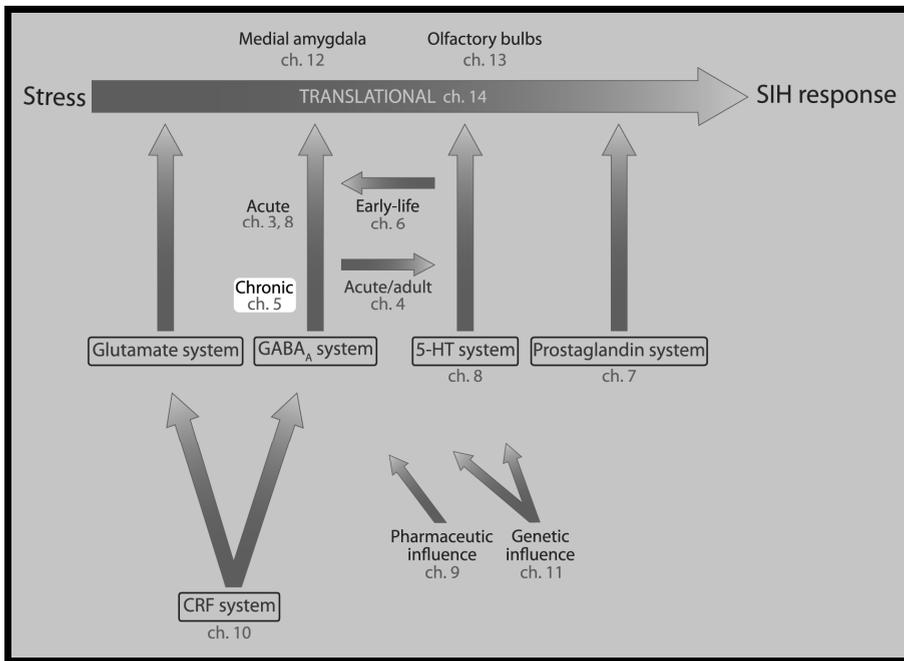
Cor J. Kalkman

Berend Olivier

5

*Submitted*

## Abstract



**Background:** A major disadvantage of chronic benzodiazepine treatment is the development of tolerance to their effects. The mechanisms underlying this loss of efficacy are little understood. The GABA<sub>A</sub> receptor  $\alpha_5$  subunit has already been shown to be pivotal in the development of tolerance to the sedative effects of diazepam. An important issue is whether  $\alpha$ -subtype selectivity curtails tolerance development. As distinct GABA<sub>A</sub> receptor  $\alpha$  subtypes generate the different benzodiazepine effects, the question also remains whether tolerance to acute benzodiazepine effects is mediated by the same GABA<sub>A</sub> receptor subtype that is involved in the acute effect or whether more or specific  $\alpha$  subunits are involved in the development of tolerance. Therefore, the present study aimed to investigate the effects of chronic treatment with non-subunit selective (classical) benzodiazepines as well as subunit-selective GABAergic drugs on the development of tolerance to the acute anxiolytic and hypothermic effects of diazepam.

**Methods:** Chronic 28-day treatment was initiated with osmotic minipumps containing the non-selective GABA<sub>A</sub> receptor agonist diazepam, the preferential  $\alpha_1$  subunit selective GABA<sub>A</sub> receptor agonist zolpidem, the  $\alpha_{2/3}$  subunit selective agonist TPA023 and the  $\alpha_5$  subunit selective GABA<sub>A</sub> receptor agonist SH-053-2F'-R-CH<sub>3</sub>. Each week, acute diazepam (5 mg/kg, IP) or vehicle were administered using the stress-induced hyperthermia paradigm. Also, body weight was monitored and locomotor behavior was tested after 28 days.

**Results:** During chronic 28-day treatment with the classical benzodiazepine diazepam, rapid tolerance to the acute hypothermic and anxiolytic effects of acutely administered diazepam developed. Such tolerance also developed after chronic treatment with the preferential  $\alpha_1$  subunit selective GABA<sub>A</sub> receptor agonist zolpidem although at a much slower pace compared to the chronic effects of diazepam. In contrast, no tolerance developed to the acute diazepam effects after chronic treatment with the  $\alpha_{2/3}$  subunit selective TPA023 or the  $\alpha_5$  subunit selective GABA<sub>A</sub> receptor agonist SH-053-2F'-R-CH<sub>3</sub>. In support, tolerance to the acute motor-depressant effects of diazepam was only present after chronic diazepam treatment but not after chronic treatment with zolpidem, TPA023 and SH-053-2F'-R-CH<sub>3</sub>. Moreover, body weight increases were associated with the chronic use of diazepam and SH-053-2F'-R-CH<sub>3</sub>.

**Conclusions:** Our results suggest that a rapidly developing tolerance depends on a combined activation of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  subunits, whereas chronic excitation of the  $\alpha_1$  subunit may result in slowly developing tolerance. In contrast, chronic selective activation of either GABA<sub>A</sub> receptor  $\alpha_{2/3}$  or  $\alpha_5$  subunits does not produce any tolerance. Thus, a combined activation of  $\alpha$  subunits over a prolonged period may give rise to rapid tolerance to the anxiolytic and hypothermic effects of classical benzodiazepines. If the chronic activation of  $\alpha_{2/3}$  subunits does not result in any tolerance, this indicates that  $\alpha_{2/3}$ -subtype selective GABAergic compounds constitute a promising class of anxiolytic drugs which may possess a more favourable side effect profile.

## 1. Introduction

Anxiety disorders are disabling disorders that are commonly treated with selective serotonin reuptake inhibitors or benzodiazepines (Nutt 2005). Although classical benzodiazepines have proven to be of great value in acute anxiety states, their use is associated with a variety of side effects that include physical dependence, tolerance, sedation, cognitive and motor impairment and severe withdrawal symptoms (Cloos and Ferreira 2009). Classical (non-selective) benzodiazepines allosterically enhance the inhibitory actions of GABA by binding to GABA<sub>A</sub> receptors that contain  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  subunits in combination with a  $\beta$  and a  $\gamma_2$  subunit (Sieghart and Sperk 2002). Recently, genetic and pharmacological evidence has indicated that  $\alpha$  subunits may differentially contribute to the effects of benzodiazepines (Crestani et al 2001; Rudolph et al 1999). More specifically, the  $\alpha_1$  subunit is thought to mediate the sedative, hypnotic, amnesic and partly anticonvulsant actions of benzodiazepines (McKernan et al 2000; Rudolph et al 1999), whereas  $\alpha_2$  and  $\alpha_3$  subunits probably mediate the anxiolytic and myorelaxant action of benzodiazepines (Atack et al 2005; Crestani et al 2001; Dias et al 2005; Low et al 2000). There is evidence that activation of the  $\alpha_5$  subunit is involved in benzodiazepine-induced cognitive side effects (Collinson et al 2002; Dawson et al 2006), sedation (Savic et al 2008) and tolerance (van Rijnsoever et al 2004). Despite its complexity, the concept that distinct GABA<sub>A</sub> receptor  $\alpha$  subtypes generate the various benzodiazepine effects is valuable from the perspective of developing novel therapeutic agents. In line with a specific function for each GABA<sub>A</sub> receptor  $\alpha$  subunit, the search for a drug that separates the anxiolytic effects from the other side effects has focused on  $\alpha_{2/3}$  subunit-selective GABA<sub>A</sub> receptor agonists (Crawforth et al 2004; Dias et al 2005; Mirza et al 2008).

A major disadvantage of chronic benzodiazepine treatment is the development of tolerance to their effects. The mechanisms underlying this loss of efficacy are poorly understood, but have been ascribed to the development of adaptation at the level of the GABA<sub>A</sub> receptor. Using  $\alpha$  point-mutation mice, the GABA<sub>A</sub> receptor  $\alpha_5$  subunit has already been shown to be pivotal in the development of tolerance to the sedative effects of diazepam (van Rijnsoever et al 2004). However, studies on putative changes in GABA<sub>A</sub> receptor subunit mRNA levels in response to chronic treatment are heterogeneous and suggested to be both treatment- and region-specific (Wafford 2005). A reduction of the allosteric coupling between the GABA and benzodiazepine binding site may also occur, providing an alternative explanation for the development of tolerance (Ali and Olsen 2001).

An important question is whether  $\alpha$ -subtype selectivity curtails tolerance development. For example, even though sedation is mediated through the  $\alpha_1$  subunit,  $\alpha_5$  subunits play a role in the development of tolerance to  $\alpha_1$ -mediated sedative effects (van Rijnsoever et al 2004). Also, as distinct GABA<sub>A</sub> receptor  $\alpha$  subtypes mediate the different benzodiazepine effects, the question remains whether tolerance to acute benzodiazepine effects is mediated by the same GABA<sub>A</sub> receptor subtype that is involved in the acute effect or whether more or specific  $\alpha$  subunits are involved in the development of tolerance. Therefore, the present study aimed to investigate the contributions of different GABA<sub>A</sub> receptor  $\alpha$  subunits on the development of tolerance to the acute anxiolytic and

hypothermic effects of diazepam. The tools in this study include the non-selective GABA<sub>A</sub> receptor agonist diazepam, the preferential  $\alpha_1$  subunit selective GABA<sub>A</sub> receptor agonist zolpidem (Petroski et al 2006), the  $\alpha_{2/3}$  subunit selective TPA023 (Atack et al 2006) as well as the  $\alpha_5$  subunit selective GABA<sub>A</sub> receptor agonist SH-053-2F'-R-CH<sub>3</sub> (Savic et al 2008). To repeatedly measure the acute anxiolytic and hypothermic potential of diazepam during the different chronic treatments, the stress-induced hyperthermia (SIH) paradigm was used (Vinkers et al 2008). The SIH response is the transient rise in body temperature in response to a stressor that is mediated by the autonomic nervous system which can be reduced using clinically effective anxiolytics including benzodiazepines (Bouwknicht et al 2007). The SIH response is robust and can be repeatedly reduced with anxiolytic drugs using a 1-week interval, and this makes the SIH paradigm very suitable to monitor developing tolerance to acute benzodiazepine effects.

## 2. Material and methods

### 2.1 Animals

Male 129Sv/EvTac mice (Taconic, M and B, Denmark) were group-housed at constant room temperature ( $21 \pm 2^\circ\text{C}$ ) and relative humidity (50%–60%) with PVC tubing as cage enrichment. Standard rodent food pellets (Special Diet Services, Witham, Essex, United Kingdom) and water were freely available. Mice were maintained on a 12-hour light–dark cycle (lights on at 6 AM). One week after arrival, osmotic minipumps were implanted. After recovery from surgery, mice were group-housed. All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Ethical Committee for Animal research of the Faculties of Sciences, Utrecht University, The Netherlands.

### 2.2 Drugs

Diazepam (base) and zolpidem (tartaric acid) were obtained from Sigma Aldrich. SH-053-2F'-R-CH<sub>3</sub> (the (R) stereoisomer of 8-ethynyl-4-methyl-6-phenyl-4H-2,5,10b-triazabenz[e]azulene-3-carboxylic acid ethyl ester) was synthesized by the laboratory of Dr. J.M. Cook (University of Wisconsin-Milwaukee, USA). TPA023 (7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine) was synthesized according to published methods (Carling et al 2005). An injection volume of 10 ml/kg was used for acute intraperitoneal injections of diazepam. A fresh diazepam suspension in gelatin-mannitol 0.5%/5% was prepared on each testing day.

### 2.3 Surgery

Mice were anaesthetized, and osmotic minipumps (Type 2004, Alzet, USA) delivering either vehicle or drug (in 95% PEG-400, 2.5% alcohol 100% and 2.5% alcohol 70%; Sigma) were subcutaneously (SC) implanted in the dorsal thoracic area. Minipumps were weekly checked.

**Table 1:** Overview of chronic and acute treatments in Experiment I. SC: subcutaneous. IP: intraperitoneal.

<b>Chronic (10 mg/kg.day, osmotic minipump, SC)</b>	<b>Acute (day 1,7,14,21,28)</b>
Vehicle	Vehicle
Vehicle	Diazepam (5 mg/kg, IP)
Diazepam	Vehicle
Diazepam	Diazepam (5 mg/kg, IP)

## 2.4 Experiment I: establishing tolerance after chronic diazepam exposure (Table 1)

Mice were implanted with osmotic minipumps containing either vehicle (n=20) or diazepam (10 mg/kg/day, n=20) (day 0). Starting the day after implantation (day 1), mice acutely received either vehicle or diazepam (5 mg/kg, IP) and were tested once per week in the stress-induced hyperthermia (SIH) paradigm. Tolerance was defined as the inability of acutely administered diazepam to affect either the SIH response or basal body temperature over time. Body weight was measured each week which was adjusted for the presence of the osmotic minipump. After the last testing day (day 28), mice that had received acute vehicle throughout the weeks (n=20) were decapitated; brains were removed and stored at -80°C until further use.

**Table 2:** Chronic and acute treatments in Experiment II. SC : subcutaneous. IP: intraperitoneal.

<b>Chronic (15 mg/kg/day, osmotic minipump, SC)</b>	<b>Acts on GABA<sub>A</sub> receptor</b>	<b>Acute (day 1,7,14,21,28)</b>
Vehicle	-	Vehicle
Vehicle	-	Diazepam (5 mg/kg, IP)
Diazepam	$\alpha_{1/2/3/5}$	Vehicle
Diazepam	$\alpha_{1/2/3/5}$	Diazepam (5 mg/kg, IP)
Zolpidem	$\alpha_1$	Vehicle
Zolpidem	$\alpha_1$	Diazepam (5 mg/kg, IP)
TPA023	$\alpha_{2/3}$	Vehicle
TPA023	$\alpha_{2/3}$	Diazepam (5 mg/kg, IP)
SH-053-2'F-R-CH3	$\alpha_5$	Vehicle
SH-053-2'F-R-CH3	$\alpha_5$	Diazepam (5 mg/kg, IP)

## 2.5 Experiment II: contributions of GABA<sub>A</sub> receptor $\alpha$ subunits on tolerance after chronic treatment (Table 2)

Mice were implanted with osmotic minipumps containing vehicle, diazepam, zolpidem, TPA023 or SH-053-2F-R-CH<sub>3</sub> (15 mg/kg/day, n=20/drug) (day 0). A higher chronic dose was chosen to study the putative increased tolerance onset, and identical doses for the different chronic treatments were used. Starting the day after implantation (day 1), mice acutely received either vehicle or diazepam and were tested once per week in the stress-induced hyperthermia (SIH) paradigm. Tolerance was defined as the inability of acutely administered diazepam to affect either the SIH response or basal body temperature over time. Body weight was measured each week which was adjusted for the presence of the osmotic minipump. After the last SIH testing day (day 28), mice that had received acute vehicle throughout the weeks, n=60 were decapitated; brains were removed and stored at -80°C until further use. The other mice that had received acute injections of diazepam each week (n=57) were tested in the open field.

## 2.6 Behavioral procedures

### Stress-induced hyperthermia (SIH) procedure

The SIH procedure was carried out according to standard procedures (Groenink et al 2009). Briefly, animals (n=10-13) were injected with vehicle or diazepam (5 mg/kg, IP) 60 min before the first temperature measurement ( $T_1$ ). The temperature was again measured 10 min later ( $T_2$ ), representing the stress-induced body temperature. The SIH response was calculated by subtracting  $T_1$  from  $T_2$ . A within-subject design was used, and cages were randomly and evenly allocated over daytimes (morning–afternoon). The body temperature of mice was measured by rectally inserting a thermistor probe by a length of 2 cm. Digital temperature recordings were obtained with an accuracy of 0.1 °C using a Keithley 871A digital thermometer (NiCr– NiAl thermocouple). The probe, dipped into silicon oil before inserting, was held in the rectum until a stable rectal temperature was obtained.

### Open field test

Animals receiving different chronic treatments (n=7-10) were tested in the open field during day 29-31 after osmotic minipump surgery. Animals received a diazepam (5 mg/kg, IP) or vehicle injection 60 minutes prior to the open field test. Each animal was placed in the center of the open field and allowed to explore for 5 min. The open field was dimly lit (5 lux) at the bottom of the box ( $\varnothing$  20 cm). Animals were tracked using an automatic tracking system (TSE ActiMot V7.01 (TSE systems GmbH, Bad Homburg, Germany)). Animals were tested using a within-subject design (latin-square design), with at least 1 day in between the subsequent open field tests.

## 2.7 Data analysis

For SIH experiments, a basal temperature ( $T_1$ ), an end temperature ( $T_2$ ) and the difference (SIH response =  $T_2 - T_1$ ) was determined for each individual mouse. Treatment effects on the SIH response, basal body temperature ( $T_1$ ) and body weight were evaluated using a repeated-measures analysis of variance with explanatory factors time (week) as within-subject factor, and acute treatment and chronic treatment as between-subject factors. If

indicated, post-hoc comparisons (Bonferroni *t*-test) were made. Chronic treatment effects were subsequently separately analyzed using a one-way ANOVA with acute treatment as between-subject factor and time as within-subject factor and compared to vehicle. Acute treatment effects were separately analyzed using a one-way ANOVA with chronic treatment as between-subject factor and time as within-subject factor. Time effects were subsequently separately analyzed using a two-way ANOVA with post-hoc comparisons for each week. The open field test was analyzed using a two-way ANOVA with chronic and acute treatment as between-subject factors. A probability level of  $p < 0.05$  was set as statistically significant, probability levels between  $p = 0.05$  and  $p = 0.1$  were regarded as trends. All data are displayed as mean  $\pm$  S.E.M..

### 3. Results

#### 3.1 Experiment I: establishing tolerance after chronic diazepam exposure

##### SIH response (Figure 1)

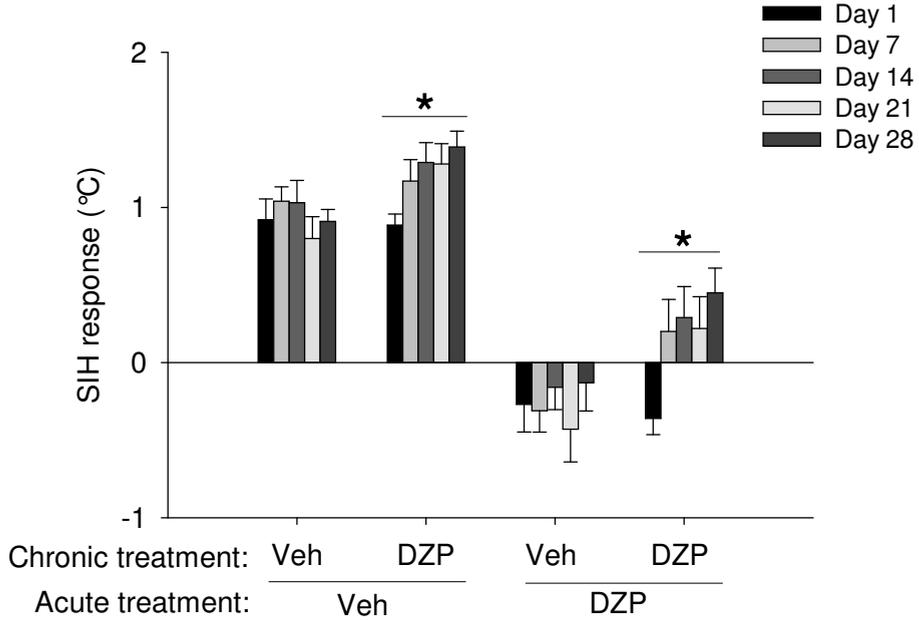
Acute diazepam administration overall reduced the SIH response ( $F_{1,36} = 144.04$ ,  $p < 0.001$ ) which was not different over the weeks (acute treatment  $\times$  time interaction  $F_{4,144} = 0.42$ ,  $p = 0.79$ , NS). Chronic diazepam treatment increased the SIH response over time (chronic treatment  $\times$  time interaction  $F_{4,144} = 3.61$ ,  $p < 0.01$ ) regardless of acute treatment (acute treatment  $\times$  chronic treatment  $\times$  time interaction  $F_{4,144} = 0.84$ ,  $p = 0.84$ , NS; acute treatment  $\times$  chronic treatment interaction  $F_{1,36} = 0.70$ ,  $p = 0.41$ , NS). Separate analysis of the different days showed that from day 7 on, acute diazepam treatment had significantly less effect in mice chronically treated with diazepam than in vehicle treated mice (day 1 ( $F_{1,39} = 0.23$ ,  $p = 0.63$ , NS); day 7 ( $F_{1,39} = 4.59$ ,  $p < 0.05$ ); day 14 ( $F_{1,39} = 5.17$ ,  $p < 0.05$ ); day 21 ( $F_{1,39} = 10.36$ ,  $p < 0.01$ ) and day 28 ( $F_{1,39} = 15.08$ ,  $p < 0.001$ )).

##### Basal body temperature (Figure 2)

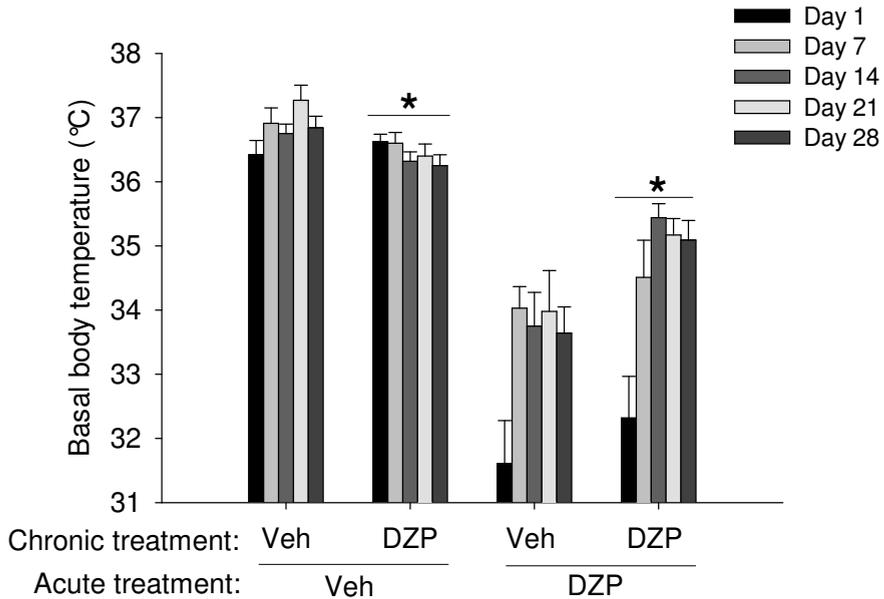
Chronic diazepam altered the hypothermic effects of acute diazepam (acute treatment  $\times$  chronic treatment  $F_{1,36} = 9.68$ ,  $p = 0.004$ ). This effect was overall not different over the different weeks (time  $\times$  acute treatment  $\times$  chronic treatment  $F_{4,144} = 1.60$ ,  $p = 0.18$ , NS). Separate analysis of the acute treatments showed that after acute vehicle administration, chronic diazepam treatment decreased basal body temperature (chronic treatment  $\times$  time interaction  $F_{4,72} = 4.78$ ,  $p < 0.01$ ), whereas after acute diazepam administration, basal body temperature increased (chronic treatment  $F_{1,18} = 6.40$ ,  $p < 0.05$ ).

##### Body weight (Figure 6A)

Body weight increased over the weeks (time effect  $F_{4,152} = 44.25$ ,  $p < 0.001$ ) and was larger in the chronic diazepam group (time  $\times$  chronic treatment interaction  $F_{4,152} = 4.60$ ,  $p < 0.01$ ). Separate analysis of the different weeks revealed a chronic diazepam treatment effect at day 14 ( $F_{1,39} = 3.98$ ,  $p < 0.05$ ), day 21 ( $F_{1,39} = 3.98$ ,  $p = 0.07$ , trend) and day 28 ( $F_{1,39} = 5.96$ ,  $p = 0.02$ ).



**Figure 1:** Effect of acute vehicle or diazepam (5 mg/kg, IP) administration on the SIH response 60 minutes later during chronic treatment with diazepam (10 mg/kg/day) or vehicle. \*:  $p < 0.05$ . DZP: diazepam; Veh: vehicle.



**Figure 2:** Effect of acute vehicle or diazepam (5 mg/kg, IP) administration on basal body temperature 60 minutes later during chronic treatment with diazepam (10 mg/kg/day) or vehicle. \*:  $p < 0.05$ . DZP: diazepam; Veh: vehicle.

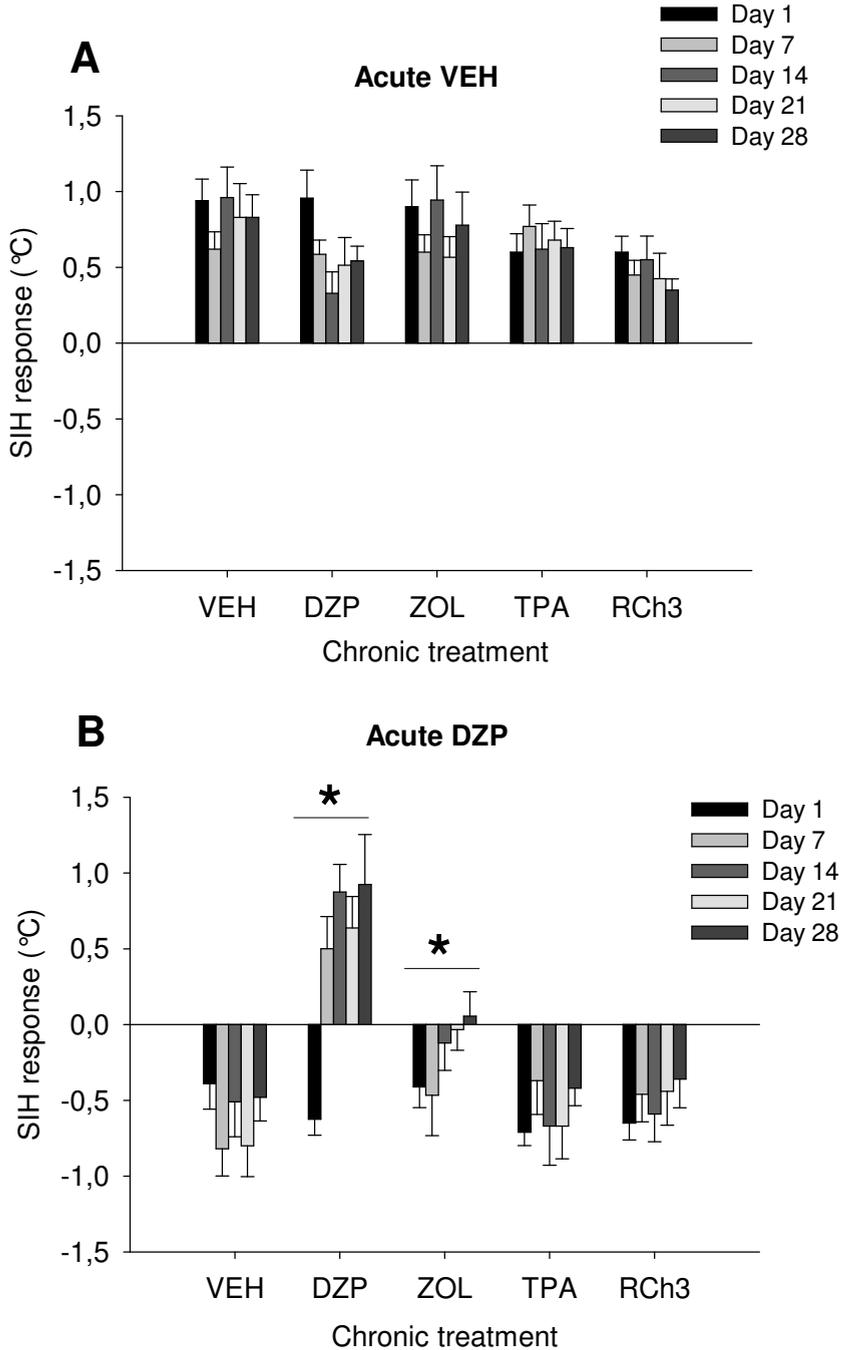
### 3.2 Experiment II: contributions of GABA<sub>A</sub> receptor $\alpha$ subunits on tolerance after chronic treatment

#### SIH response (Figure 3)

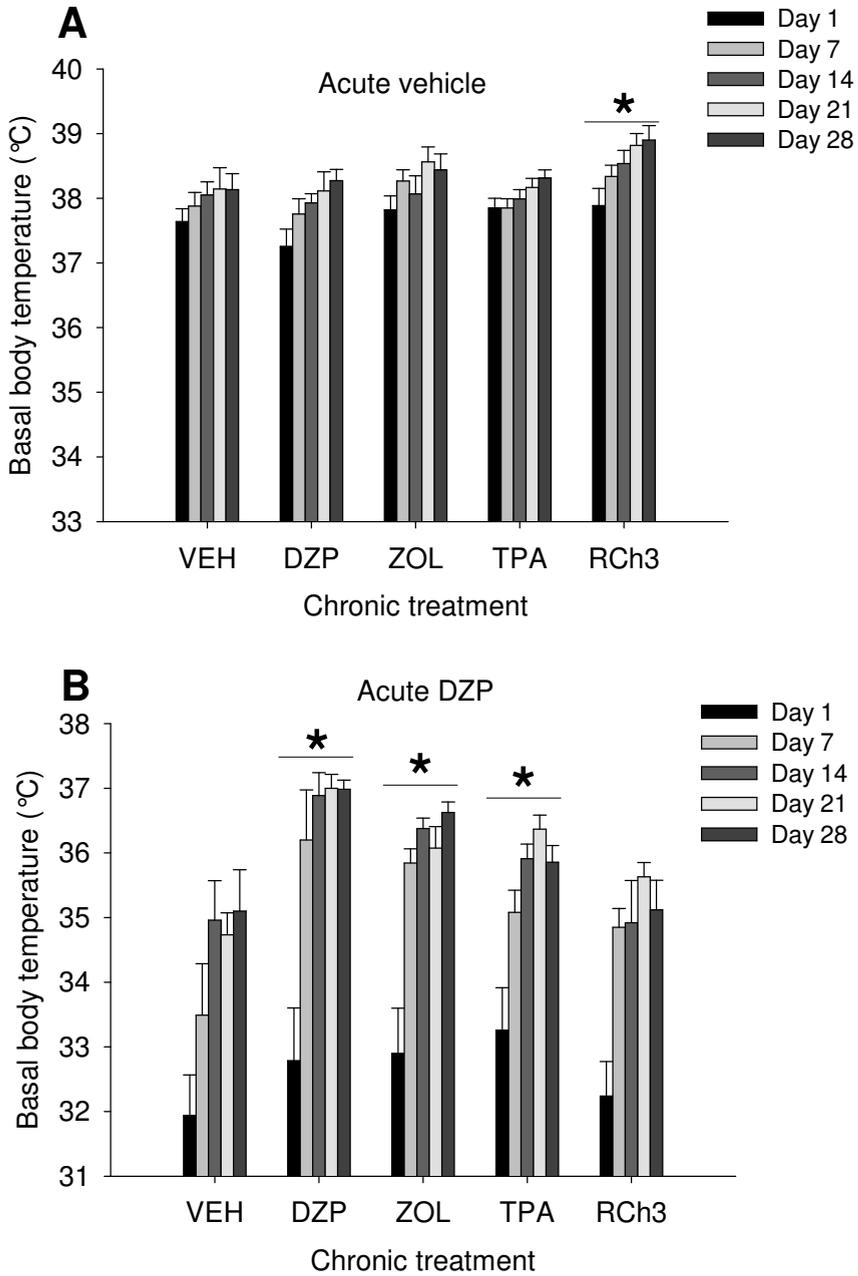
Tolerance to acute diazepam-induced reduction in the SIH response was clearly dependent on the chronic treatment mice were receiving via osmotic minipumps (chronic treatment x acute treatment interaction  $F_{4,81}=8.15$ ,  $p<0.001$ ). Further, this differential tolerance was also dependent upon time (time x chronic treatment x acute treatment interaction  $F_{16,324}=2.65$ ,  $p<0.001$ ). Separate analysis of the different chronic treatments indicated that compared to vehicle, tolerance occurred in the chronic diazepam (time x chronic x acute interaction  $F_{4,124}=7.66$ ,  $p<0.001$ ) and chronic zolpidem groups (chronic x acute interaction  $F_{1,34}=4.66$ ,  $p<0.05$ ; time x chronic x acute  $F_{4,136}=1.63$ ,  $p=0.17$ ). In contrast, compared to vehicle, no tolerance occurred after chronic TPA023 treatment (time x chronic x acute interaction  $F_{4,144}=0.69$ ,  $p=0.60$ ; chronic x acute interaction  $F_{1,36}=0.78$ ,  $p=0.38$ ) or chronic SH-053-2F'-R-CH<sub>3</sub> treatment (time x chronic x acute  $F_{4,136}=0.91$ ,  $p=0.46$ ; chronic x acute  $F_{1,34}=3.81$ ,  $p=0.08$ ).

Separate analysis of the different days showed that acute diazepam effects were present in all weeks, but that chronic treatment affected the SIH response at day 7 (chronic x acute  $F_{4,90}=3.78$ ,  $p<0.05$ ), day 14 (chronic x acute  $F_{4,90}=7.16$ ,  $p<0.01$ ), day 21 (chronic x acute  $F_{4,90}=6.52$ ,  $p<0.01$ ) and day 28 (chronic x acute  $F_{4,90}=3.64$ ,  $p<0.01$ ). In contrast, no effects of chronic treatment were present during day 1 (chronic x acute  $F_{4,90}=0.39$ ,  $p=0.82$ , NS).

Separate analysis of the acute treatments revealed that chronic treatment did not affect the SIH response in acutely vehicle-treated animals (time x chronic treatment interaction  $F_{16,156}=1.31$ ,  $p=0.21$ , NS; chronic treatment effect  $F_{4,39}=1.89$ ,  $p=0.13$ , NS), whereas chronic treatment did alter the acute diazepam effects (time x chronic treatment interaction  $F_{16,168}=2.59$ ,  $p<0.001$ ; chronic treatment  $F_{4,42}=11.25$ ,  $p<0.001$ ). Separate analysis of the different chronic treatments in the acute diazepam-treated groups showed that chronic diazepam (time x chronic treatment interaction  $F_{4,64}=7.21$ ,  $p<0.001$ ) and zolpidem treatment (chronic treatment effect  $F_{1,17}=5.62$ ,  $p<0.05$ ; time x chronic treatment interaction  $F_{4,68}=1.52$ ,  $p=0.21$ ), but not chronic TPA023 (time x chronic treatment interaction  $F_{4,72}=1.46$ ,  $p=0.22$ ; chronic treatment effect  $F_{1,18}=0.04$ , 0.84) nor chronic SH-053-2F'-R-CH<sub>3</sub> treatment (time x chronic treatment interaction  $F_{4,72}=1.51$ ,  $p=0.21$ ; chronic treatment effect  $F_{1,18}=0.34$ ,  $p=0.57$ ) induced tolerance to the acute SIH-reducing effects of diazepam.



**Figure 3:** Effect of acute vehicle or diazepam (5 mg/kg, IP) administration on the SIH response 60 minutes later during different chronic benzodiazepine treatments (vehicle or 15 mg/kg/day). \*:  $p < 0.05$  compared to the chronic vehicle treatment. VEH: vehicle; DZP: diazepam; ZOL: zolpidem; TPA: TPA023; RCh3: SH-053-2F'-R-CH<sub>3</sub>.



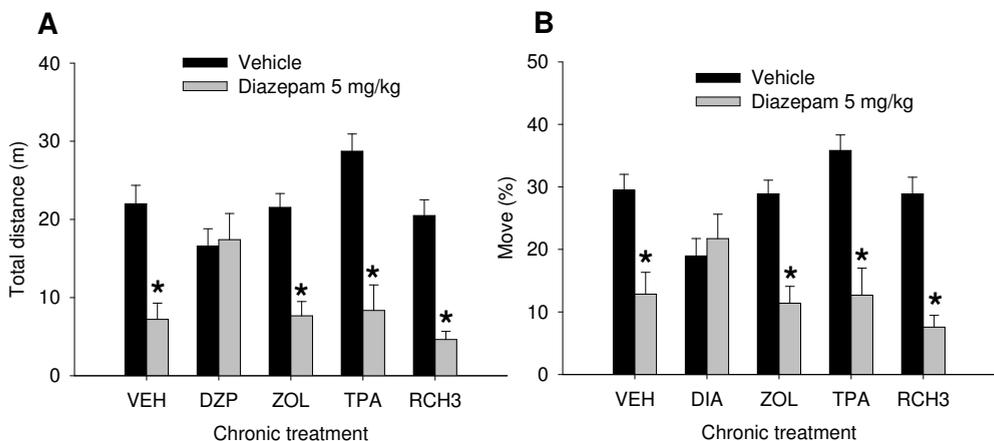
**Figure 4:** Effect of acute vehicle or diazepam (5 mg/kg, IP) administration on basal body temperature 60 minutes later during different chronic benzodiazepine treatments. \*:  $p < 0.05$  compared to the chronic vehicle treatment. VEH: vehicle; DZP: diazepam; ZOL: zolpidem; TPA: TPA023; RCh3: SH-053-2F'-R-CH<sub>3</sub>.

**Basal body temperature (Figure 4)**

The different chronic treatments affected the acute effects of diazepam on basal body temperature (chronic treatment x acute treatment interaction  $F_{4,81}=7.01$ ,  $p<0.001$ ), although these did not develop over time (time x chronic treatment x acute treatment interaction  $F_{16,324}=0.57$ ,  $p=0.88$ , NS). Separate analysis of the chronic treatments showed that, compared to vehicle, chronic diazepam (chronic x acute treatment interaction  $F_{1,31}=12.09$ ,  $p<0.01$ ), chronic zolpidem (chronic x acute treatment interaction  $F_{1,34}=5.15$ ,  $p<0.05$ ), chronic TPA023 (chronic x acute treatment interaction  $F_{1,36}=4.48$ ,  $p<0.05$ ) but not chronic SH-053-2F'-R-CH<sub>3</sub> (chronic x acute treatment interaction  $F_{1,34}=0.04$ ,  $p=0.84$ , NS) induced tolerance to the acute diazepam effects on basal body temperature.

The acute hypothermic effects of diazepam differed over the days (acute treatment x time interaction  $F_{4,324}=28.64$ ,  $p<0.001$ ). Separate analysis revealed that acute vehicle treatment altered basal body temperature levels dependent on chronic treatment (chronic treatment effect  $F_{4,39}=2.75$ ,  $p<0.05$ ), although this effect was not different over the weeks (time x chronic treatment interaction  $F_{16,156}=0.74$ ,  $p=0.75$ , NS). Post-hoc analysis showed that only chronic SH-053-2F'-R-CH<sub>3</sub> treatment (chronic treatment  $F_{1,16}=4.25$ ,  $p=0.05$ ) increased basal body temperature levels. In contrast, no effects on basal body temperature were present after chronic diazepam (chronic treatment  $F_{1,15}=0.15$ ,  $p=0.70$ ), zolpidem (chronic treatment  $F_{1,17}=0.99$ ,  $p=0.33$ ) or TPA023 (chronic treatment  $F_{1,17}=0.11$ ,  $p=0.74$ ).

Chronic treatment did affect acute diazepam-induced hypothermia (chronic treatment  $F_{4,42}=7.09$ ,  $p<0.001$ ). Separate analysis of the chronic treatment groups showed that chronic diazepam (chronic treatment  $F_{1,16}=14.69$ ,  $p=0.001$ ), chronic zolpidem (chronic treatment  $F_{1,17}=9.34$ ,  $p<0.01$ ), chronic TPA023 (chronic treatment  $F_{1,18}=6.23$ ,  $p<0.05$ ) but not chronic SH-053-2F'-R-CH<sub>3</sub> (chronic treatment  $F_{1,18}=0.77$ ,  $p=0.39$ ) resulted in tolerance to the acute hypothermic effects of diazepam.



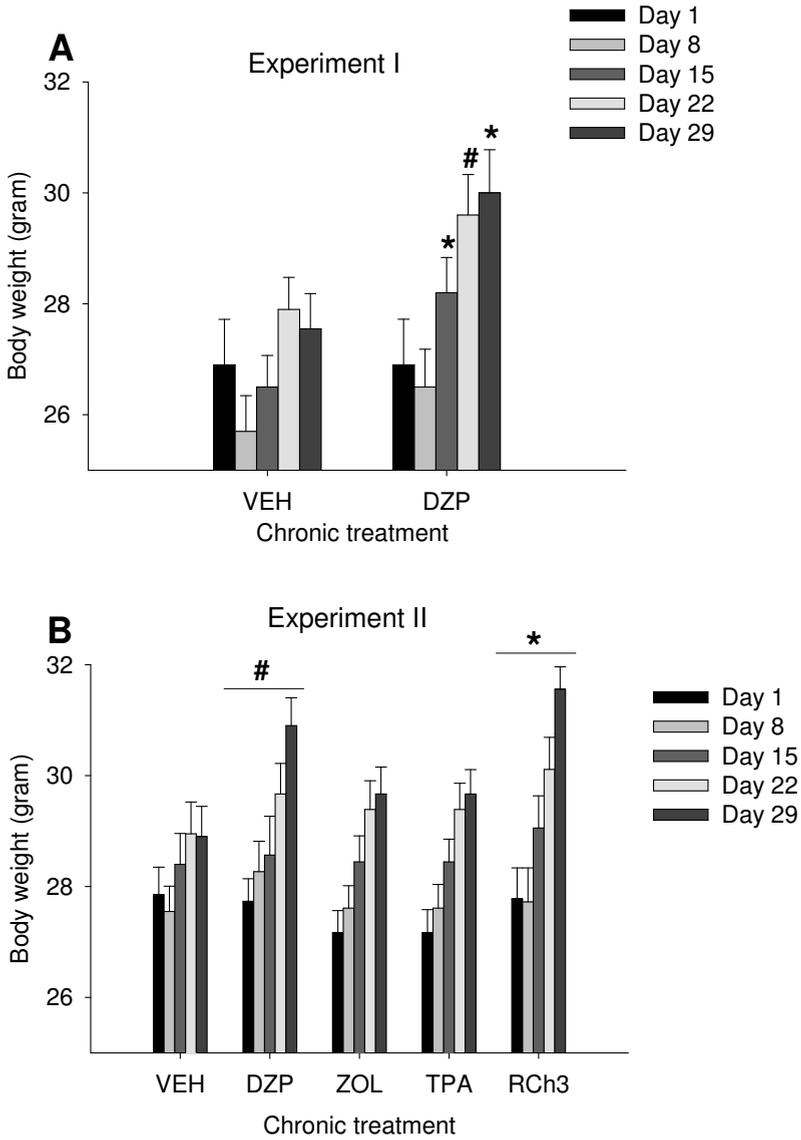
**Figure 5:** Effects of acute diazepam administration (5 mg/kg, IP) after 28 days of different chronic GABA<sub>A</sub> receptor treatments on total distance travelled (A) and percentage of time moved (B) in the open field. VEH: vehicle; DZP: diazepam; ZOL: zolpidem; TPA: TPA023; RCH3: SH-053-2F'-R-CH<sub>3</sub>.

### Open field test (Figure 5)

The acute effects of diazepam were dependent on the chronic treatment (total distance travelled: acute diazepam effect x chronic treatment interaction  $F_{4,40}=4.12$ ,  $p<0.01$ ; distance moved in center: acute diazepam effect x chronic treatment interaction  $F_{4,40}=3.64$ ,  $p=0.01$ ; percentage of time moved: acute diazepam effect x chronic treatment interaction  $F_{4,40}=4.48$ ,  $p<0.01$ ). Post-hoc analysis of the different chronic treatment groups showed tolerance to the acute locomotor sedative effects of diazepam in the chronic diazepam treatment group (acute x chronic treatment interaction  $F_{1,15}=10.80$ ,  $p<0.01$ ), but not in any other chronic treatment groups (zolpidem: acute x chronic treatment interaction  $F_{1,17}=0.06$ ,  $p=0.82$ ; TPA023: acute x chronic treatment interaction  $F_{1,17}=0.85$ ,  $p=0.37$ ; SH-053-2F'-R-CH<sub>3</sub>: acute x chronic treatment interaction  $F_{1,18}=0.13$ ,  $p=0.72$ ).

### Body weight (Figure 6B)

Body weight increased over the weeks (time effect  $F_{4,344}=123.58$ ,  $p<0.001$ ), which was dependent on chronic benzodiazepine treatment (time x chronic treatment interaction  $F_{16,344}=4.00$ ,  $p<0.001$ ). Post-hoc comparisons showed that this effect was attributable to differences between the vehicle and SH-053-2F'-R-CH<sub>3</sub> group ( $p<0.01$ ) as well as between the vehicle and diazepam group ( $p=0.07$ , trend).



**Figure 6:** Body weight during chronic diazepam (Experiment I, A) and different chronic benzodiazepine treatments (Experiment II, B). \*:  $p < 0.05$  compared to vehicle; #:  $p < 0.1$  compared to vehicle. VEH: vehicle; DZP: diazepam; ZOL: zolpidem; TPA: TPA023; RCh3: SH-053-2F'-R-CH<sub>3</sub>.

## 4. Discussion

The mechanisms underlying the development of tolerance after chronic benzodiazepine exposure are little understood. Therefore, the present study aimed to investigate the effects of chronic treatment on the development of tolerance to the acute anxiolytic and hypothermic effects of diazepam using a non-subunit selective (classical) benzodiazepine as well as  $\alpha$ -subunit selective GABA<sub>A</sub> receptor agonists. Here, we report that during chronic 28-day treatment with the classical benzodiazepine diazepam, rapid tolerance to the acute hypothermic and anxiolytic effects of acutely administered diazepam developed. Such tolerance also developed after chronic treatment with the preferential  $\alpha_1$  subunit selective GABA<sub>A</sub> receptor agonist zolpidem (Petroski et al 2006) although at a much slower pace compared to the chronic effects of diazepam. In contrast, no tolerance developed to the acute diazepam effects after chronic treatment with the  $\alpha_{2/3}$  subunit selective TPA023 (Atack et al 2006) or the  $\alpha_5$  subunit selective GABA<sub>A</sub> receptor agonist SH-053-2F'-R-CH<sub>3</sub> (Savic et al 2008). In support, tolerance to the acute motor-depressant effects of diazepam was only present after chronic diazepam treatment but not after chronic treatment with zolpidem, TPA023 and SH-053-2F'-R-CH<sub>3</sub>. Our results thus suggest that a rapidly developing tolerance depends on a combined activation of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  subunits, whereas chronic excitation of the  $\alpha_1$  subunit may result in slowly developing tolerance. In contrast, chronic stimulation of either GABA<sub>A</sub> receptor  $\alpha_{2/3}$  or  $\alpha_5$  subunits does not produce any tolerance.

GABA<sub>A</sub> receptors are inhibitory ligand-gated ion channels which are localized throughout the central nervous system. Classical benzodiazepines bind to GABA<sub>A</sub>-receptor containing  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and/or  $\alpha_5$  subunits while binding affinity to  $\alpha_4$ - and  $\alpha_6$ -containing subunits is extremely weak (Rudolph and Mohler 2004). Besides the preferred anxiolytic or anticonvulsant action, the use of benzodiazepines is associated with physical dependence, tolerance, sedation, amnesia, motor impairment and withdrawal symptoms (Cloos and Ferreira 2009). The GABA<sub>A</sub> receptor consists of five subunits that can assemble in different combinations of subunits ( $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\pi$ ) to form GABA<sub>A</sub> receptors with specific functional and pharmacological properties (Rudolph and Mohler 2006). The distinct regional and neuronal expression of GABA<sub>A</sub> receptor subtypes indicates a differentiated neuronal functionality. The contributions of the separate GABA<sub>A</sub> receptor subunits have been extensively analyzed in knock-in mice containing point-mutated subunits (Rudolph and Mohler 2004). Also, the development of subtype-specific ligands has resulted in increasing insight into the role of the various GABA<sub>A</sub> receptor subunits (Whiting 2006). These genetic and pharmacological tools have indicated that  $\alpha$  subunits differentially contribute to the various classical benzodiazepine effects with sedative, hypnotic, amnesic and partly anticonvulsant actions of the  $\alpha_1$  subunit (McKernan et al 2000; Rudolph et al 1999), anxiolytic effects of  $\alpha_2$  and/or  $\alpha_3$  subunits (Atack et al 2005; Dias et al 2005; Low et al 2000) and  $\alpha_5$  subunit involvement in cognition and memory (Collinson et al 2002; Dawson et al 2006) and sedation (Savic et al 2008). Recently, we found that GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit activation reduced the stress-induced hyperthermia (SIH) response, whereas the  $\alpha_1$  subunit activation produced hypothermia (Vinkers et al 2009f). The fact that in the present study, diazepam rapidly lost both its SIH-reducing and hypothermic effects after chronic treatment with diazepam but not TPA023

and zolpidem suggests that the development of tolerance is not mediated by the same GABA<sub>A</sub> receptor subtype that is involved in the acute effect. Moreover, higher diazepam doses during chronic treatment in experiment II resulted in more rapid and higher tolerance compared to experiment I, indicating that the choice of the benzodiazepine dose may greatly affect the development of possible tolerance.

Our results confirm and extend the important earlier finding that a concurrent activation of the  $\alpha_5$  subunit is pivotal for the manifestation of tolerance to the sedative diazepam effects, even though this effect is mediated by  $\alpha_1$  subunits (van Rijnsoever et al 2004). In support, decreased  $\alpha_5$  subunit binding in the dentate gyrus depended on the chronic activation of  $\alpha_1$  subunits. Also, our data are in line with earlier evidence that ligands which do not bind to the GABA<sub>A</sub> receptor  $\alpha_5$  subunit such as zolpidem have less but not absent propensity to result in tolerance (Ravishankar and Carnwath 1998; Zammit 2009). Importantly, these compounds fail to alter  $\alpha_5$  subunit levels (Holt et al 1997).

An important question is whether developing tolerance leads to adaptations in the expression and functionality of the GABA<sub>A</sub> receptor system. A variety of preclinical studies has assessed the effects of chronic benzodiazepine treatment on GABA<sub>A</sub> receptor subunit mRNA levels, yielding inconsistent results. For example, there are studies that report no changes in  $\alpha_1$  subunit mRNA expression after chronic benzodiazepine treatment (Fahey et al 1999; Fahey et al 2006; Ramsey-Williams and Carter 1996), whereas others report decreases (Heninger et al 1990; Impagnatiello et al 1996; Kang and Miller 1991; Tietz et al 1993). Similar heterogeneity exists in reports on other GABA<sub>A</sub> receptor subunits (Auta et al 2008; Pesold et al 1997; Tietz et al 1999; Wu et al 1994; Zhao et al 1994). Thus, putative changes in GABA<sub>A</sub> receptor subunit mRNA levels are both treatment- and region-specific and may also depend on the method to quantify mRNA levels. Moreover, the length and procedure of chronic treatment seems pivotal as differences in GABA<sub>A</sub> receptor subunit mRNA levels after chronic diazepam treatment in rats depended on the choice for either manual daily injections or osmotic minipumps (Arnot et al 2001). In support, tolerance to the acute anxiolytic diazepam effects in the elevated plus maze was dependent on the treatment regimen, even though both treatments resulted in tolerance to diazepam's sedative effects (Fernandes et al 1999). Overall, a definitive conclusion regarding the effects of chronic benzodiazepine exposure on GABA<sub>A</sub> receptor subunit levels is complex as heterogeneous results have been obtained. Alternatively, a reduced allosteric interaction between the benzodiazepine and GABA binding site may play a role in tolerance development (Ali and Olsen 2001; Hutchinson et al 1996; Primus et al 1996). This way, chronic benzodiazepine treatment reduces the acute benzodiazepine-induced GABA potentiation. Chronic exposure to benzodiazepines may also lead to compensatory changes distal to the GABA<sub>A</sub> receptor such as second messenger systems. Using point-mutated mice, chronic activation of the  $\alpha_1$  subunit was found to alter downstream signal transduction pathways, including persistent changes in calcium/calmodulin-dependent protein kinase II (Huopaniemi et al 2004).

Interestingly, we report that the non-subunit selective agonist diazepam as well as the  $\alpha_5$  subunit-selective agonist SH-053-2F'-R-CH<sub>3</sub> both resulted in increases in body weight. This suggests that the GABA<sub>A</sub> receptor  $\alpha_5$  subunit may be closely involved in body weight

regulation. The GABA system is important in the modulation of feeding behavior and energy balance within the central nervous system (Cooper and Estall 1985). Specifically, the ventromedial hypothalamus (Kimura and Kuriyama 1975) and amygdala (Minano et al 1992) may be involved. Our data are supported by earlier studies in which benzodiazepines increase food intake (Cooper and Estall 1985; Haney et al 1997) although the evidence is inconsistent (Blasi 2000; Grimm and Jancourt 1983; Oswald and Adam 1980). The reasons for these discrepancies are unclear but may be ascribed to the route of administration, the treatment duration, the applied doses and the animal species.

In conclusion, the present study shows that rapid benzodiazepine tolerance develops during the chronic combined activation of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  GABA<sub>A</sub> receptor subunits, whereas chronic  $\alpha_1$  subunit stimulation results in a slowly developing tolerance. In contrast, chronic activation of the GABA<sub>A</sub> receptor  $\alpha_{2/3}$  or  $\alpha_5$  subunit does not cause any tolerance. Thus, a combined activation of  $\alpha$  subunits over a prolonged period may give rise to rapid tolerance to the anxiolytic, hypothermic and sedative effects of classical benzodiazepines. If the chronic activation of  $\alpha_{2/3}$  subunits does not result in any tolerance, this indicates that  $\alpha_{2/3}$ -subtype selective GABAergic compounds constitute a promising class of anxiolytic drugs which may possess a more favourable side effect profile.

## Chapter 6

# Early-life blockade of 5-HT<sub>1A</sub> receptors alters adult anxiety behaviour and benzodiazepine sensitivity

Christiaan H. Vinkers

Ronald S. Oosting

Meg J. Van Bogaert

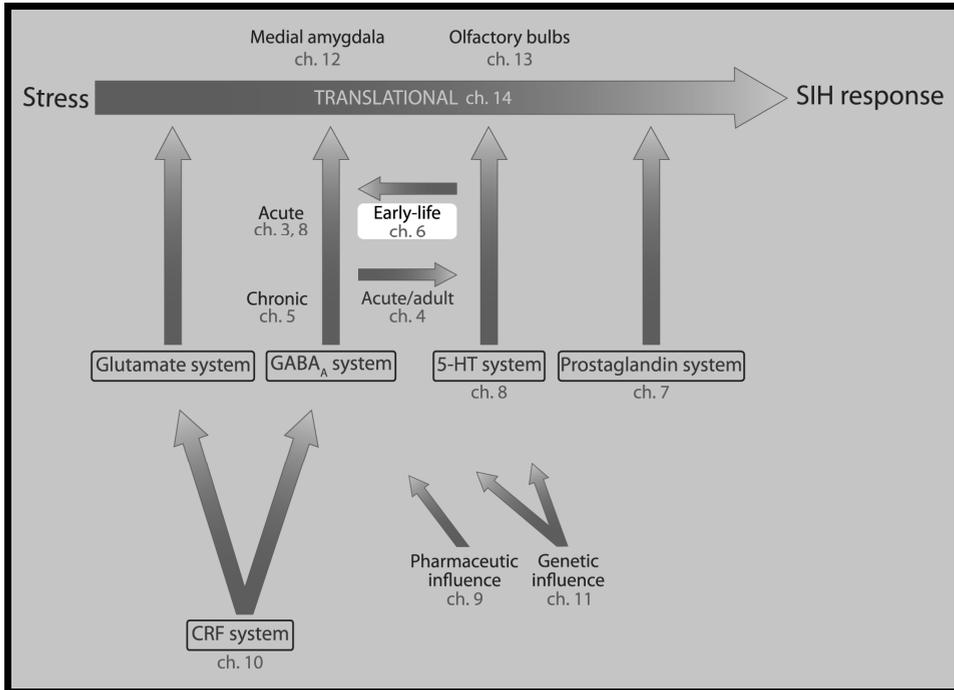
Berend Olivier

Lucianne Groenink

6

*Biological Psychiatry, in press*

## Abstract



**Background:** Early-life stress may affect 5-HT<sub>1A</sub> receptor circuitry which could result in increased anxiety in later life. An increased anxiety phenotype in 5-HT<sub>1A</sub> receptor KO mice (1AKO) mice has been ascribed to 5-HT<sub>1A</sub> receptor absence during the early postnatal period. Thus, subtle and transient serotonergic changes during the early postnatal period may lead to an increased risk to develop stress-related disorders during adulthood.

**Methods:** Wildtype and 1AKO mice on a Swiss-Webster (SW) background were treated during the early postnatal period with vehicle or the 5-HT<sub>1A</sub> receptor antagonist WAY-100635.

**Results:** Pharmacological 5-HT<sub>1A</sub> receptor blockade during the early postnatal period induced long-lasting effects on anxiety and benzodiazepine sensitivity in adolescent and adult mice on a Swiss-Webster background and resembles the SW 1AKO phenotype. Furthermore, WAY-100635-treated mice had increased cortical GABA<sub>A</sub> receptor  $\alpha_1$  and  $\alpha_3$  subunit levels and increased hippocampal GABA<sub>A</sub> receptor  $\alpha_2$  subunit levels.

**Conclusion:** Absence of 5-HT<sub>1A</sub> receptor signalling during early stages of brain maturation predisposes an organism to affective dysfunction later in life. Because early-life treatment with WAY-100635 in Swiss-Webster mice reduced diazepam sensitivity and increased GABA<sub>A</sub> receptor  $\alpha$  subunit levels in the prefrontal cortex and hippocampus, our data suggest a putative link between early-life disruption of the serotonergic system and the emergence of increased anxiety and decreased benzodiazepine responsiveness at adult age. Moreover, early-life 5-HT<sub>1A</sub> receptor functionality appears to be essential for the development of normal GABA<sub>A</sub> receptor functionality. The present study could therefore have clinical implications for psychoactive drug use during pregnancy as well as for the pharmacogenetic background of benzodiazepine sensitivity.

## 1. Introduction

Early life adversity is associated with an increased risk to develop abnormal emotional behavior later in life, including major depressive disorder and anxiety (Heim and Nemeroff 2001). Therefore, stress exposure during the postnatal period may have long-term consequences in terms of stress responsivity and emotionality (Mirescu et al 2004). As early-life stress affects 5-HT<sub>1A</sub> receptor circuitry and postnatal 5-HT<sub>1A</sub> receptor expression, postnatal disruption of the serotonergic system and increased risk to develop stress-related disorders later in life may be linked (Borella et al 1997; Gaspar et al 2003; Vazquez et al 2002). 5-HT<sub>1A</sub> receptor knockout mice (1AKO) indeed display increased anxiety levels (Heisler et al 1998; Parks et al 1998; Ramboz et al 1998). This increased anxious phenotype in 1AKO mice has been ascribed to 5-HT<sub>1A</sub> receptor absence during the early postnatal period (Gross et al 2002; Tsetsenis et al 2007). Using pharmacological 5-HT<sub>1A</sub> receptor blockade, the early postnatal period was also shown to be critical for anxiety behavior at adult age in serotonin-transporter knockout mice (Alexandre et al 2006). Thus, subtle and transient serotonergic changes during the postnatal period may be sufficient to disrupt neuronal development and cause dysfunctional emotional brain pathways at adult age. Here, we hypothesize that pharmacological 5-HT<sub>1A</sub> receptor blockade during early postnatal development will result in increased emotional behavior at adult age. In the present study, we thus inhibited the 5-HT<sub>1A</sub> receptor from P0 until P21 with the 5-HT<sub>1A</sub> receptor antagonist WAY-100635, and we compared the effects over time with vehicle-treated wildtype and vehicle-treated 1AKO mice.

The anxious phenotype and reduced benzodiazepine sensitivity of 1AKO mice on a Swiss-Webster background may result from altered GABA<sub>A</sub> receptor  $\alpha$  subunit expression (Sibille et al 2000). However, the fact that benzodiazepine insensitivity of 1AKO mice depends on the genetic background of the mouse strain (being absent in 1AKO mice on the 129Sv and C57Bl6/J genetic background), complicates the hypothesis that a postnatal disruption of 5-HT<sub>1A</sub> receptor function may influence GABAergic pathways in later life and cause reduced benzodiazepine sensitivity (Bailey and Toth 2004; Toth 2003; Van Bogaert et al 2006a). Therefore, the present study aimed to investigate the putative link between early-life 5-HT<sub>1A</sub> receptor disruption and increased anxiety levels as well as decreased benzodiazepine responsivity at adult age which are also present in various stress-related psychiatric disorders.

## 2. Materials and Methods

### 2.1 Animals

Groups of male homozygote 1AKO and wildtype mice on a Swiss-Webster (SW) background were bred within the animal facilities (GDL, Utrecht, The Netherlands). Swiss Webster mice were used in all experiments as 1AKO mice on the SW genetic background have consistently shown to be less sensitive to the anxiolytic effects of classical benzodiazepines (Bailey and Toth 2004; Pattij et al 2002b; Sibille et al 2000; Van Bogaert et al 2006a). Breeding founders were obtained from M. Toth (Cornell University, NY, USA)

and were crossbred with commercially available SW mice (Taconic, M and B, Denmark). This crossbreeding resulted in heterozygote F<sub>1</sub> generations, which were used to breed homozygote 5-HT<sub>1A</sub> receptor knockout and wildtype generations (F<sub>2</sub>). These F<sub>2</sub> generations were then used to breed the homozygote mice for this experiment. Male wildtype (WAY-100635 and vehicle treated) and 1AKO mice were used for measuring the effects of chronic WAY-100635 treatment during postnatal development (10-12 mice per group). Animals were housed under a 12-light/12-dark cycle (lights on from 0600-1800h) at controlled room temperature (20±2°C) and relative humidity (40-60%). Testing was done during the light phase and all experiments were approved by the ethical committee of the Faculty of Science, Utrecht University, The Netherlands (DEC).

## 2.2 Drugs

The 5-HT<sub>1A</sub> receptor antagonist WAY-100635 (N-{2-[4-(2-methoxy)-1-piperazinyl]ethyl}-N-(2-pyridinyl) cyclohexanecarboxamide tri-chloride; Wyeth Research, Taplow, UK) was dissolved in 0.9% saline (vehicle). Diazepam base (Brunschwig Chemie B.V., Amsterdam, The Netherlands) was suspended in 0.5% gelatin/5% mannitol (vehicle). Flesinoxan hydrochloride (Solvay Pharmaceuticals, Weesp, Netherlands) was dissolved in 0.9% saline. All drugs were injected subcutaneously except for flesinoxan (intraperitoneally). Drugs were prepared freshly every day and injected in a volume of 10 ml/kg.

## 2.3 General procedure

Nursing females were injected twice daily with WAY-100635 (3 mg/kg, 4 wildtype females) or 0.9% saline (4 wildtype females and 4 1AKO females). These injections started on the day the pups were born (P0) and continued until P21. Injections were given at 9:00 AM and 5:00 PM. Starting on P7, additional WAY-100635 (1 mg/kg) or saline injections were given to the mouse pups at 9:00 AM. This resulted in three groups of 10-12 male mouse pups: wildtype control mice (WT), wildtype WAY-100635-treated mice and 1AKO control mice (1AKO).

***Establishing sufficient WAY-100635 levels in pups.*** To determine whether WAY-100635 was passed on into mother milk in quantities high enough to block 5-HT<sub>1A</sub> receptors in pups, a separate group of WAY-100635 treated mouse pups was tested with 5-HT<sub>1A</sub>R agonist flesinoxan between 1:00 and 2:00 PM in the USV paradigm at P7.

***Acute effects of WAY-100635 on USV.*** To examine if WAY-100635 had any acute effects on USV, WAY-100635 (0-1 mg/kg) was administered to a separate group of 7-day-old wildtype mouse pups (n=10 per dose) in the ultrasonic pup vocalization test.

## 2.4 Behavioral procedures

**Determination of the effects of chronic WAY-100635 on anxiety behavior.** The groups of WT-vehicle, WT-WAY-100635 and 1AKO-vehicle mice were tested in the ultrasonic pup vocalization test at P7. At P21, they were tested in the elevated plus maze. Subsequently, mice were group-housed and allowed to age until 12 weeks of age. At this age, the mice were tested in the open field test.

**Ultrasonic pup vocalization (USV) test.** USV testing was carried out as described (Groenink 2009). Briefly, the USV apparatus consisted of an aluminium plate (Ø 19 cm) kept at a temperature of 19°C, a round transparent Plexiglas chamber (Ø 20 cm, 25 cm high) that was placed on the plate and covered with a Plexiglas top on which a microphone (SM2, Ultra Sound Advice, UK) was mounted. The microphone was connected to a bat detector (S-25, Ultra Sound Advice, UK), set at 80 kHz, which was consequently connected to an audiofilter (Noldus Inc), which translated the analogue signals into digital block pulses (Ultravox 2.0, Noldus Inc., Wageningen, The Netherlands). Total number, total duration and mean duration of ultrasounds during a 5 min test were registered.

**Elevated Plus Maze (EPM).** The EPM (black Plexiglas floor and walls) was elevated 100 cm and consisted of two open and two closed arms (30×5 cm). All arms radiated from a common centre platform (5×5 cm). Measurements started by placing a mouse on the centre platform, facing an open arm (Groenink et al 2003a). The animals were tested during between 9:00 and 14:00 under red light (5 lux). Behavior was scored using a fully automated custom-made tracking system (Ethovision®, Noldus, Wageningen, The Netherlands).

**Open field test.** The test started by placing the mice in the centre of the open field (Ø 75 cm, grey Perspex floor, height 35 cm) as described earlier (Groenink et al 2003a). The open field was divided into three regions, outer ring, inner ring and centre (Ø 25 cm). The experimental room was dimly lit by a red light. Five-minute sessions were recorded and subsequently analyzed by a blinded observer (Observer® system; Noldus Inc., Wageningen, The Netherlands).

**Determination of the effects of chronic WAY-100635 on benzodiazepine sensitivity.** The effects of diazepam (0-6 mg/kg) on the SIH response were measured using a within-subject design when animals were 3-4 weeks old. At adult age, sensitivity to diazepam (0-6 mg/kg) and flesinoxan (0-2 mg/kg) in the SIH paradigm was tested. All drug doses were counterbalanced using a Latin square design. Adolescent diazepam sensitivity was tested after the EPM test during weeks 3 and 4, and adult diazepam and flesinoxan sensitivity was tested after the open field test during weeks 13-15 of age. Consecutive SIH procedures were carried out with at least 5 days in between, as repeated daily SIH

measurements have shown to increase basal body temperature (Van der Heyden et al 1997).

**Stress-induced Hyperthermia (SIH) test.** The SIH test was carried out as previously described (Van der Heyden et al 1997). Briefly, animals were housed individually on the afternoon before the testing day. On the experimental day, mice were injected with either drug or vehicle 60 minutes before a first rectal temperature measurement ( $T_1$ , basal body temperature) and a second rectal temperature measurement 10 minutes later ( $T_2$ , stress-induced body temperature). The stress-induced hyperthermia (SIH) response was calculated ( $\Delta T = T_2 - T_1$ ). All animals received all drug doses using a randomised within animal design. Body temperature was measured by manual fixation and insertion a thermistor probe of 2 cm in length into the rectum (Digital Thermometer, Type 971A, Tegam Inc., Geneva Ohio, USA)

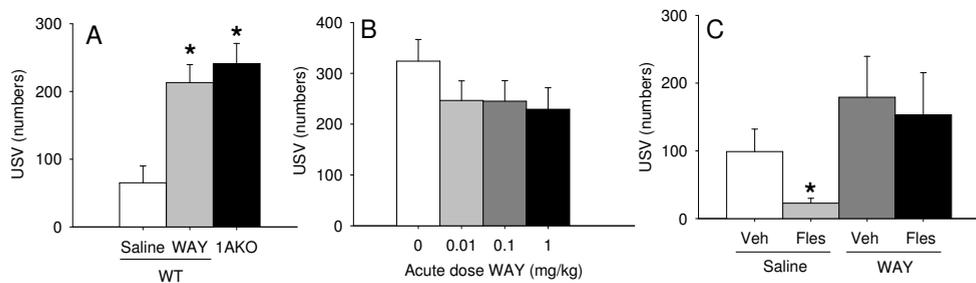
**Quantitative PCR analysis.** Levels of GABA<sub>A</sub>R subunits and 5-HT<sub>1A</sub> receptor mRNA were obtained 2 weeks after the final experiments. Mice were decapitated, brains were removed, transferred in 5 ml RNA later (Ambion) and stored at 4°C for 48 hours. Subsequently, brains were stored at -20°C until further use. After thawing, the tissue samples were sliced in 0.5 mm thick sections using a Mcllwain tissue chopper. The prefrontal cortex, hippocampus and amygdala were dissected under a binocular microscope. Then excess RNA later was removed and RNA was isolated from the sample using the GenElute RNA isolation kit (Sigma). All procedures were performed on ice and in a cooled microcentrifuge. Tissue samples were homogenized by passing 10 times through a 22G syringe. The OD<sub>260nm</sub> to OD<sub>280nm</sub> ratio (1.8-2) was used to assess the purity of the isolated RNA. In a total of 20 µl, 0.5 µg RNA was reverse transcribed using the enhanced avian first strand synthesis kit (Sigma), 0.5 units enhanced avian reverse transcriptase were used per reaction. To prevent RNA degradation, 1 µl Superase-In (Ambion) was added. The RT reaction was performed at 45°C for 2h. A real time PCR reaction was performed on 0.1 to 0.5 µl of the cDNA samples in the presence of 0.1 µM of each of the gene specific primers and the Sybr Green I dye in a total volume of 20 µl. The absolute QPCR sybr Green kit of ABgene (Epson, UK) was used. To determine the efficiency of a given PCR reaction, real time PCR reactions were performed on five 2-fold dilutions of a mixture of the obtained cDNA samples. The following protocol was used for all PCR reactions: 15 at min 95°C, followed by 40 cycles consisting of 15 s at 95°C and 60 s at 60°C. To ensure that a new developed PCR reaction resulted in the expected product, the size of the product was checked by agarose electrophoresis. Primers were developed using the Primer Express software (Applied Biosystems). The following sequences were used (FP, RP): GAPDH (GCACCCTGCATTATTTTGCA, CTTCCAGGAGCGAGACCCCA), GABA<sub>A</sub>R α1 (CCACACCCCATCAATAGTTCT, AATTCTCGGTGCAGAGGACTGAA), GABA<sub>A</sub>R α2 (GGGACGGGAAGAGTGTAGTCAA, CCGCATAGGCGTTGTTCTGT), GABA<sub>A</sub>R α3 (GCCCACTGAAGTTTGAAGCTAT, CATCCTGTGCTACTTCCACAGATT) and the 5-HT<sub>1A</sub> receptor (CCTCTATGGGCGCATCTTCA, GTGCCCGCTCCCTTCTT).

**Statistics.** USV, EPM, open field experiments and mRNA levels were analysed by a Univariate Analysis of Variance with experimental group (WT/WAY-100635/1AKO) as fixed factor. In the EPM and open field experiments, activity levels were used as covariate factor. Drug effects in the SIH paradigm were analysed using repeated measures ANOVA with dose as 'within-subject' factor and experimental group as 'between-subject' factor. Post-hoc comparisons were made using the Bonferroni correction. The level of significance was set at  $p < 0.05$ . All statistical analyses were performed using the Statistical Package for Social Sciences, version 14.0 (SPSS, Chicago, IL, USA).

### 3. Results

#### 3.1 Establishing sufficient WAY-100635 levels in pups

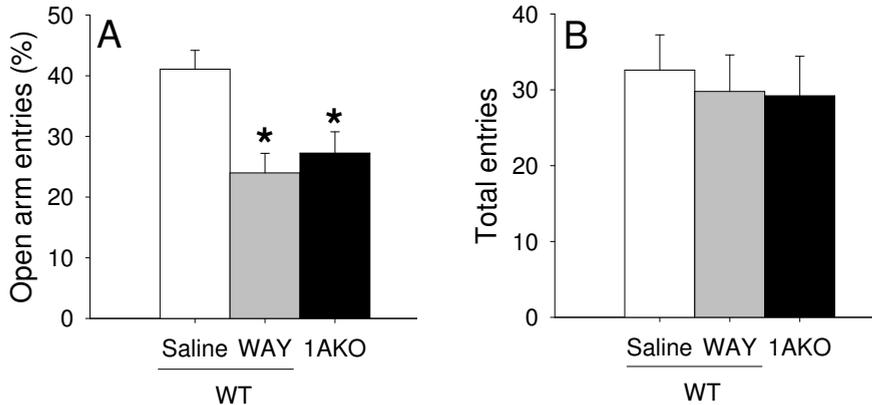
Flesinoxan reduced the number of vocalizations in chronically vehicle-treated mouse pups ( $99 \pm 33$ ;  $23 \pm 7$ ) ( $F[1,10]=14$ ,  $p < 0.01$ ), but not in chronically WAY-100635-treated mouse pups ( $179 \pm 60$ ;  $153 \pm 62$ ) ( $F[1,12] < 1$ , NS) (Fig. 1C).



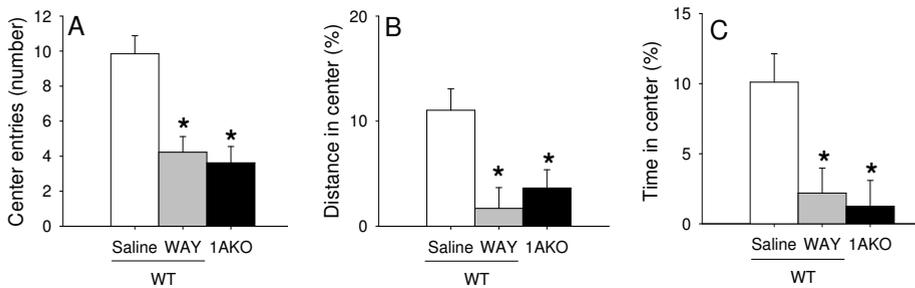
**Figure 1:** Total number of ultrasonic vocalizations (USV) at P7 in mouse pups. (A) USV after chronic WAY-100635 treatment (WAY) or vehicle treatment (WT) and in 1AKO mice at P7; (B) Effects of acute WAY-100635 treatment (SC) in wildtype mice at P7. (C) Effect of 5-HT<sub>1A</sub>R agonist flesinoxan (0.3 mg/kg, IP) on USV after chronic WAY-100635 treatment (WAY) or vehicle treatment (WT) at P7. Data are presented as mean value  $\pm$  SEM, \* indicates significant difference from WT (control),  $p < 0.05$ .

#### 3.2 Acute effects of WAY-100635 on USV

No significant changes in number of vocalizations at P7 were observed after any of the acute doses of WAY-100635 ( $F[3,35]=1.0$ , NS) (Fig. 1B).



**Figure 2:** Behavior in the elevated plus maze in wildtype mouse pups at P20 receiving chronic WAY-100635 treatment (WAY) or vehicle treatment (WT), and in 1AKO mice. Percentage of open arm entries (A) and total entry number (B) in the elevated plus maze are presented. Data are presented as mean value  $\pm$  SEM, \* indicates significant difference from WT (control),  $p < 0.05$ .

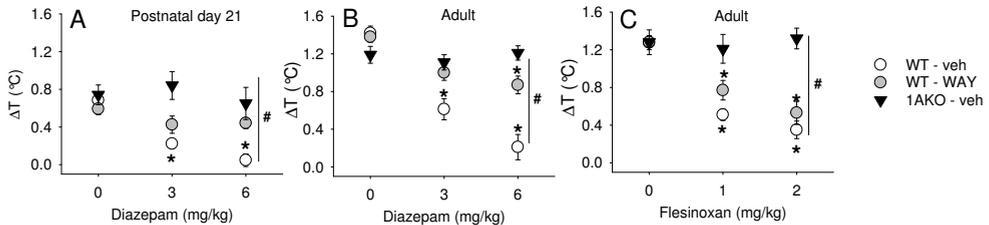


**Figure 3:** Behavior in the open field in adult wildtype mice receiving chronic WAY-100635 treatment (WAY) or vehicle treatment (WT), and in 1AKO mice. Number of centre entries (A), percentage distance in center (B) and percentage time spent in the centre (C) in the open field are presented. Data are presented as mean value  $\pm$  SEM, \* indicates significant difference from WT (control),  $p < 0.05$ .

### 3.3 Determination of the effects of chronic WAY-100635 on anxiety behavior

The number of vocalizations at P7 was significantly increased in chronically WAY-100635 treated pups ( $213 \pm 27$ ) and in 1AKO mice ( $241 \pm 30$ ) compared to WT pups ( $65 \pm 25$ ) ( $F_{[2,33]}=10.0$ ,  $p < 0.001$ ) (Fig. 1A). The same animals were tested in the elevated plus maze at P21 (Fig. 2) and in the open field at adult age (Fig. 3). In the elevated plus maze, a decrease in open arm entries was observed in WAY-100635 and 1AKO mice compared to vehicle-treated WT mice ( $F_{[2,37]}=82.6$ ,  $p=0.002$ ). Significant decreases in the WAY-100635 and 1AKO groups were also observed in the percentage of open arm entries ( $F_{[2,37]}=8.1$ ,  $p=0.001$ ). Total entry number was not significantly different between the three groups ( $F_{[2,37]} < 1$ , NS). In the open field, total entry number was different between groups ( $F_{[1,32]}=16.8$ ,  $p < 0.001$ ). Nevertheless, using the total entry number as covariate, WAY-100635 and 1AKO mice showed a significant reduction in the number of centre entries

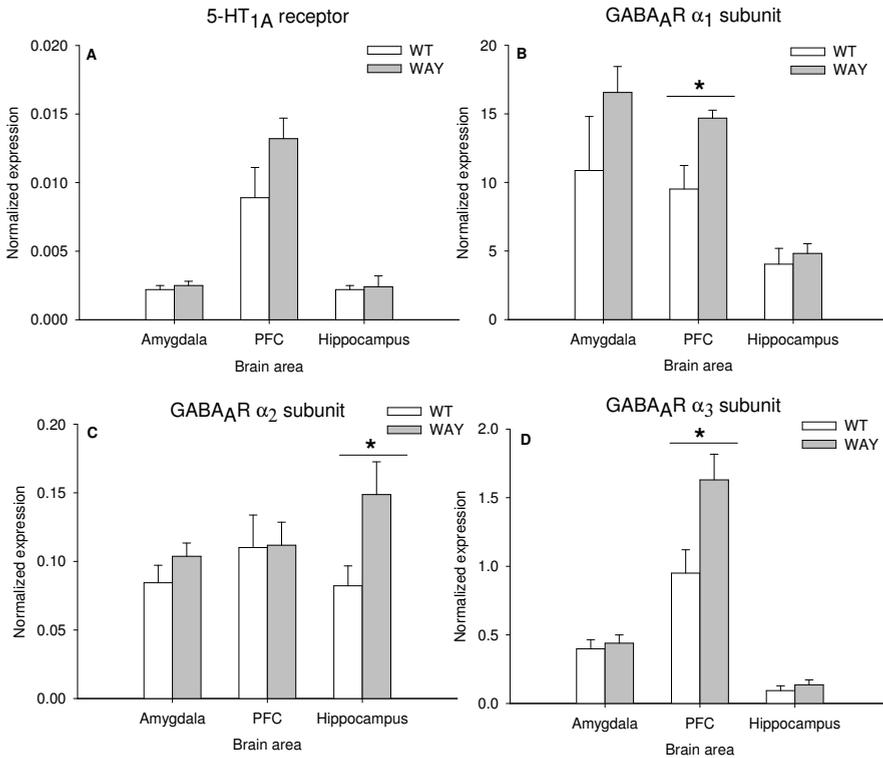
( $F[2,32]=11.7$ ,  $p<0.001$ ) as well as in the distance moved in the centre ( $F[2,32]=4.4$ ,  $p=0.021$ ). The percentage time spent in centre was also decreased in WAY-100635-treated and 1AKO mice compared to vehicle-treated WT mice ( $F[2,32]=6.2$ ,  $p=0.005$ ). Thus, WAY-100635 treatment increased the number of USVs at P7 and increased anxiety behavior in the elevated-plus maze (adolescence) and in the open field test (adult age).



**Figure 4:** Anxiolytic-like effects ( $\Delta T$ ) of diazepam at 3 weeks of age (A), diazepam at adult age (B) and flesinoxan (C) in the stress-induced hyperthermia paradigm in three treatment groups (WT-vehicle, closed circles; WT-WAY-100635, open circles; 1AKO-vehicle, open triangles). \* indicates significant decrease in  $\Delta T$  compared to vehicle condition,  $p<0.05$ . # indicates significant interaction effect between the treatment groups,  $p<0.05$ .

### 3.4 Determination of the effects of chronic WAY-100635 on benzodiazepine sensitivity

Both at three weeks and at adult age, the effect of diazepam on  $\Delta T$  depended on the experimental group (dose  $\times$  treatment interaction after 3 weeks:  $F[4,60]=4.3$ ,  $p=0.004$ ; adults:  $F[4,60]=4.1$ ,  $p=0.005$ ). At 3 weeks of age, vehicle-treated WT mice showed decreases in  $\Delta T$  after both doses of diazepam ( $F[2,7]=29.9$ ,  $p<0.001$ ), while both the WAY-100635-treated WT and vehicle-treated 1AKO groups were insensitive to these doses (WAY-100635:  $F[2,10]=1.4$ , NS; 1AKO:  $F[2,10]=0.4$ , NS). In adult vehicle-treated WT mice, diazepam decreased  $\Delta T$  after both 3 and 6 mg/kg ( $F[2,7]=27.6$ ,  $p<0.01$ ). Adult WAY-100635-treated WT mice showed only a decrease in  $\Delta T$  after 6 mg/kg ( $F[2,12]=4.9$ ,  $p=0.027$ ) and adult 1AKO mice remained insensitive to the effects of diazepam on  $\Delta T$  ( $F[2,9]=0.1$ , NS). Flesinoxan sensitivity was different between groups (dose  $\times$  treatment interaction:  $F[4,62]=3.5$ ,  $p=0.01$ ). Both vehicle-treated and WAY-100635-treated WT mice showed a dose-dependent decrease in  $\Delta T$  (WT:  $F[2,7]=19.9$ ,  $p=0.001$ ; WAY-100635:  $F[2,12]=8.8$ ,  $p=0.005$ ), but 1AKO mice were insensitive to the effects of flesinoxan on  $\Delta T$  ( $F[2,11]<1$ , NS). Thus, WAY-100635 treatment decreased diazepam sensitivity at 3 weeks and at adult age.



**Figure 5:** mRNA levels of 5-HT<sub>1A</sub> receptors and GABA<sub>A</sub> receptor subunits (mean ± SEM) in the amygdala, prefrontal cortex and hippocampus of vehicle-treated wildtype (WT) and WAY-100635-treated (WAY) mice at adult age. The mRNA expression was normalized against GAPDH level. \*:  $p < 0.05$ .

### 3.5 Quantitative PCR analysis

Results of the PCR analysis on 5-HT<sub>1A</sub>R mRNA levels showed no differences between the WT and WAY-100635 group in any brain area tested (prefrontal cortex, amygdala and hippocampus) when normalized against levels of GAPDH (Fig.5A). GABA<sub>A</sub> α<sub>1</sub> and α<sub>3</sub> subunit levels were increased in the prefrontal cortex but not in the hippocampus and amygdala. In contrast, GABA<sub>A</sub> α<sub>2</sub> subunit levels were increased in the hippocampus but not in the prefrontal cortex and the amygdala of WAY-100635-treated mice (Fig. 5B-D).

## 4. Discussion

Stressful early life experiences are major risk factors to develop anxiety and mood disorders (Heim and Nemeroff 2001). Therefore, stress exposure in the early postnatal period may result in persistent changes that render subjects more vulnerable to develop these disorders later in life (Leventopoulos et al 2009; Rosenfeld et al 1992). Early maternal separation was shown to directly alter 5-HT<sub>1A</sub> receptor (5-HT<sub>1A</sub>R) expression in pups (Vazquez et al 2002; Ziabreva et al 2003a; Ziabreva et al 2003b). Early-life stress may thus

affect 5-HT<sub>1A</sub> receptor circuitry, resulting in increased anxiety behavior at adult age (Borella et al 1997; Gaspar et al 2003). Thus, a causal link between early-life alterations in the serotonergic system and an increased risk to develop stress-related disorders in adulthood seems plausible. 5-HT<sub>1A</sub> receptor dysfunction has been implicated in major depressive disorder and other stress-related disorders (Savitz et al 2009). 5-HT<sub>1A</sub> receptor knockout mice (1AKO) indeed display increased anxiety levels (Heisler et al 1998; Parks et al 1998; Ramboz et al 1998), and it was shown that 5-HT<sub>1A</sub> receptor functionality during the early postnatal period may be crucial to develop normal anxiety-like behavior (Gross et al 2002; Tsetsenis et al 2007). Here, we show that pharmacological 5-HT<sub>1A</sub> receptor blockade during early postnatal development augments anxiety-like behavior in adolescent and adult mice mimicking the behavioral phenotype of Swiss-Webster mice lacking the 5-HT<sub>1A</sub> receptor (1AKO mice). Our pharmacological data confirm and extend the results obtained using conditional 5-HT<sub>1A</sub> receptor knockout mice, in which 5-HT<sub>1A</sub> receptor absence during the embryonic period up until postnatal day 21 (P21), but not at adult age, was pivotal in the development of increased adult anxiety levels (Gross et al 2002). In contrast to that study, we pharmacologically blocked the 5-HT<sub>1A</sub>R during the first three postnatal weeks (corresponding to the human fetal period during the second/third trimester), resulting in a physiologically more realistic, reduction in 5HT<sub>1A</sub> receptor function.

In another study, WAY-100635 treatment (3.6 mg/kg/day) during P13-P34 (but not P60-P81) was indeed sufficient to reproduce the increased anxiety-like phenotype of 1AKO mice in the open field test and the novelty-suppressed feeding test (Lo Iacono and Gross 2008). Together, our results confirm that transient serotonergic changes during the postnatal period (P15-P21) may disrupt stress-related behavior at adult age and that normal stress-related behavior at adult age is dependent on normal 5-HT<sub>1A</sub> receptor development during the early postnatal period (Gross et al 2002; Tsetsenis et al 2007). Thus, disruption of serotonergic neurotransmission during early life may predispose an organism to affective dysfunction later in life.

We also show that WAY-100635-treated mice are less sensitive to the effects of the classical non-subunit selective benzodiazepine diazepam at P21 (Fig. 4A) and at adult age (Fig. 4B). Thus, transient physiological blockade of the 5-HT<sub>1A</sub> receptor in early life not only increases emotional behavior, but also renders adult SW mice less sensitive to the anxiolytic effects of classical benzodiazepines, a pattern which has also been reported in 1AKO mice on the SW genetic background (Bailey and Toth 2004; Sibille et al 2000; Van Bogaert et al 2006a). The stress-induced hyperthermia (SIH) model was used to establish benzodiazepine sensitivity over time as this test can be repeatedly used without any habituation (Van der Heyden et al 1997). Using this test, we previously demonstrated that only 1AKO mice on a SW background were insensitive to the effects of diazepam and the  $\alpha_{2/3}$  selective GABA<sub>A</sub> receptor agonist L838,417 (Van Bogaert et al 2006a). Thus, the present study may offer pharmacogenetic evidence that benzodiazepine insensitivity in SW mice may depend on early-life modulation of genes involved in 5-HT<sub>1A</sub> receptor signalling.

Because WAY-100635-treated mice have similar 5-HT<sub>1A</sub> receptor mRNA levels compared to WT mice at adult age in the amygdala, prefrontal cortex and the hippocampus (Fig. 5A) and display normal sensitivity to the 5-HT<sub>1A</sub> receptor agonist flesinoxan (Fig. 4C), increased emotional behavior and reduced benzodiazepine sensitivity at adult age in WAY-100635-treated mice is more likely to be the result of strictly developmental 5-HT<sub>1A</sub> receptor disturbances rather than altered expression levels at adult age. However, we cannot exclude the possibility that WAY-100635-treated animals have different 5-HT<sub>1A</sub> receptor levels in other brain areas. Although adult 5-HT<sub>1A</sub> receptor mRNA levels are generally not altered after early life maternal separation, altered functionality may be present (Arborelius et al 2004; Gartside et al 2003; Neumaier et al 2002). Brief maternal separation may increase 5-HT<sub>1A</sub> receptor densities in adult rats (Vicentic et al 2006). Also, WAY-100635 treatment during early life (P13-P34, 0.3 mg/kg/day) as well as complete absence of the 5-HT<sub>1A</sub> receptor increased fear-conditioning after ambiguous-cues (Tsetsenis et al 2007). As the hippocampus plays an essential role in the regulation of these anxiety-related behavior, the increased anxiety phenotype in 1AKO mice and WAY-100635-treated mice could be consistent with an altered 5-HT<sub>1A</sub> receptor function in the hippocampus (Gross et al 2000).

Although 5-HT<sub>1A</sub> receptor mRNA levels and sensitivity were unchanged in WAY-100635-treated mice, long-lasting 5-HT<sub>1A</sub> receptor changes cannot completely be ruled out. Analysis of postmortem 5-HT<sub>1A</sub>R levels in humans after suicide have yielded inconclusive results ranging from an increase in 5-HT<sub>1A</sub> receptor binding (Arango et al 1995; Matsubara et al 1991; Meltzer 1990) to no differences (Arranz et al 1994; Dillon et al 1991; Lowther et al 1997a; Stockmeier et al 1997). A recent postmortem study found decreased WAY-100635 binding in the orbitofrontal cortex in major depressive disorder patients (Stockmeier et al 2009). Imaging studies confirm that the WAY-100635 binding potential is significantly decreased in depressed patients (for review, see (Drevets et al 2007)), although the evidence is inconsistent (Parsey et al 2006). Persistent changes in 5-HT<sub>1A</sub> receptor function in response to early life stress may also lead to compensatory changes distal to the receptor such as second messenger systems. 5HT<sub>1A</sub> receptors activate a variety of cellular processes that directly hyperpolarize the membrane. Blunted cytoplasmic protein kinase C and adenylate cyclase systems have been found in depressed patients, indicating that impaired 5-HT<sub>1A</sub> receptor function may be present without changes in receptor densities (Coull et al 2000a; Coupland et al 2005; Valdizan et al 2003). However, such altered second messenger functionality has not been consistently found, for example in phosphatidylinositol (Coull et al 2000b) or cAMP systems (Lowther et al 1997b). In mice, changes in  $\alpha$ -Ca<sup>2+</sup>/Calmoduline-dependent protein kinase-II phosphorylation were found in the hippocampus after WAY-100635 during postnatal week 3-5, which correlated with anxiety-related measures in the open field (Lo lacono and Gross 2008). 5-HT<sub>1A</sub> receptors also directly couple to tetrameric G-protein-regulated inwardly rectifying potassium (GIRK) ion channels of which three channel subunits (GIRK1-3) are widely distributed in the CNS (Colino and Halliwell 1987; Penington et al 1993). A close relationship between 5-HT<sub>1A</sub> receptor mRNA and GIRK1-3 channel mRNAs on GABAergic neurons has been found and may provide an alternative explanation for the

effects of 5-HT<sub>1A</sub> receptor blockade on GABA<sub>A</sub> receptor expression and functionality (Saenz del Burgo et al 2008). Therefore, it may be speculated that second messenger systems have changed in WAY-100635-treated mice.

Surprisingly, early life treatment with WAY-100635 (P5-19) normalized the depression-like phenotype of serotonin transporter knockout mice (5-HTTKO) (Alexandre et al 2006). In this study, WAY-100635 treatment did not modify genotype differences of mRNA 5-HT<sub>1A</sub> receptor levels in the raphe nuclei and hippocampus at adult age, even though WAY-100635 reversed depression-like behavior in 5-HTTKO mice. Combined together with the fact that 5-HTT inhibition during early development alters emotional behavior in adult mice (Ansorge et al 2004), these studies confirm our observation that modifying the early postnatal serotonergic neurotransmission may result in long lasting changes in behavior and receptor expression, putatively via other neurotransmitter systems including the GABA<sub>A</sub> receptor complex.

WAY-100635-induced postnatal disturbances could affect the GABAergic system to cause increased anxiety-like behavior and reduced benzodiazepine sensitivity. A direct serotonergic modulation of GABA<sub>A</sub> receptor is present in the amygdala which is affected by stress (Jiang et al 2009; Rainnie 1999), and genetic variations of the serotonin transporter expression influence the GABA-driven stress responsivity in the amygdala (Hariri et al 2005; Hariri et al 2002). Although the monoamine theory has dominated research on stress-related psychiatric disorders, a large body of preclinical and clinical literature supports the idea that GABA neurotransmission is actually involved in the pathophysiology of anxiety and mood disorders (Brambilla et al 2003; Kalueff and Nutt 2007). A dysfunctional GABAergic system is recognized in different stress-related psychiatric disorders among which anxiety disorders (Nutt and Malizia 2001), and reduced sensitivity to benzodiazepines and altered GABA<sub>A</sub> receptor expression has been found in patients suffering from panic disorder and posttraumatic stress disorder (Bremner et al 2000a; Bremner et al 2000b; Cameron et al 2007; Kaschka et al 1995; Roy-Byrne et al 1996). The GABA<sub>A</sub> receptor  $\alpha_1$  subunit (present in over 50% of all GABA<sub>A</sub> receptors) is thought to mediate the sedative and amnestic actions of benzodiazepines, whereas  $\alpha_2/\alpha_3$  subunits (present in 10–20% of all GABA<sub>A</sub> receptors) probably mediate the anxiolytic action of benzodiazepines (Dias et al 2005; Low et al 2000; McKernan et al 2000). In 1AKO mice, a decrease in GABA<sub>A</sub> receptor  $\alpha_1$  and  $\alpha_2$  subunits but not the  $\gamma_2$  subunit expression in 1AKO mice emerges from P14 to P28, suggesting specific  $\alpha$  subunit deficits in 1AKO mice during early development (Bailey and Toth 2004). In the present study, GABA<sub>A</sub> receptor  $\alpha_1$ ,  $\alpha_3$  but not  $\alpha_2$  subunit mRNA levels were increased in the prefrontal cortex of WAY-100635-treated mice, whereas chronic WAY100635 treatment increased GABA<sub>A</sub> receptor  $\alpha_2$  subunit but not  $\alpha_1$  and  $\alpha_3$  mRNA levels in the hippocampus (Fig. 5B). In the amygdala, GABA<sub>A</sub>R subunit levels were generally unchanged compared to vehicle-treated mice. Thus, altered GABA<sub>A</sub> receptor composition and  $\alpha$  subunit levels in the hippocampus and prefrontal cortex of WAY-100635-treated mice may be related to the reduced benzodiazepine sensitivity. Our results are in line with increased  $\alpha_1$  subunit levels in the prefrontal cortex in 1AKO mice (Sibille et al 2000), although a later study in these mice

found decreased  $\alpha_1$  subunit levels (Bailey and Toth 2004). In contrast, both studies found decreased amounts of  $\alpha_2$  subunit expression. Different 5-HT<sub>1A</sub> receptor blocking strategies (constitutional absence vs. physiological blockade) may at least partially explain these differences in mRNA expression levels. An increased level of  $\alpha$  subunit mRNA could be attributable to a transcriptional and/or posttranscriptional mechanism, in which increased transcription and/or increased decay would lead to increased mRNA levels. The stability of  $\alpha_1$  and  $\alpha_2$  subunit mRNA appeared to be comparable between 1AKO and WT mice on the SW background, suggesting that a transcriptional mechanism is more likely even though no compensatory increase in translational capacity was observed in 1AKO SW mice (Bailey and Toth 2004). Increased  $\alpha$  subunit levels may reflect altered GABA<sub>A</sub> receptor composition, and this changed receptor composition may be related to the anxious phenotype and benzodiazepine insensitivity. To our knowledge, no studies have been carried out investigating WAY-100635-induced effects on maternal care. Reduced maternal care leads to increased expression of GABA<sub>A</sub> receptor  $\alpha_3$  subunit mRNA in the amygdala and the locus coeruleus, and we cannot exclude the possibility that WAY-100635 directly or indirectly altered maternal behavior (Caldji et al 2004).

Caution should be taken to directly relate mRNA GABA<sub>A</sub> receptor findings to benzodiazepine sensitivity or anxiety phenotype in WAY-100635-treated mice. The fact that the anxious 1AKO phenotype but not benzodiazepine insensitivity is present on three different genetic backgrounds indicates that GABA<sub>A</sub> receptor levels alone cannot account for increased anxiety levels. In different 1AKO strains, no correlation was found between benzodiazepine sensitivity and  $\alpha_2$  subunit expression in the amygdala (Bailey and Toth 2004). More complex mechanisms are likely to play a role, and WAY-100635-treatment may remodel the developing brain or alter central pathways to cause reduced benzodiazepine responsiveness.

WAY-100635 has a relatively short half-life, and systemic levels will be phasic, even after administration of multiple relatively high doses. Both pups (1 mg/kg WAY-100635/day starting from P7) as well as the nursing mothers (3 mg/kg WAY-100635, b.i.d.) were injected with WAY-100635 to ensure complete 5-HT<sub>1A</sub> receptor blockade. However, we cannot exclude the possibility that 5-HT<sub>1A</sub> receptor occupancy was not completely maintained throughout the entire period. However, from our data it appears that the WAY-100635 dosing produced sufficient 5-HT<sub>1A</sub> receptor occupancy to prevent fleroxan-induced reduction of ultrasonic vocalizations 4 hours after the WAY administration (Fig. 1C). In support, Alexandre and co-workers found effects of early life 5-HT<sub>1A</sub> receptor blockade after WAY-100635 administration at a dose of 1 mg/kg twice daily (Alexandre et al 2006). Moreover, there are studies in which even phasic WAY-100635 at lower doses compared to the present study produced sufficient plasma levels to prevent any detectable fluoxetine effects (which possesses a long half-life). Therefore, complete 5-HT<sub>1A</sub> receptor antagonism for a limited period each day, or, alternatively, a longer lasting partial occupancy may be sufficient to block the 5-HT<sub>1A</sub> receptor. An *in vivo* microdialysis study shows that WAY-100635 already blocks 5-HT<sub>1A</sub> receptor autoreceptors at doses between 0.01 and 0.3 mg/kg (Hjorth et al 1997). Chronic administration of WAY-100635 alone does not change in the functional status of the 5-HT<sub>1A</sub> receptor (Dawson et al 2002).

In support, chronic WAY-100635 treatment at adult age generally does not modify anxiety levels in wildtype mice (Cao and Rodgers 1997). Our studies cannot distinguish between pre- vs. postsynaptic alterations in 5-HT<sub>1A</sub> receptors, as WAY-100635 blocks both receptor types. At P7, increased anxiety in WAY-100635-treated mice is unlikely to be the direct result of WAY-100635 treatment as acute WAY-100635 administration does not affect USVs in WT mice (Fig. 1B). However, USVs in WT mice seem to increase at higher WAY-100635 dose, and we cannot completely exclude the possibility that increased anxiety behavior during chronic WAY-100635 treatment is the result of acute 5-HT<sub>1A</sub> receptor antagonism. The difference in USV levels between chronically and acutely vehicle-treated pups may be explained by the fact that postnatal handling affects the serotonergic system and increases the ability of handled animals to cope with stressful stimuli (Panagiotaropoulos et al 2004). Such a coping strategy may be absent in 1AKO or WAY-100635-treated animals, which underlines the idea that the 5-HT<sub>1A</sub> receptor may be closely involved in early life stress management.

The main finding from the present studies is that transient postnatal pharmacological blockade of the 5-HT<sub>1A</sub> receptor results in consistent and life-long increased anxiety, reduced benzodiazepine sensitivity and increased GABA<sub>A</sub> receptor  $\alpha$  subunit levels in the prefrontal cortex and hippocampus. This suggests a putative link between early life modification of the serotonin system and adult changes in the GABAergic system in Swiss-Webster mice. The increased anxious phenotype after WAY-100635 treatment closely resembles the 1AKO phenotype, and, as a result, this phenotype may stem from specific developmental 5-HT<sub>1A</sub> receptor disruption that continues into adult age. Our findings may thus suggest and confirm a link between early-life disruption of the serotonergic system and the emergence of increased adult anxiety levels and decreased benzodiazepine responsiveness that are also present in various stress-related psychiatric disorders. If chronic drug exposure during developmental periods produces changes in adult anxiety levels, this could have clinical implications for psychoactive drug use during pregnancy.

### **Acknowledgements**

The authors would like to thank the following people for their technical assistance during the experiments: Erik Hendriksen, Ruud van Oorschot, Dolf Meier, Koen Westphal, Natalia del Campo and Emilie Bloemaerts.



# Chapter 7

## **Stress-induced hyperthermia and infection-induced fever: Two of a kind?**

Christiaan H. Vinkers

Lucianne Groenink

Meg J. Van Bogaert

Koen G.C. Westphal

Cor J. Kalkman

Ruud van Oorschot

Ronald S. Oosting

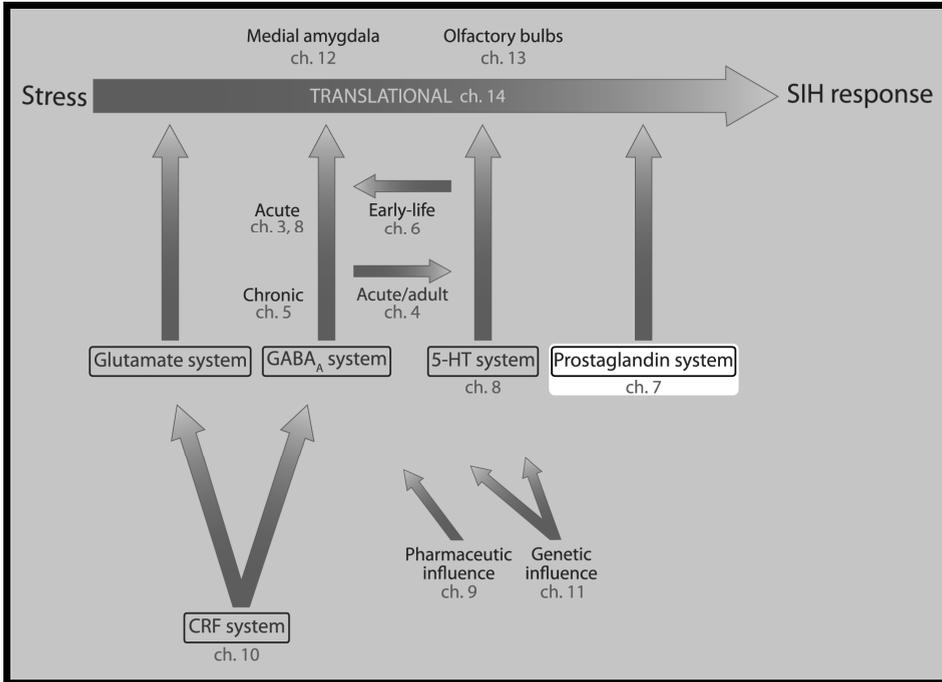
Berend Olivier

S. Mechiel Korte

# 7

*Physiology and Behavior (2009), 98:37-43*

## Abstract



Stress exposure activates the autonomic nervous system and leads to an increase in body temperature (stress-induced hyperthermia, SIH). On the other hand, an activation of the immune system in response to an infection leads to fever. Both processes increase body temperature, and the relation between SIH and infection-induced fever has been subject to debate. It is not clear whether SIH is a form of fever, or whether both processes are more or less distinct. We therefore examined the relation between SIH and infection-induced fever by looking at the effects of a GABA<sub>A</sub> receptor agonist (diazepam) and a prostaglandin-synthesis blocking drug (acetylsalicylic acid, Aspirin®) on both the SIH response and fever in rats and mice. The present study shows that the benzodiazepine diazepam but not the prostaglandin-synthesis blocking drug aspirin dose-dependently attenuated the SIH response in both rats and mice. In contrast, aspirin reduced both LPS-induced and IL-1 $\beta$ -induced fever, whereas diazepam had little effect on these fever states. Altogether, our findings support the hypothesis that stress-induced hyperthermia and infection-induced fever are two distinct processes mediated largely by different neurobiological mechanisms.

## 1. Introduction

Stress exposure causes an activation of the autonomic nervous system which leads to a consistent increase in body temperature. This stress-induced hyperthermia (SIH), also referred to as “psychogenic fever” (Oka et al 2001), is a transient stress response comparable across all species (Vinkers et al 2008). In the SIH paradigm, anxiolytic drugs such as GABA<sub>A</sub> receptor agonists and 5-HT<sub>1A</sub> receptor agonists result in a reduction of the SIH response, whereas peripheral administration of non-anxiolytic dopaminergic or noradrenergic drugs, as well as acute administration of SSRIs and TCAs generally do not affect the SIH response (Olivier et al 2003).

The exact neuronal pathways subserving the SIH response have not been elucidated yet (Vinkers et al 2008). Specifically, the relation between SIH and other changes in body temperature such as fever caused by immune system activation (“fever”) has been subject to debate (Oka et al 2001). Some argue that SIH is a passive rise in body temperature above set point in contrast to fever which is generally thought to be an active raise in body temperature set point (Oka et al 2001). However, both SIH and fever result in a higher body temperature accompanied by shivering and cutaneous vasoconstriction (Briese and Cabanac 1991), are identical in warm and cool environments (Long et al 1990a) and come along with activation of both HPA axis and sympathetic nervous system (Connor and Leonard 1998; Croiset et al 1987; Leonard and Song 1999). In contrast, differences exist between SIH and infection-induced fever. Infection-induced fever is initiated by exogenous pyrogens like bacteria which in turn induce endogenous cytokines, but any form of physical or psychological stress can cause SIH (Bouwknicht et al 2007). Moreover, the hypothalamic pre-optic area plays a major role in fever (Blatteis and Sehic 1998; Boulant 2000), whereas the amygdala is involved in the acute stress response (Davis 1997). As a SIH response only occurs after exposure to acute stress, a role for the amygdala in the initiation of autonomic stress responses including the SIH response seems plausible. Also, differences exist regarding the pharmacological substrates involved in SIH and fever. There is ample evidence for a role of the GABA<sub>A</sub> receptor in the SIH response (Vinkers et al 2008), while evidence points to a role for prostanoid EP3 receptors in fever (Nakamura et al 1999). Indeed, fever can be effectively reversed by prostaglandin-blocking drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) which block the synthesis of prostaglandins (Stitt 1986). However, conflicting reports exist on the effects of NSAIDs on the SIH response. While some studies report no anxiolytic effects of NSAIDs in the SIH paradigm in mice and ducks (Gray et al 2008; Lecci et al 1990b; Olivier et al 2003; Zethof et al 1994), contradictory reports exist in which administration of salicylate or indomethacin was to at least partially attenuate the SIH response in rats and pigs (Kluger et al 1987; Morimoto et al 1991; Parrott and DM 1995; Singer et al 1986; Vellucci and Parrott 1995).

Altogether, it is not clear whether SIH is a form of fever, or whether both processes are more or less distinct. If prostaglandin-blocking drugs such as NSAIDs and GABA<sub>A</sub> receptor agonists such as benzodiazepines have similar effects on both the SIH response and fever induced by the immune system, this indicates that pharmacologically, the SIH response could be regarded as a specific form of fever. Therefore, the present study aimed to investigate the relation between SIH and infection-induced fever by looking at the effects

of a GABA<sub>A</sub> receptor agonist (diazepam) and a prostaglandin-synthesis blocking drug (acetylsalicylic acid, Aspirin®) on both the SIH response and fever in rats and mice.

## 2. Methods and materials

### 2.1 Animals

#### *General*

Mouse and rat experiments were carried out in different rooms at different times and days. All experiments were carried out with approval of the ethical committee on animal experiments of the Faculties of Sciences, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki.

#### *Mice*

Male mice of different genetic backgrounds (SW and C57BL/6J, Taconic M&B, Ry, Denmark) were used in the experiments as they have been extensively used in the SIH paradigm (Olivier et al 2003; Van Bogaert et al 2006a; van Bogaert et al 2006b). Mice were housed socially in a controlled environment under a 12 h light-12 h dark cycle (lights on from 6:00 AM until 6:00 PM) at controlled temperature ( $20 \pm 2$  °C) and relative humidity (40-50%) with free access to standard food pellets and tap water. One week after arrival, telemetry transmitters were implanted. After surgery, mice were singly housed in Macrolon® Type 2 cages (22x16x14 cm) enriched with bedding and nesting material. Food (standard lab chow) and tap water were available ad libitum. Once a week, an experimental procedure was carried out.

#### *Rats*

Male Wistar rats (Harlan Zeist, the Netherlands) were housed socially in a controlled environment under a 12 h light-12 h dark cycle (lights on from 6:00 AM until 6:00 PM) at controlled temperature ( $20 \pm 2$  °C) and relative humidity (40-50%) with free access to standard food pellets and tap water. One week after arrival, telemetry transmitters were implanted. After recovery from surgery, rats were housed in groups of four in type IV Macrolon® cages with a plastic tube as cage enrichment. Food (standard lab chow) and tap water were available ad libitum. Once a week, an experimental procedure was carried out.

### 2.2 Telemetry transmitter surgery

Radio telemetry transmitters (Data Sciences International (DSI), type ETA-F20, St. Paul, MN, USA) were implanted in the abdominal cavity as earlier described (Olivier et al 2002). Animals received Rimadyl, (5 mg/kg, subcutaneously) post-surgically twice a day for 2 days, as well as a solid energy drink (Triple A Trading, The Netherlands) and soaked food pellets. Animals were allowed to recover during a period of 2 weeks.

### 2.3 Radiotelemetry system

The radio telemetry system consisted of an implanted transmitter with two flexible leads (DSI, type ETA-F20), a receiver placed under the cage (DSI model RPC-1) and a data exchange matrix collecting receiver signals and subsequently sending them to a

computer where all raw data was stored. Data were collected using Dataquest Gold A.R.T. software (DSI, version 2.2). Raw data consisted of temperature responses collected for 10 seconds every 5 minutes.

## 2.4 Drugs

All drug solutions were freshly prepared each testing day and injected intraperitoneally (IP) or orally (PO) in a volume of 10 ml/kg (mice) or 2 ml/kg (rats). Aspirin (acetylsalicylic acid) and diazepam base (Sigma Aldrich) were suspended in a 0.5% gelatine/ 5% mannitol solution. Lipopolysaccharide (LPS, serotype 01111:B4, Sigma Aldrich) and mouse IL-1 $\beta$  (Sigma Aldrich) were dissolved in saline and injected intraperitoneally.

## 2.5 Experimental procedures

### General

On the afternoon before an experimental day, animals were weighed and put in a type II (mice) or type III (rats) Macrolon<sup>®</sup> cage, located on a telemetric receiver. Cage transfer the day before the experimental day was carried out in accordance with the standardized SIH procedure (Van der Heyden et al 1997). The telemetric transmitters were activated by a magnet. Data collection of body temperature was subsequently started. The day after, an experimental procedure was carried out. At the end of the experimental day, subjects were either singly (mice) or group-housed (rats) and transmitters were turned off. All stress and fever induction procedures were initiated between 9-12 AM.

**Table 1:** Time table for for stress and fever induction

<b>A. Stress induction in C57/BL6, SW mice and Wistar rats</b>	
Time (min)	Treatment
t= -60	Aspirin 0-300 mg/kg PO Diazepam 0-4 mg/kg IP
t= 0	Novel cage
<b>B. Fever induction in C57/BL6 mice</b>	
Time (min)	Treatment
t= -120	IL-1 $\beta$ 10 $\mu$ g/kg or vehicle IP
t= 0	Aspirin 300 mg/kg IP Diazepam 4 mg/kg IP Rectal temperature measurement No stress
<b>C. Fever and stress induction in Wistar rats</b>	
Time (min)	Treatment
t= -240	LPS 50 $\mu$ g/kg or vehicle IP
t= 0	Vehicle Aspirin 300 mg/kg IP Diazepam 4 mg/kg IP
t= 60	Novel cage

**Stress induction (Table 1)**

The standardized SIH procedure (Van der Heyden et al 1997) consisted of an intraperitoneal (IP) injection or oral (PO) administration with vehicle or a different dose of either diazepam or aspirin. Aspirin was administered orally in mice in accordance with earlier studies (Lecci et al 1990b; Olivier et al 2003; Zethof et al 1995). Immediately after injection, animals were placed back into their individual cage. One hour after injection, animals were placed in a novel cage (clean cage with fresh bedding and a paper tissue) and left undisturbed for approximately two hours afterwards. To prevent habituation to the novel cage procedure, the interval between two experiments was set to be at least one week. For stress induction, a within subject design was used, and all animals received all (combined) doses of the drugs.

**Fever induction (Table 1)***General*

Aspirin or diazepam was administered 2 hours (mice) or 4 hours (rats) after LPS/IL-1 $\beta$  administration to enable a standard SIH procedure to be carried out under stable (normal or febrile) body temperatures, and to quantify the effects of both aspirin and diazepam on (a)febrile body temperatures.

*Mice*

C57BL/6J mice were used for fever induction. Animals received an injection of IL-1 $\beta$  (10  $\mu$ g/kg, IP) or vehicle. Two hours later, animals received an injection (IP or PO) with vehicle or either diazepam (4 mg/kg, IP), aspirin (300 mg/kg, PO), a rectal temperature measurement (stress) or "no stress". A within subject design was used, and all animals received all treatments with a one-week interval.

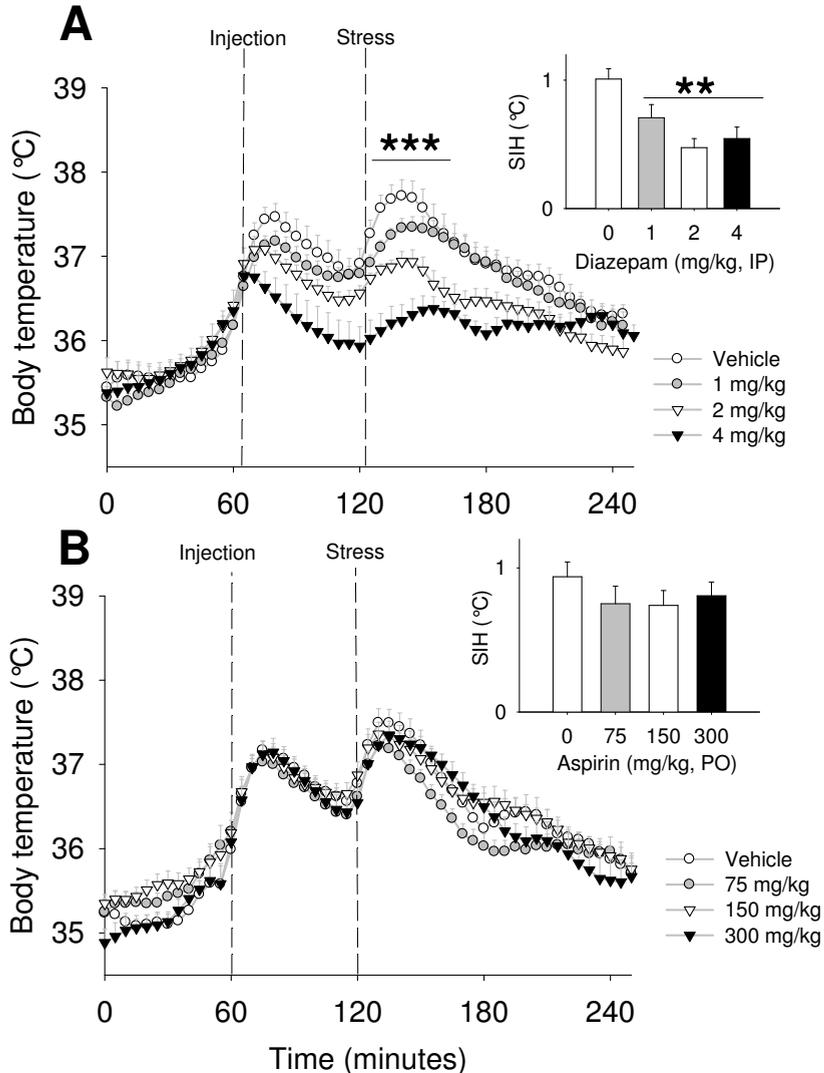
*Rats*

Animals received an injection of LPS (50  $\mu$ g/kg, IP) or vehicle. Four hours later, animals received an injection (IP) with vehicle, diazepam (4 mg/kg) or aspirin (300 mg/kg). After one hour, animals were placed in a novel cage (clean cage with fresh bedding and a paper tissue). A between subject design was used, and animals were evenly distributed over all treatment groups.

**2.6 Data analysis**

All data were collected in 5-minute blocks and are displayed as means  $\pm$  SEM. Experiments were carried out with a between-subject (fever induction in rats) or a within-subject design (fever induction in mice and all SIH experiments). For all experiments, body temperature data during the first 60 minutes after injection or novel cage were analyzed. As planned for all fever experiments, fever and non-fever states were also separately analyzed. All data were analyzed using a univariate repeated measures analysis of variance (ANOVA) with manipulation time as within-subject factor. Treatment (aspirin or diazepam at three doses) was a within-subject factor in the experiments on the effects of aspirin and diazepam on the SIH response (Figure 1-3). Treatment (stress/no stress, aspirin/vehicle, diazepam/vehicle) was a between-subject factor in the fever experiments (Figure 4-5). The SIH response was also calculated as  $\Delta T$ , by subtracting the maximum temperature in the first 30 minutes after stress from the basal temperature 5 minutes

before stress. For the dose-response experiments, simple contrast tests were used to compare drug with vehicle conditions whenever a significant main effect for drug was observed. A probability level of  $p < 0.05$  was set as statistically significant. All reported results were corrected by the Greenhouse Geisser procedure where appropriate, which is indicated by an adjustment of the degree of freedom.



**Figure 1:** Effects of (A) diazepam (0-4 mg/kg, IP) and (B) aspirin (0-300 mg/kg, PO) on the SIH response in **C57 mice** (n=11). \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ . At the 2 hour timepoint, the animals were placed in a new cage.

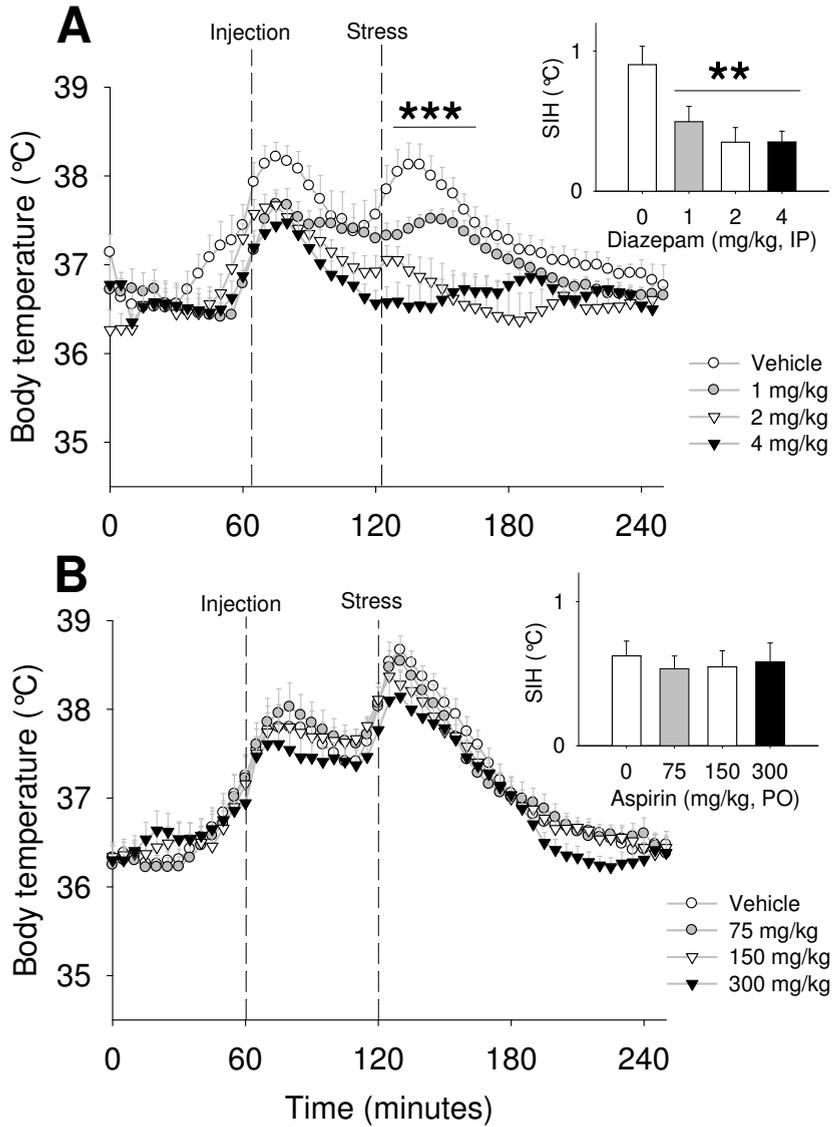
## 3. Results

### 3.1 Effects of aspirin and diazepam on the SIH response

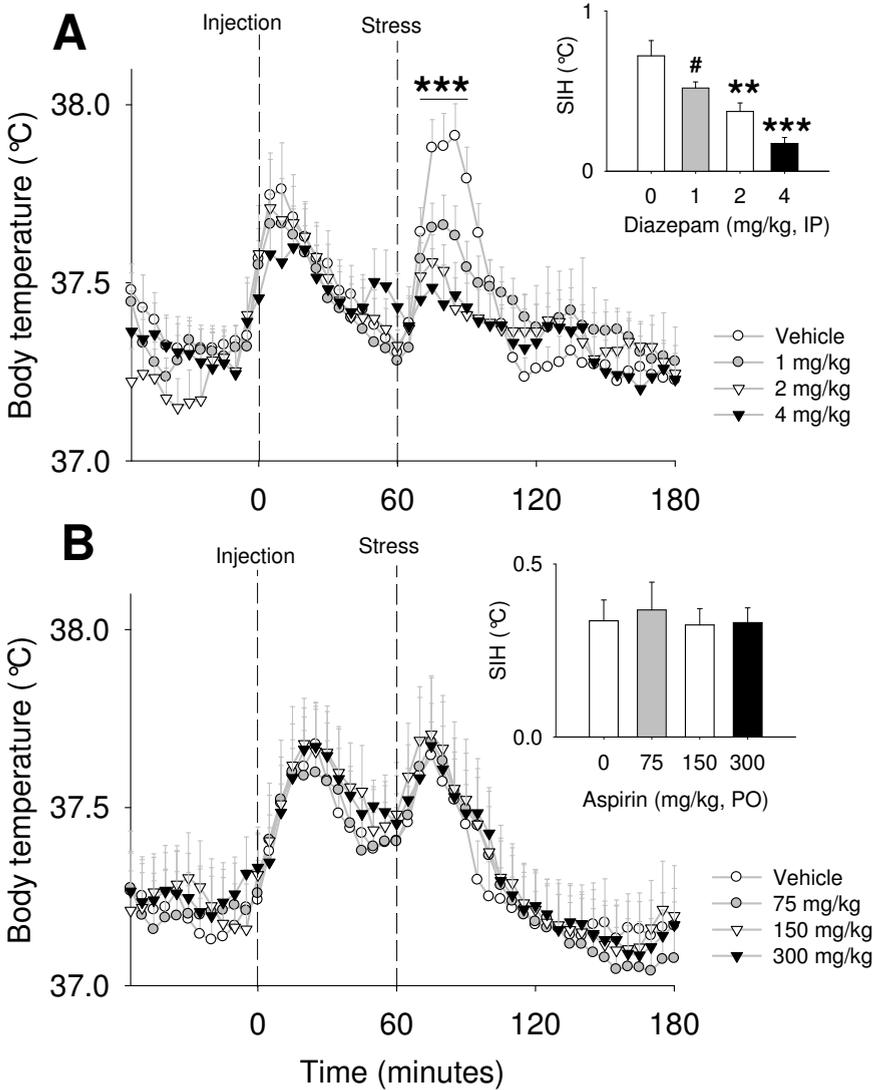
#### Mice

Diazepam dose-dependently reduced the SIH response in C57 mice (n=11) (diazepam x time interaction  $F_{33,330}=2.72$ ,  $p<0.001$ ) and in SW mice (n=10) (diazepam x time interaction  $F_{33,297}=2.19$ ,  $p<0.001$ ) (Figure 1A and 2A). Also, diazepam reduced the injection and novel cage-induced body temperature in C57 mice (diazepam effect  $F_{3,30}=11.50$ ,  $p<0.001$ ) and SW mice (diazepam effect  $F_{3,27}=7.14$ ,  $p<0.001$ ). Analysis of the SIH response confirmed that diazepam reduced the SIH response in C57 mice (diazepam effect  $F_{3,30}=9.59$ ,  $p<0.001$ ) and in SW mice (diazepam effect  $F_{3,27}=10.65$ ,  $p<0.001$ ) (Figure 1A and 2A, inset). Simple contrasts revealed that all diazepam doses reduced the SIH response in C57 mice (simple contrasts: veh-1mg/kg:  $F_{1,10}=11.18$ ,  $p<0.01$ ; veh-2mg/kg:  $F_{1,10}=56.29$ ,  $p<0.01$ ; veh-4mg/kg:  $F_{1,10}=12.07$ ,  $p<0.01$ ). Comparatively, all diazepam doses reduced the SIH response in SW mice (simple contrasts: veh-1mg/kg:  $F_{1,9}=10.72$ ,  $p=0.01$ ; veh-2mg/kg:  $F_{1,9}=23.90$ ,  $p<0.01$ ; veh-4mg/kg:  $F_{1,9}=17.38$ ,  $p<0.01$ ).

Aspirin did neither influence the SIH response in C57 mice (n=12) (aspirin x time interaction  $F_{33,363}=1.84$ ,  $p=0.11$ , NS) nor in SW mice (n=10) (aspirin x time interaction  $F_{33,297}=1.07$ ,  $p=0.37$ , NS) (Figure 1B and 2B). Also, aspirin did not influence the injection and novel cage-induced body temperature in C57 mice (aspirin effect  $F_{3,33}=0.31$ ,  $p=0.82$ , NS) or in SW mice (aspirin effect  $F_{3,27}=0.94$ ,  $p=0.44$ , NS). Analysis of the SIH response confirmed that aspirin did not influence the SIH response in C57 mice (aspirin effect  $F_{3,33}=0.68$ ,  $p=0.57$ , NS) or in SW mice (aspirin effect  $F_{3,27}=0.11$ ,  $p=0.96$ , NS) (Figure 1B and 2B, inset).



**Figure 2:** Effects of (A) diazepam (0-4 mg/kg, IP) and (B) aspirin (0-300 mg/kg, PO) on the SIH response in SW mice (n=10). \*\*: p<0.01; \*\*\*: p<0.001. At the 2 hour timepoint, the animals were placed in a new cage.



**Figure 3:** Effects of (A) diazepam (0-4 mg/kg, IP, n=11) and (B) aspirin (0-300 mg/kg, PO, n=12) in the SIH test in Wistar rats. \*\*: p<0.01; \*\*\*: p<0.001; #: p=0.06. At the 1 hour timepoint, the animals were placed in a new cage.

## Rats

Diazepam dose-dependently reduced the SIH response in rats ( $n=11$ ) (diazepam x time interaction  $F_{33,330}=4.42$ ,  $p<0.001$ ) (figure 3A). Diazepam did not influence the injection and novel cage-induced body temperature (diazepam effect:  $F_{3,30}=1.34$ ,  $p=0.28$ , NS). Reduction of the SIH response was confirmed by separate SIH response analysis (Diazepam effect  $F_{2,20}=18.18$ ,  $p<0.001$ ) (figure 3A, inset). Simple contrasts revealed that diazepam doses of 2 and 4 mg/kg reduced the SIH response, while the 1 mg/kg almost did so (Simple contrasts: veh-1mg/kg:  $F_{1,10}=4.62$ ,  $p=0.06$ , trend; veh-2mg/kg:  $F_{1,10}=11.01$ ,  $p<0.01$ ; veh-4mg/kg:  $F_{1,10}=40.68$ ,  $p<0.001$ ).

Aspirin had neither effect on the SIH response in rats ( $n=12$ ) (aspirin x time interaction  $F_{6,65}=0.41$ ,  $p=0.87$ , NS), nor affected injection and novel cage-induced body temperature (aspirin effect  $F_{3,33}=0.07$ ,  $p=0.97$ , NS) (figure 3B). Separate SIH response analysis confirmed that aspirin did not influence the SIH response (aspirin effect  $F_{3,33}=0.11$ ,  $p=0.95$ , NS) (figure 3B, inset). Simple contrasts revealed that none of the aspirin doses used affected the SIH response (simple contrasts: veh-75mg/kg:  $F_{1,11}=0.21$ ,  $p=0.66$ , NS; veh-150mg/kg:  $F_{1,11}=0.02$ ,  $p=0.89$  NS; veh-300mg/kg:  $F_{1,11}=0.004$ ,  $p=0.95$ , NS).

## **3.2 Effects of stress, aspirin and diazepam in fever states**

### IL-1 $\beta$ -induced fever in C57 mice ( $n=8$ )

*SIH in fever and non-fever states (Figure 4A,  $t=240-300$  min):*

IL-1 $\beta$  increased body temperature (IL-1 $\beta$  effect  $F_{1,7}=6.01$ ,  $p<0.05$ ), and stress increased body temperature (stress effect  $F_{1,7}=9.37$ ,  $p<0.05$ ; stress x time interaction  $F_{11,77}=8.76$ ,  $p<0.001$ ). This SIH response was independent of fever state (IL-1 $\beta$  x stress interaction  $F_{1,7}=3.44$ ,  $p=0.11$ , NS; IL-1 $\beta$  x stress x time interaction  $F_{11,77}=1.58$ ,  $p=0.12$ , NS). When analyzing fever and non-fever state separately, stress increased body temperature in the non-fever state (stress effect  $F_{1,7}=21.04$ ,  $p<0.01$ ; stress x time interaction  $F_{11,77}=5.94$ ,  $p<0.001$ ) and the IL-1 $\beta$ -induced fever state (stress x time interaction  $F_{11,77}=3.23$ ,  $p<0.01$ ). However, when comparing the SIH response in fever and non-fever state, the SIH response was significantly larger in the non-fever state (stress x time interaction  $F_{11,77}=3.31$ ,  $p<0.01$ ), probably due to a ceiling effect. Analysis of the calculated SIH response confirmed that stress increased the SIH response compared to no stress (stress effect  $F_{1,7}=25.24$ ,  $p<0.01$ ) and that the SIH response was different in the fever state (IL-1 effect  $F_{1,7}=5.71$ ,  $p<0.05$ ). (Figure 4A, inset). Stress did increase the SIH response regardless of fever state (IL-1 x stress interaction,  $F_{1,7}=3.89$ ,  $p=0.1$ , NS).

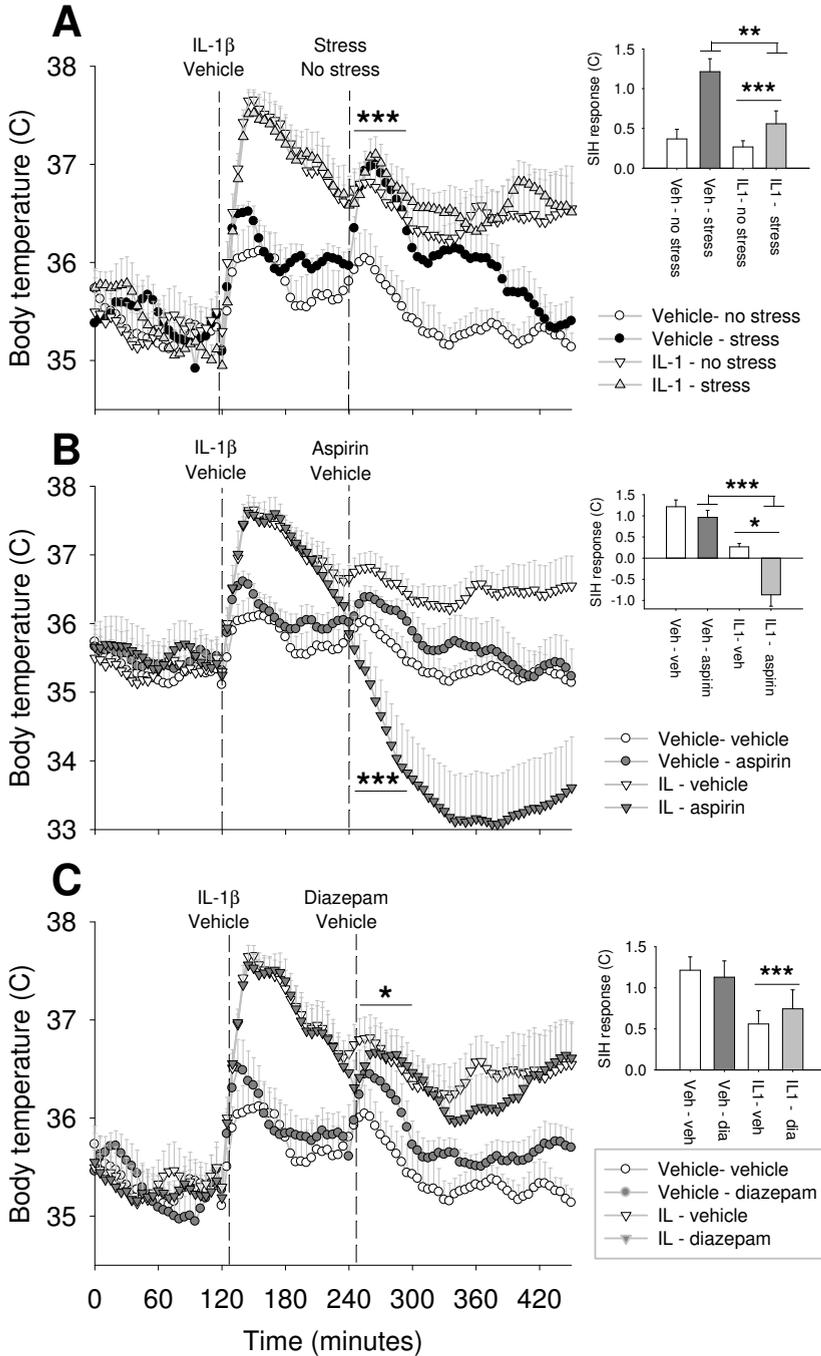
*Effects of aspirin on IL-1 $\beta$ -induced fever (Figure 4B,  $t=240-300$  min):*

Aspirin had profound effects on body temperature (aspirin effect  $F_{1,7}=16.58$ ,  $p<0.001$ ; aspirin x time interaction  $F_{11,77}=2.99$ ,  $p<0.01$ ), but this effect was limited to the IL-1 $\beta$ -induced fever state (IL-1 $\beta$  x aspirin interaction  $F_{1,7}=16.60$ ,  $p<0.01$ ; IL-1 $\beta$  x aspirin x time interaction  $F_{11,77}=7.98$ ,  $p<0.001$ ). This aspirin effect in the fever state made that IL-1 $\beta$  almost not had no overall fever effect anymore (IL-1 $\beta$  effect  $F_{1,7}=5.02$ ,  $p=0.06$ ). Planned separate analysis of the fever state and non-fever state confirmed that aspirin effects were limited to the fever state (aspirin effect  $F_{1,7}=24.10$ ,  $p<0.001$ ; IL-1 $\beta$  x aspirin interaction  $F_{11,77}=8.65$ ,  $p<0.001$ ), while aspirin effects in the non-fever state were absent (aspirin effect  $F_{1,7}=1.66$ ,  $p=0.24$ , NS; aspirin x time interaction  $F_{11,77}=0.49$ ,  $p=0.90$ , NS). Analysis of the calculated SIH response confirmed that acute aspirin administration affected the SIH

response (aspirin effect  $F_{1,7}=20.03$ ,  $p<0.001$ ) and that the SIH response was different in the fever state (IL-1 effect  $F_{1,7}=57.82$ ,  $p<0.001$ ). Aspirin affected the SIH response only in the fever states (IL-1 x aspirin interaction,  $F_{1,7}=5.14$ ,  $p<0.05$ ). (Figure 4B, inset).

*Effects of diazepam on IL-1 $\beta$ -induced fever (Figure 4C,  $t=240-300$  min):*

Diazepam had no overall effect on body temperature (diazepam effect  $F_{1,7}=0.19$ ,  $p=0.68$ , NS), although it influenced temperature over time (diazepam x time interaction  $F_{11,77}=2.22$ ,  $p<0.05$ ). Diazepam showed a trend to affect body temperature differently in fever states (IL-1 $\beta$  x diazepam interaction  $F_{1,7}=5.08$ ,  $p=0.06$ , trend). When analyzing the fever and non-fever states separately, diazepam overall reduced body temperature in the fever state (diazepam effect  $F_{1,7}=6.53$ ,  $p<0.05$ ), while diazepam increased body temperature due to injection stress in the non-fever state (diazepam x time interaction  $F_{11,77}=1.91$ ,  $p=0.05$ ) without overall affecting body temperature (diazepam effect  $F_{1,7}=0.80$ ,  $p=0.40$ , NS). Analysis of the calculated SIH response confirmed that acute diazepam administration did not have effects on the SIH response (diazepam effect  $F_{1,7}=0.09$ ,  $p=0.78$ , NS) and that the SIH response was different in the fever state (IL-1 effect  $F_{1,7}=10.27$ ,  $p<0.05$ ). Acute diazepam administration did not affect the SIH response in any of the fever states (IL-1 x diazepam interaction,  $F_{1,7}=0.25$ ,  $p=0.64$ , NS). (Figure 4C, inset).



**Figure 4:** Effects of aspirin (300 mg/kg, PO) and diazepam (4 mg/kg, IP) on IL-induced fever in **C57 mice**. **A:** The SIH response is present in IL-induced fever, although smaller. Inset: the calculated SIH response is increased after stress and is smaller in fever states. **B:** Aspirin has profound effects only in IL-induced fever states. Inset: the calculated SIH response is smaller in fever states and decreased after aspirin administration as aspirin decreases the body temperature in fever states **C:** Diazepam has almost no effect on IL-induced fever states. Inset: the calculated SIH response is smaller in fever states. \*\*\*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*  $p < 0.05$ .

### LPS-induced fever in Wistar rats

#### *Effects of aspirin on LPS-induced fever (Figure 5A, t=390-450 min):*

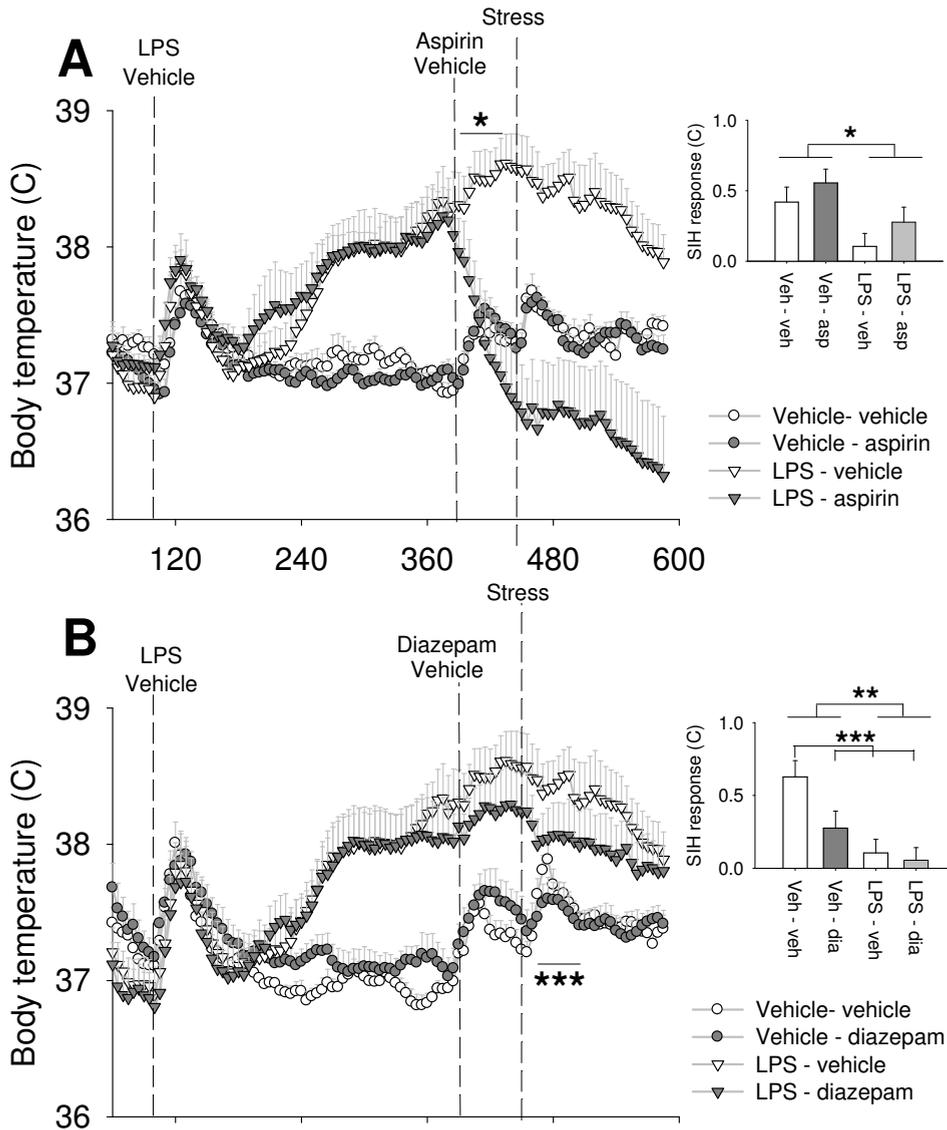
LPS increased body temperature (LPS effect  $F_{1,24}=11.27$ ,  $p<0.001$ ; LPS x time interaction  $F_{2,58}=9.36$ ,  $p<0.001$ ). Aspirin decreased body temperature (aspirin effect  $F_{1,24}=5.95$ ,  $p<0.05$ ; aspirin x time interaction  $F_{2,58}=6.45$ ,  $p<0.01$ ), but only did so in the fever state (LPS x aspirin interaction  $F_{1,24}=7.68$ ,  $p=0.01$ ; LPS x aspirin x time interaction  $F_{2,58}=8.78$ ,  $p<0.001$ ). The fever state was separately analyzed, and effects of aspirin on fever states were confirmed (aspirin effect  $F_{1,13}=8.77$ ,  $p=0.01$ ; aspirin x time interaction  $F_{2,22}=12.94$ ,  $p<0.001$ ), while there were no aspirin effects in the non-fever state (aspirin effect  $F_{1,11}=0.32$ ,  $p=0.58$ , NS; aspirin x time interaction  $F_{3,34}=0.55$ ,  $p=0.66$ , NS). Analysis of the calculated SIH response showed that aspirin administration did not affect the SIH response (aspirin effect  $F_{1,24}=1.66$ ,  $p=0.20$ , NS) but that the SIH response was different in the fever state (LPS effect  $F_{1,24}=6.86$ ,  $p<0.05$ ). Aspirin did not affect the SIH response in the fever as well as the non-fever state (LPS x aspirin interaction,  $F_{1,24}=0.11$ ,  $p=0.75$ , NS). (Figure 5A, inset).

#### *Effects of diazepam on LPS-induced fever (Figure 5B, t=390-450 min):*

LPS induced fever (LPS effect  $F_{1,24}=26.98$ ,  $p<0.001$ ), and diazepam did not affect overall body temperature (diazepam effect  $F_{1,24}=1.51$ ,  $p=0.23$ , NS; diazepam x time interaction  $F_{3,62}=0.75$ ,  $p=0.51$ , NS), nor specifically in fever and non-fever states (LPS x diazepam interaction  $F_{1,24}=0.11$ ,  $p=0.75$ , NS; LPS x diazepam x time interaction  $F_{3,62}=0.45$ ,  $p=0.69$ , NS). Separate analysis of fever and non fever states confirmed that diazepam did not affect body temperature in fever (diazepam effect  $F_{1,12}=0.77$ ,  $p=0.40$ , NS; diazepam x time interaction  $F_{2,28}=0.30$ ,  $p=0.77$ , NS) and non-fever states (diazepam effect  $F_{1,12}=0.92$ ,  $p=0.36$ , NS; diazepam x time interaction  $F_{2,29}=0.86$ ,  $p=0.46$ , NS). Analysis of the calculated SIH response showed that diazepam administration did reduce the SIH response (diazepam effect  $F_{1,24}=4.62$ ,  $p<0.05$ ) but that the SIH response was different in the fever state (LPS effect  $F_{1,24}=12.39$ ,  $p<0.01$ ). Diazepam effects were present regardless of the fever/non-fever state (LPS x diazepam interaction,  $F_{1,24}=2.00$ ,  $p=0.17$ , NS). (Figure 5B, inset).

#### *Effects of aspirin and diazepam on the SIH response in the non-fever state (separate analysis of the non-fever state, figure 5A-B, t=450-510 min):*

Aspirin did not have effects on the SIH response (aspirin effect  $F_{1,11}=0.10$ ,  $p=0.76$ , NS; time x aspirin interaction  $F_{12,24}=0.38$ ,  $p=0.71$ , NS). However, diazepam reduced the SIH response (time x diazepam interaction  $F_{3,37}=3.33$ ,  $p<0.05$ ) in the absence of effects on basal body temperature (diazepam effect  $F_{1,12}=0.31$ ,  $p=0.59$ , NS).



**Figure 5:** Effects of aspirin (300 mg/kg, PO) and diazepam (4 mg/kg, IP) on LPS-induced fever and subsequent stress in **Wistar rats**. **A:** Aspirin has profound effects only in LPS-induced fever states, but has no effect on stress-induced hyperthermia response. Inset: the calculated SIH response is smaller in fever states, and diazepam decreases the SIH response. **B:** Diazepam has no effect on LPS-induced fever states, but reduced the stress-induced hyperthermia response. Inset: the calculated SIH response is smaller in fever states. \*\*\*:  $p < 0.01$ ; \*:  $p < 0.05$ .

## 4. Discussion

The main finding of this study is that the peripheral administration of prostaglandin synthesis-blocking drug aspirin reduced body temperature in infection-induced (IL-1 $\beta$  and LPS) fever, but did not influence the SIH response in rats and mice. While the anxiolytic drug diazepam robustly blocked the SIH response, it had no or minimal effects on infection-induced fever. Therefore, we conclude that prostaglandins are essential in maintaining the fever state after activation of the immune system in rodents, but that peripheral administration of prostaglandin synthesis-blocking drug aspirin does not or minimally influence the stress-induced rise in body temperature. This is in line with known antipyretic effects of NSAIDs (Rotondo et al 1988).

Oppositely, we show that the GABA<sub>A</sub> receptor is closely involved in the SIH response, but plays no or a minimal role in fever states, confirming the anxiolytic effects of diazepam (Korte et al 1990; Olivier et al 2002). To our knowledge, this is the first study to complementarily confirm the differential effects of aspirin and diazepam on SIH and infection-induced fever. Rats as well as mice were studied to confirm that both species responded similarly to all interventions. Body temperature increased before injection stress in mice (Figure 1 and 2) and in rats (Figure 3), which is a known phenomenon (van Bogaert et al 2006b) and may be attributed to early animal disturbance prior to the actual injection stress. The manually calculated SIH response from the time graphs is generally in complete agreement with the time graphs (figure 1-3). Only in fever states or when drugs markedly decrease body temperature, there appears to be a small difference between the calculated SIH response and the time graphs. This difference is attributable to the fact that the calculated SIH response is based on the maximum temperature during the first 30 min after stress. In this way, the calculated SIH response in these cases is likely to yield a positive value. The differences, however, are small and do not change the interpretation of our data. The fact that in figure 4A and B, the “no stress” procedure leads to an apparent SIH response can be explained by the fact that even in the absence of novel cage stress, all animals are exposed to minor disturbance stress, consisting of the investigator entering the room (van Bogaert et al 2006b).

Stress exposure also led to a superimposed SIH response in fever states in mice (figure 4A), indicating that a SIH response can be initiated regardless of the fever state. However, the SIH amplitude is smaller in the fever state which may be attributed to a ceiling effect above which no further temperature rise is possible. Such ceiling effects could be stress-specific, and typically lie around 2-2.5 °C above baseline temperature in non-fever states. Nonetheless, we cannot totally exclude the possibility that higher temperature values can be reached after novel cage stress. In rats, such a superimposed SIH response in fever states is not discernable, indicating that either such a response is not present or not visible due to a ceiling effect. Febrile responses were observed after administration of both LPS and IL-1 $\beta$ , both depending on microsomal prostaglandin E synthase 1 (mPGES-1), the inducible terminal PGE<sub>2</sub> synthesizing enzyme to induce a febrile response (Engblom et al 2003; Saha et al 2005). A febrile response was present in both rats and mice even though the ambient temperature was below the thermoneutral zone (20 $\pm$ 2 °C). Some studies have increased the ambient temperature in order to ensure fever

production (Soszynski et al 1998), although others have been able to produce fever in similar ambient temperatures (Soszynski and Chelminiak 2007). This might depend on animal age, as older rats developed significantly lower LPS and IL-1 $\beta$ -induced fever at 20 °C compared to younger rats (Buchanan et al 2006; Peloso et al 2003).

The different effects of peripheral aspirin and diazepam administration on fever and SIH states, respectively, indicate that although SIH and fever both increase body temperature in rats and mice, they have distinct physiological mediators. The notion of SIH and infection-induced fever as two independent centrally mediated phenomena is supported by studies in which SIH remains present in animals in which fever states can no longer be evoked, such as prostaglandin EP3 receptor null mutation mice (Oka et al 2003; Saha et al 2005) and in lipopolysaccharide tolerant animals (Soszynski et al 1998). Also, antiserum against IL-1 $\alpha$  and IL-1 $\beta$  does not affect the SIH response (Long et al 1990b), and direct corticosterone injection into the anterior hypothalamus attenuates LPS-induced fever but not the SIH response (Morrow et al 1996). Moreover, intracerebroventricular administration of a corticotropin-releasing factor receptor antagonist is able to reduce the SIH response without influencing IL-1 $\beta$ -induced fever (Nakamori et al 1993).

Some studies reported that NSAIDs were able to at least partially attenuate the SIH response (Kluger et al 1987; Morimoto et al 1991; Parrott and DM 1995; Singer et al 1986). The reasons for differences among NSAIDs in modifying the SIH response are unclear. It has been shown that salicylates can decrease normal body temperatures (Satinoff 1972), and in forementioned studies, peripheral administration of NSAIDs only partially reversed the SIH response, allowing other factors to play role in the SIH response. Furthermore, the fact that prostaglandin E2 also attenuates the SIH response complicates interpretation (Long et al 1991). Oka and colleagues suggest that SIH responses caused by conventional psychological stressors such as an open field test respond to NSAID treatment (e.g. (Morimoto et al 1991; Singer et al 1986)), whereas SIH induced by anticipatory anxiety stress is immune to such interventions (Oka et al 2001). This is an interesting suggestion, although the question remains whether conventional stress tests completely lack an anticipatory stress component, and whether cage switch stress can be truly be distinguished from open field stress. Such issues need further investigation. The choice of species may also account for different NSAID sensitivity to SIH. However, this can only offer a partial explanation, as we show that rats do not respond to NSAID treatment in response to stress-induced changes in temperature, and another study did not find effects of NSAIDs on the SIH response in ducks (Gray et al 2008). Moreover, the fact that restraint stress in rats can also lead to hypothermia which is insensitive to NSAIDs shows that stress does not always lead to an increased body temperatures and is a complex phenomenon (Chen and Herbert 1995). However, we cannot exclude the possibility that mice are less sensitive to NSAID-attenuating effects on the SIH response, or that NSAID sensitivity is species-dependent. A final explanation for the apparent differences in NSAID effectiveness in reducing the SIH response may be attributed to differences in administration route. NSAIDs were able to completely reduce the SIH response when they were administered either intravenously or intracerebroventricularly, whereas other studies that administered drugs orally, subcutaneously or intraperitoneally did find no or

at the most partial effectiveness of NSAIDs on the SIH response (Gray et al 2008; Lecci et al 1990b; Olivier et al 2003; Zethof et al 1994).

Even though our data point to different pharmacological mechanisms in SIH and infection-induced fever, both temperature-increasing processes are not completely independent. Chronic stress directly influences the immune system (Leonard and Song 1999), and stress states diminish the duration and magnitude of LPS-induced fever (Gray et al 2008). Also, stress can seriously compromise the effectiveness of the adrenocortical response in containing some immunological defense (Carobrez et al 2002), and the adrenocortical glucocorticoid response itself directly affects LPS-induced fever (Cabrera et al 2000). LPS-induced fever can be influenced by behavioral conditioning, making a limbic input to fever plausible (Ader 2003). Also, IL-1 $\beta$  augments the effects of GABA at GABA<sub>A</sub> receptors by promoting chloride transport (Kang and Miller 1991).

The hypothalamic preoptic area induces fever by activating pathways that include neurons in the dorsomedial hypothalamus (DMH) and the rostral raphe pallidus (Boulant 2000; Cerri and Morrison 2006; Nakamura et al 2005b). DMH activation results in both vasoconstriction and shivering via neurons that project directly to the rostral raphe pallidus (for reviews: (DiMicco et al 2006; Dimicco and Zaretsky 2007)). The rostral raphe pallidus directly controls sympathetic preganglionic neurons in the intermediolateral cell column of the thoracic spinal cord (Nakamura et al 2004; Nakamura et al 2005a). Thus, infectious fever is the result of DMH activation by the hypothalamic preoptic area. The SIH response is an acute stress response and is mediated by limbic brain areas, including various amygdala nuclei (Carrasco and Van de Kar 2003). Especially, the medial amygdala nucleus is involved in the stress reaction (Davis 1997). Local administration of muscimol into the medial amygdala attenuates restraint stress-induced responses (Kubo et al 2004), and the medial amygdala displays c-fos activation after different kinds of acute stressors (Cullinan et al 1995; Dayas et al 2001; Dayas et al 1999; Emmert and Herman 1999; Figueiredo et al 2003a; Kollack-Walker et al 1997; Pezzone et al 1992). A connection between the amygdala and the DMH to cause a SIH response is plausible. Microinjection of bicuculline, a GABA<sub>A</sub> receptor antagonist, into the DMH results in a SIH response, whereas microinjection with muscimol, a GABA<sub>A</sub> receptor agonist into the DMH seems to entirely ablate this temperature increase (unpublished observations in the reference) (Dimicco and Zaretsky 2007). Altogether, a common activation of the DMH by both stress and infection to increase body temperature seems present and might explain overlapping properties and interdependence.

In conclusion, the present study shows that the benzodiazepine diazepam but not the prostaglandin-blocking aspirin attenuated the SIH response in rodents. In contrast, aspirin reduced LPS- and IL-1 $\beta$  induced fever, whereas diazepam had little effect on these fever states. Altogether, our findings support the hypothesis that stress-induced hyperthermia and infection-induced fever are two distinct processes mediated largely by different physiological mechanisms.

## Chapter 8

# **Stress-induced hyperthermia is reduced by rapid-acting anxiolytic drugs independent of injection stress in rats**

Christiaan H. Vinkers

Noëlle M. De Jong

Cor J. Kalkman

Koen G.C. Westphal

Ruud van Oorschot

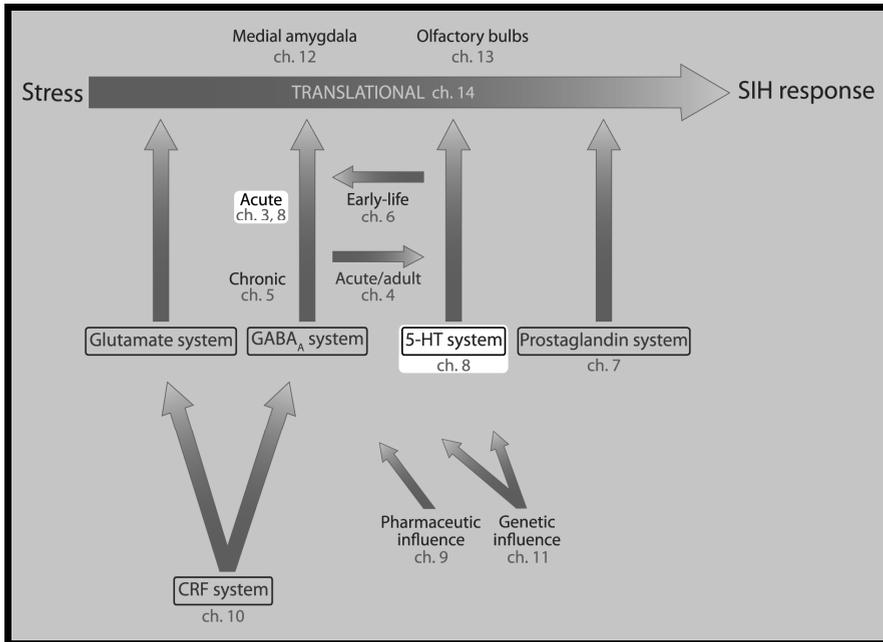
Berend Olivier

S. Mechiel Korte

Lucianne Groenink

8

## Abstract



**Background:** Stress-induced hyperthermia (SIH) is the transient rise in body temperature after encountering a stressor. The SIH response can be blocked by administration of various anxiolytic drugs prior to inducing stress. However, a drug injection involves handling and injection stress and therefore induces a SIH response itself. In the standard SIH test, drugs are therefore injected 60 minutes before stress induction to allow injection-induced hyperthermia to decline. This makes it difficult to study putative anxiolytic compounds with a short half-life. The present study therefore aimed to compare the effects of standard (stressful) and stress-free anxiolytic drug administration on the subsequent SIH response with a 10 minute injection-stressor interval.

**Methods:** Anxiolytic drugs with short half-lives (midazolam, 8-OH-DPAT, nicotine) were injected subcutaneously in rats using either a stressful (manual injection) or stress-free injection (subcutaneous cannula) method 10 minutes before novel cage stress. Body temperature and locomotor activity were measured using telemetric transmitters.

**Results:** Stressful and stress-free drug administration resulted in comparable drug effects on the stress-induced hyperthermia and locomotor responses in rats.

**Conclusion:** The present study shows that both stressful and stress-free drug injection shortly before a stressor results in reproducible attenuation of the SIH response in rats. In rats, a short injection-stressor interval can therefore be applied using the SIH paradigm, enabling the study of putative anxiolytic drugs with short half-lives.

# 1. Introduction

Stress-induced hyperthermia (SIH) is a transient rise in body temperature in response to stress and is comparable across all species (Vinkers et al 2008). Anxiolytic drugs including benzodiazepines and 5-HT<sub>1A</sub> receptor agonists block the SIH response (Olivier et al 2002; Olivier et al 2003). In contrast, non-anxiolytic drugs including dopaminergic and noradrenergic drugs do not affect the response, and the SIH paradigm therefore possesses excellent predictive validity (Bouwknrecht et al 2007).

Administration of a drug involves handling and injection of animals and therefore induces a SIH response itself (Van der Heyden et al 1997). In the classic SIH test using rectal temperature measurements, drugs are therefore injected 60 minutes before a stressor when injection-induced hyperthermia has sufficiently declined (Van der Heyden et al 1997). In mice, an injection-stressor interval shorter than 60 minutes leads to a smaller SIH response because body temperature is increased after injection stress (Van der Heyden et al 1997). This makes it difficult to study putative anxiolytic compounds with a short half-life. For example, injection of nicotine ( $t_{1/2}=6$  min (Petersen et al 1984)) 10 minutes prior to stress led to false-positive results in the SIH test due to an elevated 'basal' temperature in vehicle-treated mice (Bouwknrecht et al 2007). In the same experiment, nicotine had no effects on the SIH response after an injection-stressor interval of 30 minutes, indicating that such an interval extension is not always possible (Bouwknrecht et al 2007).

It is therefore of interest to study the effects of injection stress on anxiolytic drug outcome in the SIH paradigm. Also, the effects of injection stress on the subsequent stress response are unknown. We therefore aimed to compare the effects of standard (stressful) and stress-free administration of various anxiolytic drugs with short half-lives on the SIH and locomotor responses in rats using a 10 minute injection-stressor interval. Locomotor activity was measured to compare the temperature effects of injection stress to locomotor responses. Relatively stress-free drug injection was achieved using a tether-swivel combination connected to a subcutaneous catheter, minimizing handling and injection stress. The anxiolytic midazolam is a benzodiazepine with rapid onset of action and a high metabolic clearance ( $t_{1/2}=27$  min) (Mandema et al 1991; Reves et al 1985). The 5-HT<sub>1A</sub> receptor agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) also possesses anxiolytic effects (Shields and King 2008), and has a half-life of around 30 minutes (Yu and Lewander 1997). Nicotine acts swiftly ( $t_{1/2}=6$  min) on nicotinic receptors known to be involved in anxiety processes (Petersen et al 1984; Picciotto et al 2002).

## 2. Methods and materials

### 2.1 Animals

Male Wistar rats (Harlan Zeist, the Netherlands) were housed socially (four rats per cage) in a controlled environment with a non-reversed 12 hour light/dark cycle (white lights on from 7am-7pm). Animals had unlimited access to food (standard lab chow) and water. One week after arrival, telemetry transmitters were implanted and a subcutaneous cannula was implanted. The implantation of a telemetric transmitter and a subcutaneous cannula were combined into one surgical procedure. After recovery from surgery, rats

were singly housed in type III Macrolon® cages with a plastic tube as cage enrichment. Food (standard lab chow) and tap water were available ad libitum. Once a week, an experimental procedure was carried out. All experiments were carried out with approval of the ethical committee on animal experiments of the Academic Biomedical Center, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki (6<sup>th</sup> revision, 2008).

## **2.2 Surgeries**

### *Telemetry transmitter surgery*

Telemetric devices (type ETA-F20, Data Sciences International, St Paul, MN, USA) were implanted in the abdominal cavity as described earlier (Pattij et al 2001). Prior to surgery, rats received a subcutaneous (s.c.) injection (2ml/kg) of the antibiotic Baytrill® (2.5% enrofloxacin). Rats were anaesthetized using O<sub>2</sub>/NO<sub>2</sub>/Isoflurane gas anesthesia. Carprofen (5 mg/kg, s.c.) was given as an analgetic immediately after surgery and twice daily for two days after surgery. After surgery, animals were housed individually and recovery from surgery was monitored (body weight). Also, all rats had access to wet food and solid drinks for two days after surgery. Wound recovery was regularly checked.

### *Subcutaneous cannula surgery*

Rats were equipped with a subcutaneous cannula that was placed subcutaneously approximately 9 centimeters along the right flank of the animal. Cannulas were made of polyurethane tubing (Instech Laboratories, Plymouth Meeting, PA, USA), and the last 3 centimeters of each cannula were perforated with a needle at every 2 millimeters to allow fluid to spread evenly and to prevent cannula obstruction. The subcutaneous cannula was connected to a Vascular Access Harness (Instech Laboratories, Plymouth Meeting, PA, USA).

## **2.3 Radiotelemetry system**

The radiotelemetry system consisted of an implantable transmitter with two flexible leads (type ETA-F20, Data Sciences International, St Paul, MN, USA), a telemetric receiver (model RPC-1) and a Data Exchange Matrix collecting input from the receivers, all purchased from Data Sciences International (St. Paul, MN, USA). The matrix was connected to a Compaq computer. Signals from the transmitters were passed on *via* a radio signal to the receiver, localized under the animal cage, transforming it into a digital signal. Digital information from the telemetry receivers was collected by the data matrix and provided to the computer where all raw data were stored. Data were collected using Dataquest Gold A.R.T. software (DSI, version 2.2). Raw data consisted of locomotor activity and body temperature responses collected for 10 seconds every 2 minutes.

## **2.4 Experimental procedure**

### *General*

Rats received a stressful or stress-free subcutaneous injection with vehicle or a certain drug dose 10 minutes before novel cage stress. 10 minutes later, rats were placed in a novel cage (clean cage with fresh bedding) and left undisturbed. To prevent habituation to the novel cage procedure, the interval between two experiments was set to be at least

one week. Overall, rats generally received two different treatment with a testing interval of at least one week, in accordance with a one week testing interval in the SIH paradigm to wash out acute drug effects (Vinkers et al 2008). Stress-free vehicle (n=10), midazolam (n=8), nicotine (n=6) and 8-OH-DPAT were administered, as well as stressful vehicle (n=6), midazolam (n=8), nicotine (n=3) and 8-OH-DPAT (n=5).

#### *Stress-free injection method*

The vascular access harness of each rat was connected to a tether (Instech Laboratories, Plymouth Meeting, PA, USA) which was connected to a lever arm with a swivel that was mounted on top of the cage. This setup made it possible to inject drugs via the tubing extending from the swivel at some distance from the cage without any animal handling. All tethers were filled with physiological saline at room temperature before connecting. Rats were connected to the tethers at least 2 hours before the SIH test.

#### *Stressful injection method*

Drugs were injected using a standard subcutaneous injection method on the flank with a needle and syringe.

## **2.5 Drugs**

Midazolam HCl,  $\pm$ -8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and nicotine-tartrate were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) and dissolved in saline. The amount of nicotine-di-tartrate was adjusted to obtain the concentration of free base nicotine as indicated in the literature (Matta et al 2007). An injection volume of 1 ml/kg was used and all drugs were injected subcutaneously. Fresh solutions and suspensions were prepared each testing day, and all drugs were injected at room temperature.

## **2.6 Data analysis**

All data were collected in 2-minute blocks and are displayed as mean  $\pm$  SEM. All experiments were carried out with a between-subject design. Drug effects on body temperature and locomotor activity were analyzed during the first 60 minutes after novel cage stress using a univariate repeated measures analysis of variance (ANOVA) with time as within-subject factor and drug as between-subject factor. In the vehicle conditions, stressful and stress-free injection methods were compared using a univariate repeated measures analysis of variance (ANOVA) with time as within-subject factor and injection method as between-subject factor. Cumulative activity levels were obtained by summation of locomotor activity either during the 10 minute period after injection (reflecting locomotor responses to injection stress) or during the first 60 minutes after the novel cage procedure (reflecting stress-induced locomotor responses), and were compared using a one way ANOVA. A probability level of  $p < 0.05$  was set as statistically significant, probability levels between  $p = 0.05$  and  $p = 0.1$  were regarded as trends. To ensure sufficient power of drug effects on the SIH response, a repeated measures power analysis was conducted based on literature (D'Amico et al 2001). Using a standard deviation of 0.35 (based on our current results), the power during the first 60 minutes after the novel cage stress was over 95%, independent of correlation between the time points (data not shown).

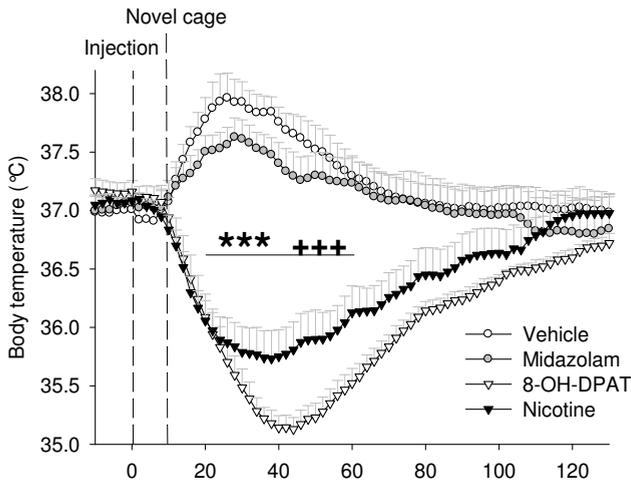
### 3. Results

#### 3.1 Midazolam (3 mg/kg)

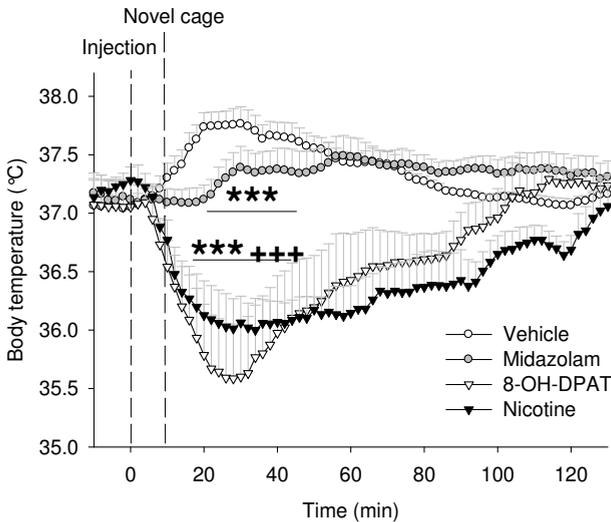
##### Body temperature

Midazolam did not influence basal body temperature (stressful: drug effect  $F_{1,12}=1.05$ ,  $p=0.33$ , NS; stress-free: drug effect  $F_{1,16}=2.07$ ,  $p=0.17$ , NS). Stressful injection of midazolam did not significantly reduce the SIH response (drug x time interaction  $F_{29,348}=1.07$ ,  $p=0.37$ , NS), whereas stress-free injection did reduce the SIH response (drug x time interaction  $F_{29,464}=4.51$ ,  $p<0.001$ ) (Figure 1).

#### A: stressful



#### B: stress-free



**Figure 1:** Effects of stressful (A) and stress-free (B) subcutaneous injection of midazolam (3 mg/kg), 8-OH-DPAT (0.4 mg/kg) and nicotine (1 mg/kg) on the novel cage-induced stress-induced hyperthermia (SIH) response. \*: time x drug interaction compared to vehicle (\*\*\*:  $p<0.001$ ). +: overall drug effect on body temperature compared to vehicle (+++:  $p<0.001$ )

*Locomotor activity*

Midazolam reduced stress-induced locomotor activity after both stressful and stress-free injection (*stressful*: drug x time interaction  $F_{29,348}=2.92$ ,  $p<0.001$ ; drug effect  $F_{1,12}=20.64$ ,  $p<0.001$ ; *stress-free*: drug x time interaction  $F_{29,464}=0.68$ ,  $p=0.90$ , NS; drug effect  $F_{1,16}=5.92$ ,  $p<0.05$ ). Midazolam also decreased cumulative locomotor levels after novel cage stress (*stressful*:  $F_{1,13}=20.64$ ,  $p<0.001$ ; *stress-free*:  $F_{1,17}=5.92$ ,  $p<0.05$ ), but not directly after injection (*stressful*:  $F_{1,13}=0.11$ ,  $p=0.74$ , NS; *stress-free*:  $F_{1,17}=2.30$ ,  $p=0.15$ , NS). (Figure 2, right panel).

**3.2 8-OH-DPAT (0.4 mg/kg)***Body temperature*

8-OH-DPAT reduced the SIH response and basal body temperature after both the stressful and the stress-free injection method (*stressful*: drug x time interaction  $F_{29,319}=32.20$ ,  $p<0.001$ ; drug effect  $F_{1,11}=97.64$ ,  $p<0.001$ . *Stress-free*: drug x time interaction  $F_{29,377}=18.11$ ,  $p<0.001$ ; drug effect  $F_{1,13}=21.09$ ,  $p=0.001$ ) (Figure 1).

*Locomotor activity*

8-OH-DPAT increased stress-induced and overall locomotor activity after both injection methods (*stressful*: drug x time interaction  $F_{29,319}=2.99$ ,  $p<0.001$ ; drug effect  $F_{1,11}=20.35$ ,  $p=0.001$ . *Stress-free*: drug x time interaction  $F_{29,377}=4.23$ ,  $p<0.001$ ; drug effect  $F_{1,13}=6.39$ ,  $p<0.05$ ). 8-OH-DPAT also increased the calculated cumulative locomotor levels under both conditions after injection (*stressful*:  $F_{1,12}=54.17$ ,  $p<0.001$ ; *stress-free*:  $F_{1,14}=19.78$ ,  $p<0.001$ ) and after novel cage stress (*stressful*:  $F_{1,12}=20.354$ ,  $p<0.001$ ; *stress-free*:  $F_{1,14}=6.39$ ,  $p<0.001$ ) (Figure 2, right panel).

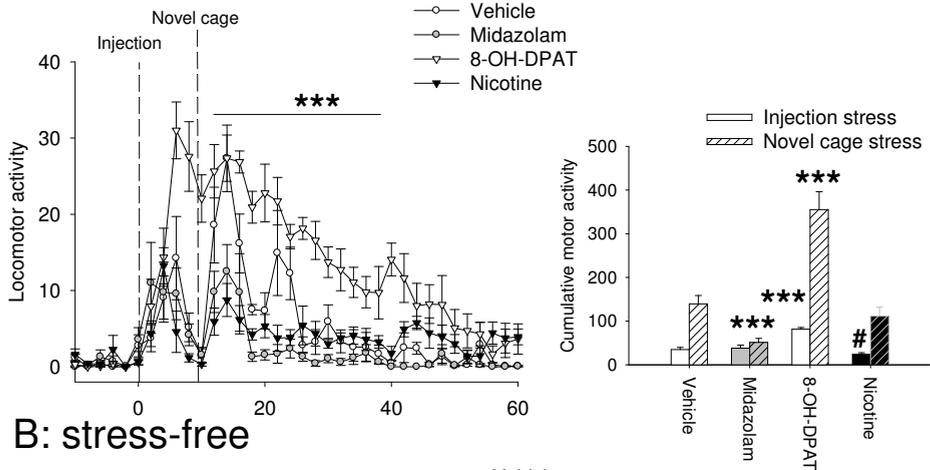
**3.3 Nicotine (1 mg/kg)***Body temperature*

Nicotine reduced the SIH response and basal body temperature after stressful and stress-free injection (*stressful*: drug x time interaction  $F_{29,290}=16.70$ ,  $p<0.001$ ; drug effect  $F_{1,10}=40.87$ ,  $p<0.001$ . *Stress-free*: drug x time interaction  $F_{29,390}=4.56$ ,  $p<0.001$ ; drug effect  $F_{1,11}=38.55$ ,  $p<0.001$ ) (Figure 1).

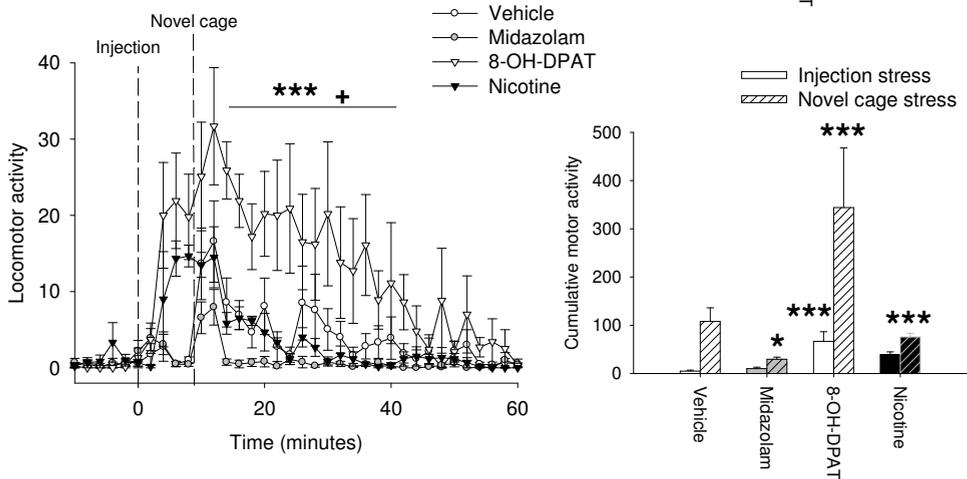
*Locomotor activity*

Nicotine reduced stress-induced locomotor levels after stressful injection (drug x time interaction  $F_{29,290}=5.35$ ,  $p<0.001$ ) but not after stress-free injection (drug x time interaction  $F_{29,319}=0.21$ ,  $p=0.97$ , NS). Overall locomotor activity levels after novel cage stress were however not affected by nicotine (*stressful*:  $F_{1,10}=1.00$ ,  $p=0.34$ , NS; *stress-free*: drug effect  $F_{1,11}=0.36$ ,  $p=0.56$ , NS). Nicotine did also not affect the calculated cumulative activity after novel cage stress relative to vehicle (*stressful*: NC:  $F_{1,11}=1.00$ ,  $p=0.34$ , NS; *stress-free*:  $F_{1,12}=0.36$ ,  $p=0.56$ , NS) (Figure 2, inset). In contrast, cumulative locomotor activity levels were increased immediately after nicotine injection independent of injection method (*stressful*:  $F_{1,11}=3.11$ ,  $p=0.09$ , trend; *stress-free*:  $F_{1,12}=53.60$ ,  $p<0.001$ ) (Figure 2, inset).

## A: stressful



## B: stress-free



**Figure 2:** Effects of stressful (A) and stress-free (B) subcutaneous injection of midazolam (3 mg/kg), 8-OH-DPAT (0.4 mg/kg) and nicotine (1 mg/kg) on the novel cage-induced locomotor response. \*: time x drug interaction compared to vehicle (\*\*\*: 8-OH-DPAT,  $p < 0.001$ ) +: overall drug effect on body temperature compared to vehicle (+: midazolam,  $p < 0.05$ ). **Inset Figure 2 A and B:** Cumulative activity response after injection and after novel cage stress. \*\*\*:  $p < 0.001$ ; \*:  $p < 0.05$ ; #:  $p = 0.09$  compared to vehicle.

### 3.4 Stressful and stress-free vehicle injection compared

Stressful and stress-free vehicle injection did not differ in basal body temperature during the 10 minutes after injection (method effect  $F_{1,14} = 0.92$ ,  $p = 0.35$ , NS; time x method interaction  $F_{1,19} = 0.02$ ,  $p = 0.23$ , NS), whereas locomotor activity levels were increased only after stressful injection (method effect  $F_{1,14} = 44.26$ ,  $p < 0.001$ ; time x method interaction  $F_{2,26} = 3.38$ ,  $p = 0.05$ ). Cumulative activity levels confirmed that stressful injection led to increases locomotor activity after injection (method effect  $F_{1,15} = 44.26$ ,  $p < 0.001$ ). Although the SIH response in the stressful injection group was larger after novel cage stress (time x

method interaction  $F_{29,406}=1.77$ ,  $p=0.01$ ), both groups had a similar basal body temperature (method effect  $F_{1,14}=0.01$ ,  $p=0.98$ , NS). Stressful vehicle injection led to higher locomotor activity levels after novel cage stress relative to the stress-free vehicle injection (method x time interaction  $F_{29,406}=3.39$ ,  $p<0.01$ ), although overall locomotor levels were not different (method effect  $F_{1,14}=0.63$ ,  $p=0.44$ , NS). Cumulative activity levels confirmed that overall activity was similar after novel cage stress (method effect  $F_{1,15}=0.63$ ,  $p=0.44$ , NS).

## 4. Discussion

The present study compared standard (stressful) and stress-free drug injection shortly before novel cage stress in the stress-induced hyperthermia (SIH) model. The SIH paradigm uses the transient body temperature increase in response to stress that can be blocked by various anxiolytic drugs. However, administration of a drug involves handling and injection of animals and thus induces a autonomic stress response itself in both rats and mice (Van der Heyden et al 1997). This makes it difficult to study the autonomic stress response when putative anxiolytic drugs are injected shortly before a stressor (Bouwknrecht et al 2007). Using a swivel-tether combination connected to a subcutaneous catheter, we were able to reduce the stress associated with manual (stressful) drug injections as stress-free injections did not increase locomotor responses and led to no apparent behavioral responses in the rat (Figure 2B).

Both stressful and stress-free injection of anxiolytic drugs with a short half-life (8-OH-DPAT and nicotine) resulted in a robust attenuation of the SIH response in rats (Figure 1). This indicates that a short injection-stressor interval can be used to study the effects of anxiolytic drugs on the autonomic stress response. In this way, compounds with a short half-life or lower doses of a compound can be assessed. In contrast, midazolam did not reduce the SIH response in the stressful injection method, although comparison by the eye might suggest otherwise (Figure 1). This suggests that the stress-free injection method may be more sensitive to register anxiolytic effects on the SIH response. The GABA<sub>A</sub> receptor agonist midazolam, the nicotine receptor agonist nicotine and the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT all led to a robust decrease in stress-induced and basal body temperature. The effects of both 8-OH-DPAT and nicotine on body temperature are in line with known hypothermic and stress-induced hyperthermia reducing effects at similar doses in rats (Gordon et al 2002; Rusyniak et al 2007). Furthermore, we found that nicotine at a dose of 0.25 mg/kg reduced the SIH response without causing hypothermia independent of injection method (data not shown), which is again in line with nicotine effects on body temperature at lower doses (Gordon et al 2002).

In general, anxiolytic drugs that attenuate the SIH response also lead to hypothermia and disturb thermoregulatory processes (Vinkers et al 2008). Therefore, in the current study, a complete distinction between an attenuation of the SIH response and a general reduction of the basal body temperature cannot be made. Stressful drug injection led to an overall less variable response, probably due to a better and more consistent drug delivery after manual injection (figure 1 and 2). In contrast to our study, injection stress in mice results

in an almost maximal hyperthermia after 10 minutes (Van der Heyden et al 1997). This difference may be attributed to a more controlled thermoregulation in rats, leading to a less reactive and less pronounced SIH response in reaction to injection stress. In support, we earlier found that handling stress in three different mouse strains led to a consistent SIH response of around 2 °C (van Bogaert et al 2006b), whereas handling stress in a rat leads to a SIH response of maximally 1°C (Vinkers et al 2008). The fact that stress does not immediately increase body temperature cannot be ascribed to physical transmitter delay as in the aforementioned study, various stressors increased body temperature in three different mouse strains within 2 minutes using identical telemetry transmitters (van Bogaert et al 2006b).

To our knowledge, this is the first study in which nicotine reduced the SIH response. In an earlier study in mice that used a similar injection-stressor interval, nicotine did not attenuate the SIH response (Bouwknicht et al 2007). In this study, injection stress itself increased baseline temperature in mice and, as body temperature had not returned to baseline values, consequently reduced the SIH amplitude after vehicle treatment. 8-OH-DPAT was also able to reduce the SIH response, an effect that was already earlier found in mice using 6- to 25-fold higher doses which were injected 30 minutes before a stressor (Borsini et al 1989). The effects of the benzodiazepine midazolam and the 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT in the present study are in general agreement with known SIH-attenuating effects of similar acting drugs with longer half-lives, such as diazepam and flesinoxan (Vinkers et al 2009f; Vinkers et al 2008). Midazolam led to overall sedation regardless of injection method (Figure 2), which is in line with known sedative effects (Lau et al 1996). Also, both stressful and stress-free injection of 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT resulted in direct locomotor stimulant effects (Figure 2), which is attributed to presynaptic 5-HT<sub>1A</sub> receptor activation (Chen and Reith 1995; Karamanakos et al 2004).

Interestingly, stressful vehicle injection subsequently led to a larger SIH and locomotor response to novel cage stress compared to stress-free vehicle injection (Figures 1 and 2). This is an interesting phenomenon, which may be explained by the fact that a stressful event (manual injection) is followed by another (relative) stressful event (novel cage stress). In animals, stress exacerbates subsequent anxiety-like responses in a number of anxiety models even immediately after an acute stressor (File et al 1992; MacNeil et al 1997; Vinkers et al 2008), and also in humans, unconditioned anxiety is enhanced by prior stress (Lissek et al 2005). In addition, there is a link in rodents between prior stress and increased subsequent locomotor responses to psychostimulants (Veening et al 2005).

In conclusion, the present study shows that both stressful (manual) and stress-free administration of anxiolytic drugs with short half-lives shortly before novel cage stress lead to a reduced SIH response. Thus, manual drug administration combined with a short injection-stressor interval can be applied to study in the SIH paradigm in rats. This opens up possibilities to study lower doses of anxiolytic drugs or to assess putative anxiolytic drugs with short half-lives in the SIH paradigm.

## Chapter 9

# The rapid hydrolysis of chlordiazepoxide to demoxepam has consequences for chronic minipump applications

Christiaan H. Vinkers

Gerdien A.H. Bouws-Korte

Javier Sastre Toraño

Naheed R. Mirza

Elsebet Ø. Nielsen

Philip K. Ahring

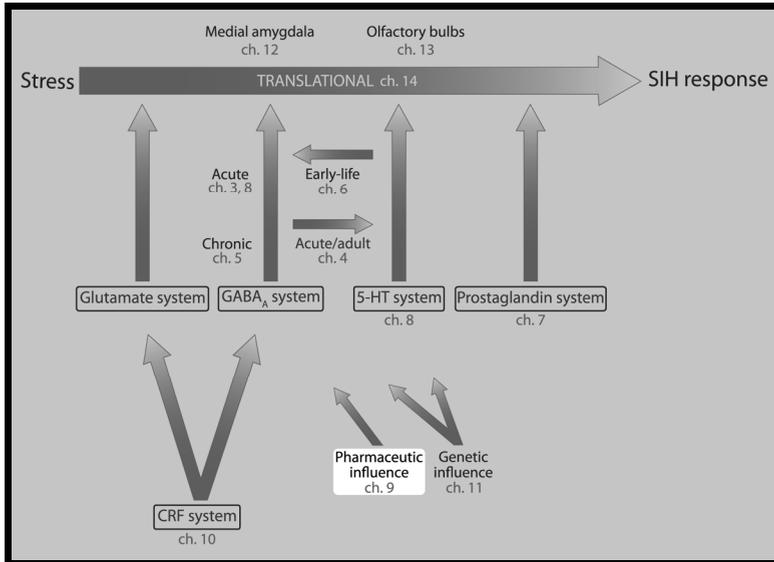
Gerhardus J. de Jong

Berend Olivier

*Submitted*

9

## Abstract



**Background:** When designing chronic studies using benzodiazepines, chlordiazepoxide (CDP) is often the preferred drug of choice because, in contrast to most other classical benzodiazepines, it is soluble in water. However, the rapid hydrolysis of CDP in aqueous solution to the active ketone product demoxepam has been described. This would diminish CDP plasma levels in minipump studies over time and introduce one or more active compounds that would be co-released from the minipump.

**Methods:** Therefore, the present study aimed to explore the putative hydrolysis of the classical benzodiazepine CDP in aqueous solution and in minipumps over time and to identify the hydrolysis products using mass spectrometry. Moreover, we aimed to characterize the hydrolysis products for their *in vitro* ( $^3\text{H}$ -flunitrazepam binding and oocyte electrophysiology) and *in vivo* (stress-induced hyperthermia paradigm) GABA<sub>A</sub> receptor potency.

**Results:** Chlordiazepoxide (CDP) in solution rapidly hydrolyzed to the ketone structure demoxepam with a half-time of around 9 days at 37 °C. Using mass spectrometry, we confirmed that the single hydrolysis product is demoxepam which is the result of oxidative deamination of CDP. The CDP hydrolysis was concentration dependent (following first-order kinetics) as well as temperature dependent. In osmotic minipumps, hydrolysis to demoxepam was again apparent. Using the mouse stress-induced hyperthermia paradigm, our *in vivo* data suggest that CDP may exert greater acute potency compared to demoxepam. In support, CDP showed increased efficacy and affinity for GABA<sub>A</sub> receptors containing  $\alpha_1$  (but not  $\alpha_3$ ) subunits. *In vitro*  $^3\text{H}$ -flunitrazepam binding was not different between CDP and demoxepam.

**Conclusions:** The present study shows that the classical benzodiazepine CDP is rapidly hydrolyzed in solution to the active compound demoxepam with a reduced activity at the GABA<sub>A</sub> receptor. The fact that CDP is readily soluble in water makes it an attractive choice for minipump applications, but the separate effects of CDP and its active hydrolysis product demoxepam are hard to dissect. Thus, chronic studies that use dissolved CDP should be interpreted with caution. In chronic studies, drug stability and release should always be considered and, when needed, carefully investigated.

## 1. Introduction

The GABA<sub>A</sub> receptor is the main inhibitory receptor in the central nervous system known to be closely involved in stress and anxiety processes. Classical (non-selective) benzodiazepines are clinically relevant anxiolytic drugs that act on GABA<sub>A</sub> receptors, allosterically enhancing the inhibitory GABA actions by binding to  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  subunits (Rudolph and Mohler 2006). Besides the preferred anxiolytic action, benzodiazepines possess a wide variety of adverse effects, including dependence, tolerance, withdrawal, sedation and cognitive impairing effects. Recently, the concept that distinct GABA<sub>A</sub> receptor  $\alpha$  subtypes generate specific (side) effects has led to an increasing body of research studying the acute and chronic effects of classical benzodiazepines (Crestani et al 2001; Rudolph et al 1999).

In clinical practice, benzodiazepines are usually prescribed for a longer period (ranging from weeks, months to even years). Animal studies that aim to elucidate the largely unknown mechanisms underlying the chronic benzodiazepine (side) effects either administer drugs manually or use osmotic minipumps (hereafter referred to as "minipumps"). The main advantage of osmotic minipumps is a continuous drug release for a longer period whereas no animal handling is needed during the entire delivery period. However, when loaded into a minipump, a drug needs to be dissolved in order to reach the desired controlled delivery. If a compound cannot be dissolved or is unstable in solution, this may have enormous consequences for the interpretation of minipump studies (van der Zwaal et al 2008).

When designing chronic minipump studies using classical benzodiazepines, chlordiazepoxide (CDP) is often the preferred drug of choice (e.g. (Kas et al 2008; West and Weiss 2005)), because it - in contrast to most other classical benzodiazepines - is soluble in water (1:10) (Moffat et al 2004). However, the rapid hydrolysis of CDP in aqueous solution to the active ketone product demoxepam has been described (Han et al 1976). This would diminish CDP plasma levels in minipump studies over time and introduce an active second compound that would be co-released from the minipump. Therefore, the present study aimed to explore the putative hydrolysis of the classical benzodiazepine CDP in aqueous solution and in minipumps over time and to identify the hydrolysis products using mass spectrometry. Moreover, we characterized the hydrolysis products for their *in vitro* (<sup>3</sup>H-flunitrazepam binding and oocyte electrophysiology) and *in vivo* (stress-induced hyperthermia paradigm) GABA<sub>A</sub> receptor potency.

## 2. Materials and methods

### 2.1 Drugs

Chlordiazepoxide HCl (Pharbita, Zaandam, the Netherlands) was freshly dissolved at each testing day. Demoxepam was obtained by keeping CDP at a concentration of 2 mg/ml at a temperature of 37 °C for 40 days. Subsequently, it was filtered through a 0.2  $\mu$ m filter and stored at -80 °C. Frozen demoxepam aliquots were defrosted shortly before experiments.

## 2.2 Identification of the chlordiazepoxide hydrolysis products using LC-MS/MS

To confirm the identity of the CDP hydrolysis products, a LC-MS/MS setup was used. Chlordiazepoxide HCl (throughout the present study, CDP in the salt form was used) at a concentration of 2 mg/ml was kept for 40 days at a temperature of 37 °C and subsequently stored at -80 °C. The solution was then analyzed using a liquid chromatography system and an Agilent 1100 Series LC/MSD SL ion-trap mass spectrometer equipped with an electrospray ionization source (MS, Agilent Technologies). The mobile phase consisted of 100mM ammonium formate (pH 4.5)-methanol (1:1, v/v) and the flow rate was 0.5 ml/min. The MS was operated in positive ion mode, the electrospray voltage was optimized and set to +3.5 kV and lens voltages were optimized for maximal signal intensity. The nebulizing gas pressure was adjusted to 50 psi (345kPa) and the flow of the drying gas to 10 L/min with a drying temperature of 350 °C. The scan range was 140–310 m/z and 5 scans were averaged for one spectrum. Collision-induced dissociation (CID) with helium as collision gas was performed on the protonated molecular ion ( $[M+H]^+$ ) of the degradation product. The m/z-values of the precursor ion was manually selected, and the collision energy was set to the instrument default value of 100% (1.00 V), resulting in significant presence of fragmentation ions.

## 2.3 Stability of chlordiazepoxide in solution

To investigate the hydrolysis kinetics under various concentrations and environmental temperature conditions, the stability of a standard CDP solution was studied using a 10 µg/ml CDP solution that was stored in a thermostated autosampler at a temperature of 37°C. These samples were analyzed over time in the next 45 days. Furthermore, the influence of environmental temperature on CDP hydrolysis was studied by storing a 2 mg/ml CDP solution at various temperatures with subsequent analysis over time. Concentration-dependent hydrolysis was studied by storing CDP solutions at three different concentrations (0.02, 0.2 and 2 mg/ml) at 37°C during 35 days which were subsequently analyzed. For all samples, CDP and putative hydrolysis products were detected simultaneously by HPLC with UV detection at a wavelength of 254 nm (Spectroflow 757 Kratos Analytical). The system consisted of a pump (SpectraSeries P100 Thermo Separation products), a vacuumdegasser (ERC-3113 Erma CR Inc., Tokyo), an autosampler (SpectraSeries AS300 Thermo Separation products), a column (Hypersil BDS 150 mm x 4.6 mm x 5µm) and a detector. The mobile phase consisted of 50 mM Phosphoric acid (pH 6.5) in 50 % methanol and was pumped at 0.5 ml/min. From each sample, 100 µl was injected onto the column. The chromatograms were recorded and analyzed using the Atlast 2003 chromatography data handling system (Thermo Election Corporation, Altrincham, UK). For degradation plots, the peak area of demoxepam after 35 days was set at 100 – (% CDP).

## 2.4 Chlordiazepoxide delivery rates from osmotic minipumps

To confirm that chlordiazepoxide delivery would indeed decline over time, three osmotic minipumps (Alzet, 2004 model, pumping rate 0.25 µg/h during 28 days) were filled with a CDP solution (2 mg/ml). Osmotic minipumps were put into separate tubes containing 7.0 ml of saline and were kept at 37°C. Minipumps were transferred regularly to novel saline tubes for 5 weeks. The concentrations CDP and demoxepam were determined using an identical HPLC with UV setup as above.

## 2.5 In vitro <sup>3</sup>H-flunitrazepam binding and oocyte electrophysiology

To assess the in vitro affinity and efficacy of CDP and demoxepam for the GABA<sub>A</sub> receptor, in vitro <sup>3</sup>H-flunitrazepam and oocyte electrophysiology experiments were carried out. In vitro <sup>3</sup>H-flunitrazepam binding was done as described previously (Mirza et al 2008). Shortly, aliquots of 500 µl of rat cortical membranes were added to 25 µl of test compound, and 25 µl (1 nM, final concentration) of [<sup>3</sup>H]flunitrazepam (88 Ci/mmol; GE Healthcare) were mixed and incubated for 40 min at 2°C. Binding was terminated by rapid filtration, and the amount of radioactivity on the filters was determined by conventional liquid scintillation counting. Electrophysiological responses from *X. laevis* oocytes were measured using the two-electrode-voltage clamp technique as described previously (Mirza et al 2008). Briefly, GABA was freshly dissolved in OR2 in a concentration known to elicit EC<sub>5</sub>-EC<sub>25</sub> currents for a given GABA<sub>A</sub>R subtype combination (0.5-5 µM) and this solution was used for controls as well as a stock solution for dissolving demoxepam. Modulatory effects of demoxepam were calculated by comparing demoxepam traces to a prior GABA control trace. To enable comparison of effects between individual oocytes, demoxepam potentiations were normalized to a control 0.5 µM diazepam potentiation on the same oocyte.

## 2.6 In vivo comparison of chlordiazepoxide and demoxepam

### The stress-induced hyperthermia (SIH) procedure

To screen for the anxiolytic and hypothermic potency of a CDP and demoxepam, the SIH paradigm was applied. The SIH paradigm uses the stress-induced increase in body temperature which is considered to be a functional body response in anticipation to physiological demands (preparation for fight or flight) (Vinkers et al 2008). In the SIH paradigm, classical as well as subunit-selective benzodiazepines consistently reduce the SIH response as well as basal body temperature levels at higher doses. In the present study, the SIH tests were carried out according to the standard procedures (Vinkers et al 2009g). Briefly, the rectal basal body temperature (T<sub>1</sub>) is measured and functions as a stressor as well, and is followed 10 min later by a second rectal temperature measurement T<sub>2</sub> that reflects the stress-induced body temperature. The difference (ΔT=T<sub>2</sub>-T<sub>1</sub>) is the SIH response. A between-subject design was used. Cages were randomly and evenly allocated over daytimes (morning-afternoon). The temperature of mice was measured by rectally inserting a thermistor probe by a length of 2 cm. Digital temperature recordings were obtained with an accuracy of 0.1 °C using a Keithley 871A digital thermometer (NiCr- NiAl thermocouple). The probe, dipped into silicon oil before inserting, was held in the rectum until a stable rectal temperature had been obtained for 20 s. Animals were injected intraperitoneally with either CDP, demoxepam or vehicle (intraperitoneally, 10 ml/kg) 60

min before the first temperature measurement ( $T_1$ ). The temperature was again measured 10 min later ( $T_2$ ).

### Animals

For in vivo experiments, male mice (129Sv/EvTac) were obtained from Taconic M&B, Ry, Denmark. Animals were housed in Macrolon type 3 cages enriched with bedding and nesting material under a 12-h light/12-h dark cycle (lights on from 0600 to 1800 h) at controlled temperature ( $20 \pm 2$  °C) and relative humidity (40–60%) with free access to standard food pellets and tap water. Experiments were carried out with approval of the ethical committee on animal experiments of the Faculties of Sciences, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki.

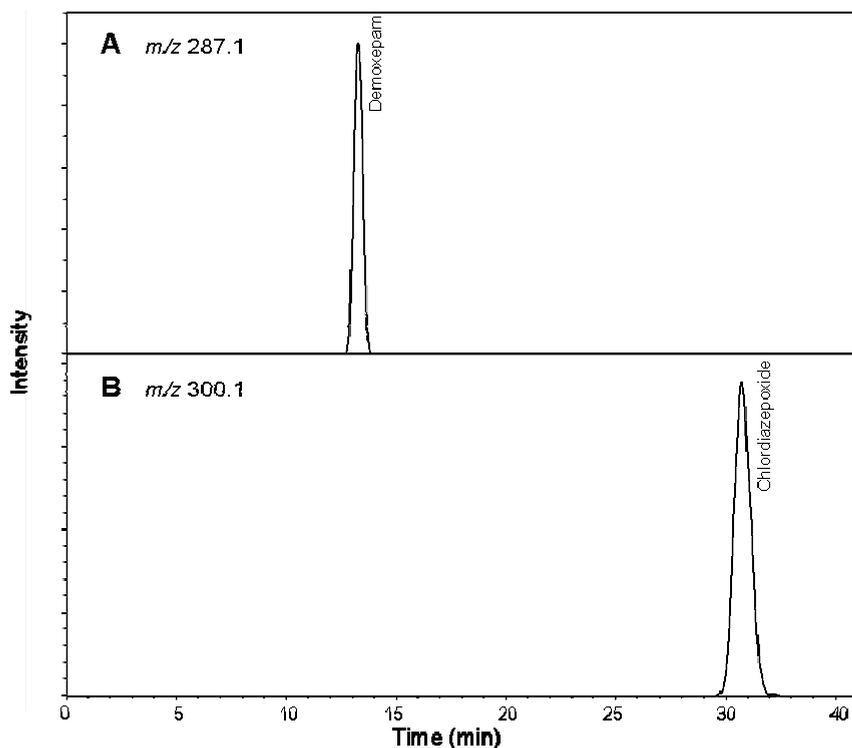
### Statistics

For each individual mouse, a basal temperature ( $T_1$ ), an end temperature ( $T_2$ ) and the difference (SIH response =  $T_2 - T_1$ ) was determined. Treatment effects were evaluated using a one-way analysis of variance with explanatory factor treatment. If the overall analysis of variance appeared significant, post hoc tests (Dunnet's post hoc test) were used to identify significant differences.

## **3. Results**

### **3.1 Identification of the chlordiazepoxide hydrolysis products using LC-MS/MS**

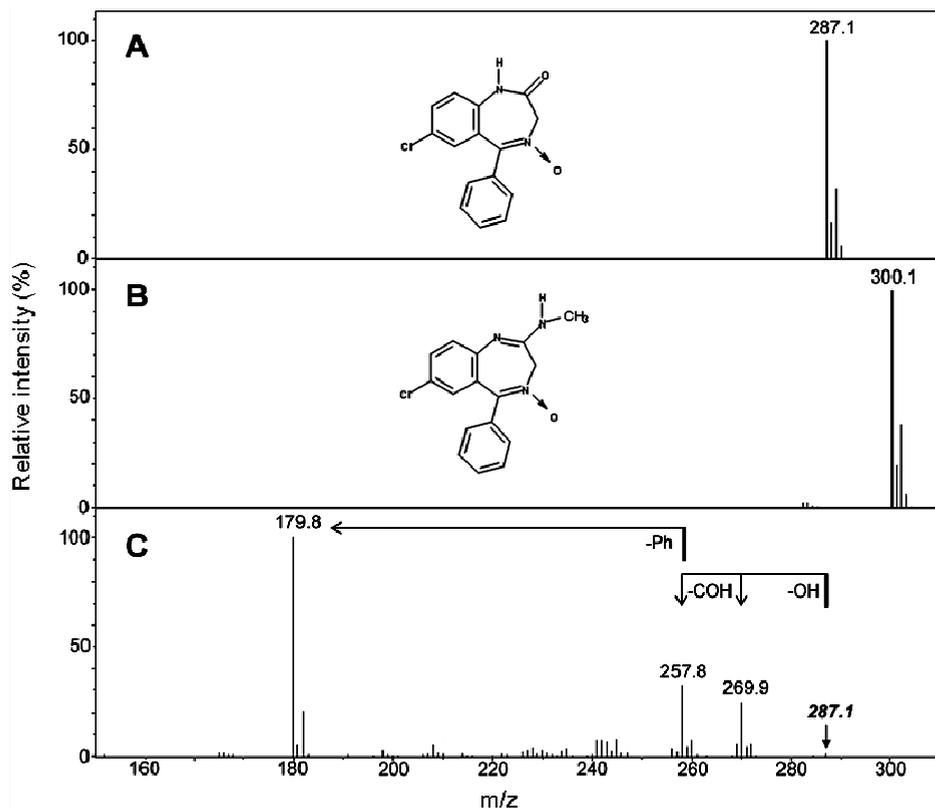
The extracted ion chromatogram at  $m/z$  300.1 showed the presence of CDP (Fig. 1B). The first and only eluting degradation product with  $m/z$  287.1 was demoxepam, the CDP hydrolysis product after oxidative deamination (Fig. 1A). The typical mass spectra of CDP ( $[M+H]^+ = 300.1$ ) and the hydrolyzed product demoxepam ( $[M+H]^+ = 287.1$ ), both with a typical chloride isotopic distribution pattern, are presented in figure 2. The identification of demoxepam (with a calculated monoisotopic mass of 286.1) was performed by MS/MS of  $m/z$  287.1. The resulting fragments (Fig. 2C) showed the loss of a hydroxyl (corresponding to a N-oxide benzodiazepine), a  $-COH$  (corresponding to a benzodiazepine with carbonyl group) and a phenyl group. The interpretation of the various fragments of demoxepam is in line with literature (Nedved et al 1996; Risoli et al 2007; Smyth et al 2000).



**Figure 1:** Extracted ion traces of demoxepam (A) and chlordiazepoxide (B) obtained by LC-MS of a 2 mg/ml chlordiazepoxide solution kept for 35 days at 37°C.

### 3.2 Stability of chlordiazepoxide in solution

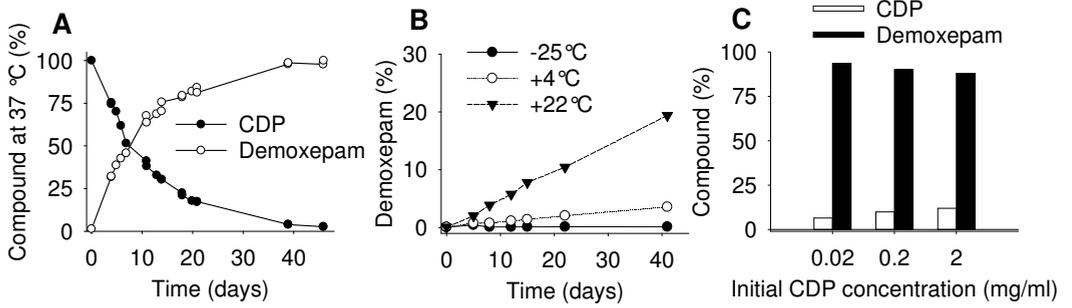
At 37°C, CDP was rapidly degraded into demoxepam with a half-time of around 8.8 days (Fig. 3A). CDP hydrolysis strictly followed first order kinetics ( $[CDP]_t = [CDP]_0 \times e^{-kt}$  with  $t$  being time and  $k$  being the first order rate constant) and was temperature dependent. At storage conditions below 0°C, no apparent hydrolysis occurred (Fig. 3B). Identical to the results at -25 °C, storage conditions at -80 °C did not result in any hydrolysis (data not shown). In line with first order kinetics, CDP hydrolysis was concentration dependent (Fig. 3C).



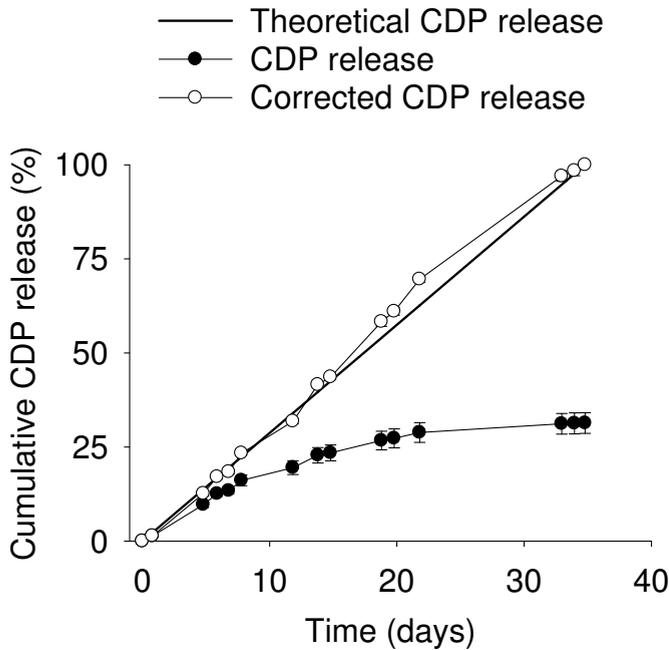
**Figure 2:** Mass spectra and molecular structures of demoxepam with  $[M+H]^+$  287.1 (A) and chlordiazepoxide with  $[M+H]^+$  300.1 (B) obtained by LC-MS analysis of the mixture of chlordiazepoxide and demoxepam in the 2 mg/ml chlordiazepoxide solution kept for 35 days at 37°C. The CID mass spectrum of  $m/z$  287.1 confirmed the presence of demoxepam (C).

### 3.3 Chlordiazepoxide delivery rates from osmotic minipumps

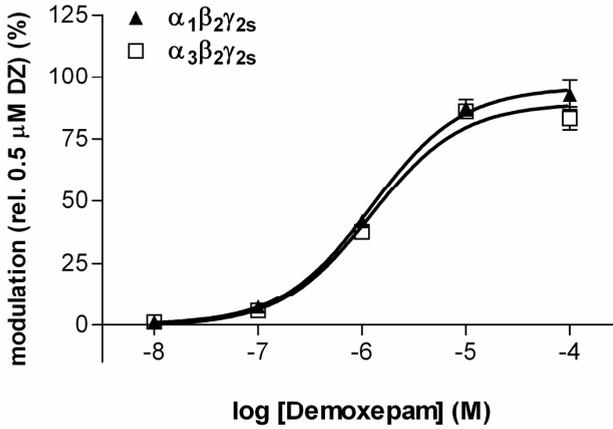
CDP release from the minipump ( $n=3$ ) declined over time (Fig. 4A, black symbols). After 28 days, only 25% of all CDP was released from the minipumps. When the cumulative CDP concentration over time was corrected for its hydrolysis, drug release from the minipumps followed the theoretical release profile over time (white symbols), suggesting that CDP hydrolysis completely accounted for the declined CDP release over time.



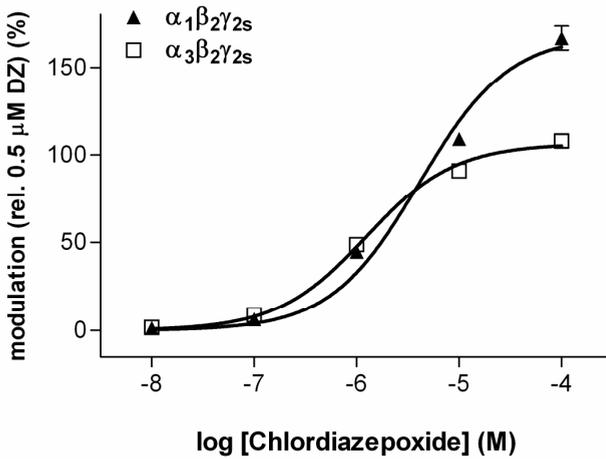
**Figure 3:** Chlordiazepoxide (2 mg/ml) rapidly hydrolyzes into demoxepam at 37 °C (A). This hydrolysis rate is absent at lower temperatures (B), and concentration-dependent (C, compound after 35 days at 37°C)



**Figure 4:** Release of chlordiazepoxide (2 mg/ml) from osmotic minipumps (n=3) over time. CDP release declines over time (black symbols). When CDP hydrolysis is taken into account, CDP release follows the theoretical curve (white symbols), indicating that it is CDP hydrolysis that leads to declined CDP release.



Demoxepam	$\alpha_1\beta_2\gamma_2s$	$\alpha_3\beta_2\gamma_2s$
$E_{Max}$ %	96	89
$EC_{50}$ nM	1240	1220
# oocytes	8	5



CDP	$\alpha_1\beta_2\gamma_2s$	$\alpha_3\beta_2\gamma_2s$
$E_{Max}$ %	168	106
$EC_{50}$ nM	4100	1200
# oocytes	15	11

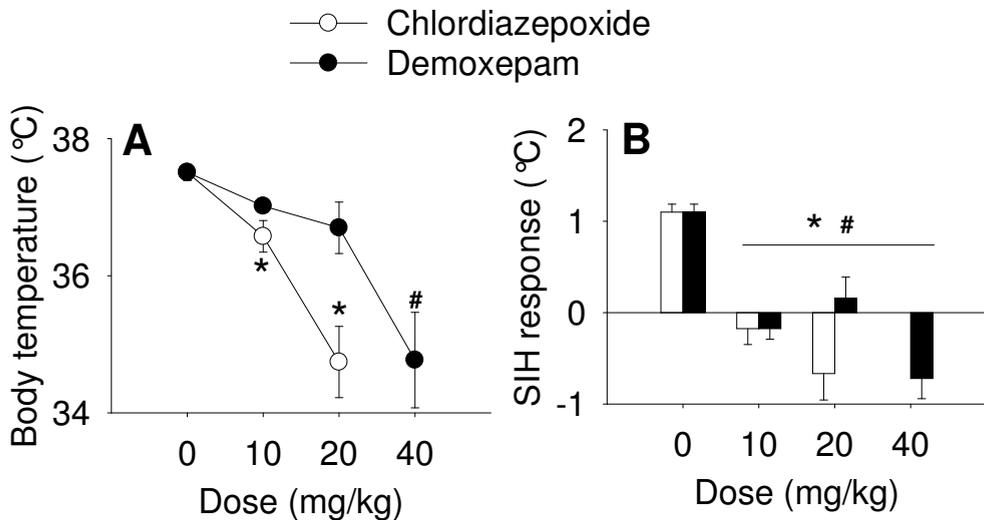
**Figure 5:** Efficacy (compared to diazepam) of demoxepam (upper figure) and chlordiazepoxide (lower figure) in oocytes expressing GABA<sub>A</sub> receptors containing  $\alpha_1$  and  $\alpha_3$  subunits.

**Table 1:** Inhibition of  $^3\text{H}$ -flunitrazepam ( $^3\text{H}$ -FNM) binding to rat cortical membranes by chlordiazepoxide and demoxepam. Data are mean  $\pm$  SEM, n = 3-4 experiments.

Compound	$^3\text{H}$ -FNM binding (K <sub>i</sub> , $\mu\text{M}$ )
Chlordiazepoxide	0.67 $\pm$ 0.10
Demoxepam	0.44 $\pm$ 0.02

### 3.4 In vitro $^3\text{H}$ -flunitrazepam binding and oocyte electrophysiology

Chlordiazepoxide and demoxepam both displayed comparable affinity for rat GABA<sub>A</sub> benzodiazepine binding sites labeled by [ $^3\text{H}$ ]flunitrazepam (Table 1). However, demoxepam showed less efficacy for  $\alpha_1\beta_2\gamma_2$  GABA<sub>A</sub> receptors compared to CDP, whereas efficacy at  $\alpha_3\beta_2\gamma_2$  GABA<sub>A</sub> receptors was comparable (Figure 5)

**Figure 6:** In vivo efficacy of chlordiazepoxide (0-20 mg/kg, IP) and demoxepam (0-40 mg/kg, IP) on the basal body temperature (A) and the stress-induced hyperthermia response (B) in 129Sv mice (n=10-12). \*: p<0.05 CDP compared to vehicle. #: p<0.05 demoxepam compared to vehicle.

### 3.5 In vivo comparison of chlordiazepoxide and demoxepam

CDP and demoxepam overall reduced stress-induced hyperthermia (SIH) (drug effect  $F_{5,69}=11.91$ ,  $p<0.001$ ) as well as basal body temperature (drug effect  $F_{5,69}=9.46$ ,  $p<0.001$ ) (Figure 6). Post-hoc testing revealed that CDP lowered basal body temperature at 10 mg/kg ( $p<0.05$ ) as well as 20 mg/kg ( $p<0.001$ ), whereas demoxepam lowered basal body temperature only at its highest dose of 40 mg/kg ( $p<0.05$ ). Planned comparisons showed that CDP at a dose of 20 mg/kg had a stronger effect on basal body temperature ( $F_{1,23}=9.49$ ,  $p<0.01$ ) as well as SIH attenuation ( $F_{1,23}=6.35$ ,  $p<0.05$ ) compared to demoxepam 20 mg/kg. When we compared CDP 20 mg/kg to demoxepam 40 mg/kg, there were no differences in basal body temperature ( $F_{1,21}=0.001$ ,  $p=0.97$ , NS) nor SIH reduction ( $F_{1,21}=0.01$ ,  $p=0.98$ , NS). CDP thus more potently reduced basal body

temperature as well as the SIH response compared to demoxepam. Demoxepam reached the anxiolytic and hypothermic effects of CDP at higher doses.

## 4. Discussion

The present study shows that chlordiazepoxide (CDP) in solution rapidly hydrolyzes to the ketone structure demoxepam with a half-time of around 9 days at 37 °C, thus confirming earlier reports on CDP hydrolysis (Han et al 1976). Using mass spectrometry, we confirmed that the single hydrolysis product is indeed demoxepam, arising from oxidative deamination of CDP (Figs. 1-2). The CDP hydrolysis was concentration dependent (following first-order kinetics) as well as temperature dependent (Fig. 3B-C). In osmotic minipumps, hydrolysis to demoxepam was again apparent (Fig. 4). Demoxepam is already known as an active metabolite of CDP with a half-life markedly longer than that of CDP (Schwartz et al 1971), and with steady-state concentrations exceeding those of CDP (Roy-Byrne et al 1996). In contrast to another study, we did not find a parallel degradation of demoxepam to a 2-amino-5-chlorobenzophenone and a glycine derivative (Han et al 1976), although we cannot exclude the possibility that hydrolysis products were present which were not eluted in time to be detected.

Using the stress-induced hyperthermia model (Vinkers et al 2008), our data in mice suggest that CDP may exert greater acute potency compared to demoxepam (Fig. 5). Interestingly, a study that found significant dependence after chronic CDP administration showed low CDP blood levels and high metabolite levels, indicating that demoxepam is indeed active (Chan et al 1989). Another study in subjects suffering from anxiety disorder found a significant correlation between anxiety reduction and demoxepam plasma levels but not CDP plasma levels, suggesting that after chronic exposure, demoxepam may possess anxiolytic effects that surpass those of CDP itself (Lin and Friedel 1979). Demoxepam binding data to oocyte GABA<sub>A</sub> receptors containing  $\alpha_1$  and  $\alpha_3$  receptors showed that demoxepam displayed lower efficacy at  $\alpha_1$  containing GABA<sub>A</sub> receptors, even though in vitro <sup>3</sup>H-flunitrazepam binding was not different. Thus, the active metabolites (including demoxepam) may significantly contribute to the various effects of CDP.

The apparent hydrolysis of CDP in solution was previously not taken into consideration in chronic experiments that used osmotic minipumps. Results obtained from chronic CDP administration using osmotic minipumps may be (partially) ascribed to demoxepam or the combination of both rather than to only CDP itself. Studies that have tested dissolved CDP in osmotic minipumps have yielded variable results. In one study, chronic minipump treatment with CDP (10 mg/kg/day) prevented hyperalgesia in rats subjected to a social-defeat procedure (Alexandre et al 2006), and in another study, 7-14-day minipump treatment with CDP (10 mg/kg/day) increased GABA<sub>A</sub> receptor desensitization in rats, as well as tolerance in an elevated-plus-maze test (Cash et al 1997). One week CDP minipump treatment (5-10 mg/kg/day) in C57BL/6J mice reduced sheltered feeding preference without altering motor activity levels (Kas et al 2008). In contrast, 14-day CDP minipump treatment (2 mg/kg/day) did not prevent the stress-induced decrease in swim-test struggling in rats unlike chronic treatment with different antidepressant drugs (West and Weiss 2005). Only in one study, plasma concentrations of CDP and its metabolites were determined after 14 days (Cash et al 1997).

An alternative to minipump administration is the chronic application of repeated daily injections. However, adequate plasma levels are not easily established, and repetitive injections may affect experimental outcomes, especially in stress-related research. In a study that compared daily injections with minipumps, chronic diazepam administration resulted in sedative tolerance under both conditions, although tolerance to the anxiolytic effects was only present after the daily injections (Fernandes et al 1999). This suggests that both ways of establishing chronic benzodiazepine exposure may not yield identical results. To complicate things even further, withdrawal anxiety from chronic diazepam treatment by manual daily injections was found to depend on administration route as it was present after subcutaneous but not after intraperitoneal injections (Allison and Pratt 2006).

In conclusion, the present study indicates that the classical benzodiazepine CDP is rapidly hydrolyzed in solution to the active compound demoxepam. The fact that CDP is readily soluble in water makes it an attractive choice for minipump applications, but it immediately poses the researcher for some challenges. As the exact anxiolytic contributions of CDP and its active hydrolysis product demoxepam are hard to dissect and the ratio CDP:demoxepam is not stable over time in solution, we advise that chronic CDP applications in which CDP is dissolved should be interpreted with caution. The possibility of using more lipophilic classical benzodiazepines (e.g. diazepam) in minipumps with solid PEG-400 dispersions may present a valid alternative (Verheyen et al 2002). In general, the use of osmotic minipumps presents a valid and attractive alternative to the labour-intensive daily injections, although caution should be exerted that drug stability and release is carefully investigated.



# Chapter 10

## **Corticotropin-releasing factor overexpression influences GABA<sub>A</sub> and metabotropic glutamate<sub>2/3</sub> receptor systems**

Christiaan H. Vinkers

Hendrikus Hendriksen

Una Campbell

Ruud van Oorschot

James M. Cook

Sundari Rallipalli

Shengming Huang

Mark J. Millan

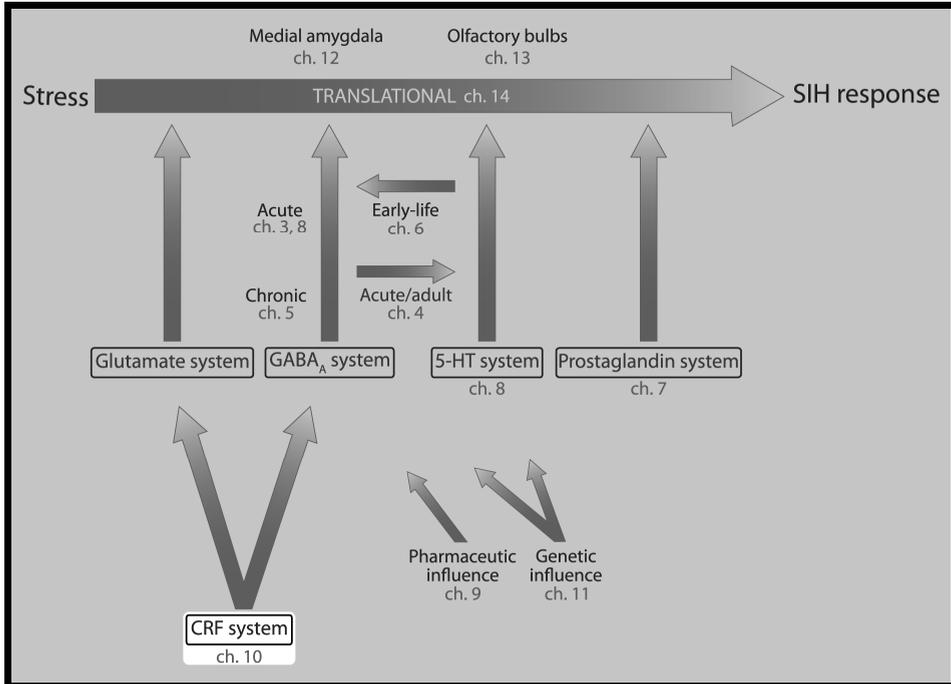
Berend Olivier

Lucianne Groenink

# 10

*Submitted*

## Abstract



**Background:** Corticotropin-releasing factor (CRF) modulates glutamate and GABAergic transmission in limbic regions, and CRF receptors are located on GABAergic and glutamatergic neurons. As CRF dysfunction is associated with stress-related disorders, the present study aimed to investigate the putative link between chronic central CRF overexpression and subsequent alterations in GABA<sub>A</sub> and glutamate receptor responsiveness.

**Methods:** Using a mouse model of central CRF overexpression, we tested CRF<sub>1</sub>, GABA<sub>A</sub> and metabotropic glutamate (mGlu) receptor sensitivity using the stress-induced hyperthermia (SIH) paradigm. Moreover, we examined mRNA expression levels of GABA<sub>A</sub> receptor  $\alpha$  subunits as well as mGlu receptor in the amygdala and hypothalamus.

**Results:** Chronic postnatal CRF overexpression decreased GABA<sub>A</sub> and metabotropic glutamate 2/3 receptor (mGluR<sub>2/3</sub>) sensitivity. CRF-overexpressing mice were less sensitive to the effects of the non-selective GABA<sub>A</sub> receptor agonist diazepam (0-4 mg/kg), the preferential  $\alpha_1$ -subunit selective GABA<sub>A</sub> receptor agonist zolpidem (0-10 mg/kg), the  $\alpha_3$ -subunit selective TP003 (0-3 mg/kg) and the mGluR<sub>2/3</sub> receptor agonist LY379268 (0-10 mg/kg). In contrast, no genotype differences were found using the  $\alpha_5$ -subunit selective compound SH-053-2F-R-CH<sub>3</sub> and the mGluR<sub>5</sub> antagonists MPEP and MTEP. CRF-overexpressing mice were less sensitive to the SIH-reducing effects of the CRF<sub>1</sub> receptor antagonists CP154,526 and DMP695. In the amygdala, CRF-overexpressing mice displayed decreased GABA<sub>A</sub> receptor  $\alpha_2$  subunit and mGluR<sub>3</sub> receptor mRNA levels, whereas no differences were found in GABA<sub>A</sub> receptor  $\alpha_1/\alpha_5$  subunits and mGluR<sub>2</sub>/mGluR<sub>5</sub> expression. In contrast, increased mRNA levels of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_5$  GABA<sub>A</sub> subunits as well as mGluR<sub>3</sub> receptors were present in the hypothalamus of CRF-overexpressing mice.

**Conclusions:** The present study shows that life-long CRF overproduction reduces GABA<sub>A</sub> receptor and mGluR<sub>2/3</sub> sensitivity which is accompanied with concomitant altered mRNA receptor levels in the amygdala and hypothalamus. Moreover, a reduced sensitivity to CRF<sub>1</sub> receptor antagonists is present in CRF-overexpression mice. These data provide a putative link between an increased central CRF drive and the emergence of dysfunctional GABA<sub>A</sub> and mGluR<sub>2/3</sub> receptor pathways which may include the amygdala and hypothalamus. As CRF dysfunction seems to be present in major depression and anxiety disorders, it may be speculated that CRF-induced changes in GABAergic and glutamatergic pathways may contribute to the development of stress-related disorders.

## 1. Introduction

The neuropeptide corticotropin-releasing factor (CRF) was initially characterized as the principal HPA-axis modulator in response to stress (Vale et al 1981). However, CRF has been found to modulate autonomic, immune and behavioral stress-related responses via central CRF<sub>1</sub> and CRF<sub>2</sub> receptors (De Souza 1995; Owens and Nemeroff 1991). These non-endocrine CRF brain circuits extend outside the hypothalamus and include cortical, limbic, striatal and brainstem areas (Hauger et al 2006). In line with a pivotal role in modulating stress responses, CRF dysfunction appears to be present in various psychiatric disorders including anxiety disorders, drug addiction, major depressive disorder and schizophrenia (Baker et al 1999; Gold and Chrousos 2002; Reul and Holsboer 2002; Risbrough and Stein 2006; Sarnyai et al 2001).

A transgenic mouse model of long-term CRF overexpression has been proposed to model CRF dysfunction. In these animals, chronically elevated CRF levels are associated with neurochemical, autonomic and physiological changes, including altered HPA axis activity, dexamethasone non-suppression, reduced heart rate variability and increased body temperature (Dirks et al 2002; Groenink et al 2002; Stenzel-Poore et al 1996). These data suggest that chronic postnatal CRF overproduction in these mice leads to alterations mimicking findings that are associated with anxiety disorders, major depression and schizophrenia (Holsboer 2000; Licht et al 2009; Ludewig et al 2002). Exogenous CRF administration into the basolateral amygdala elicits arousal and anxiety, putatively through CRF<sub>1</sub> receptor activation (Heinrichs et al 1997; Matsuzaki et al 1989; Sutton et al 1982). Thus, the amygdala plays an important role in the effects of CRF.

As has been shown with repeated central infusion of CRF and related ligands (Rainnie et al 2004), the function and structure of neurotransmitter systems other than the CRF system may adjust in response to (sub)chronic CRF receptor stimulation (Buwalda et al 1997; Linthorst et al 2002; Rainnie et al 2004). Indeed, CRF has been shown to be able to modulate glutamate and GABAergic transmission in limbic regions (Bagosi et al 2008; Liu et al 2004; Nie et al 2004; Rainnie et al 2004). Moreover, CRF receptors are located on GABAergic and glutamatergic neurons (Chen et al 2004b). Therefore, the present study aimed to investigate the putative link between chronic central CRF overexpression and subsequent alterations in GABA<sub>A</sub> and glutamate receptor responsivity which are also present in various stress-related psychiatric disorders. To this end, we used the stress-induced hyperthermia (SIH) paradigm. This paradigm is based on the rise in body temperature in response to stress (Vinkers et al 2008). This SIH response and, at higher doses, basal body temperature levels, can be reduced using clinically effective anxiolytics including GABA<sub>A</sub> receptor agonists, metabotropic glutamate receptor (ant)agonists and CRF<sub>1</sub> receptor antagonists (Griebel et al 2002; Olivier et al 2002; Spooren et al 2002). If chronic CRF release elicits long-lasting changes in other neurotransmitter systems it would provide a putative mechanism by which CRF dysfunction could result in stress-related disorders. Here, we report that chronic CRF overexpression in the central nervous system leads to blunted GABA<sub>A</sub> and metabotropic glutamate receptor systems as well as altered mRNA levels of these receptors in the amygdala and the hypothalamus.

## 2. Materials and methods

### 2.1 Animals

Transgenic mice overexpressing neural CRF were generated as described previously (Dirks et al 2002). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (.6-kb fragment), which was inserted into a 8.2-kb genomic DNA-fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence. The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons. Subsequent breeding at the local breeding facilities (Utrecht, the Netherlands) consisted of matings between heterozygous transgenic males (C57BL/6J background) and C57BL/6Jlco females (Charles River, the Netherlands).

Male transgenic CRF-overexpressing mice (line 2122, fifteenth generation) were used in these experiments. Littermate WT mice served as control mice. Animals were group-housed at constant room temperature ( $21 \pm 2^\circ\text{C}$ ) and relative humidity (50%–60%) with PVC tubing as cage enrichment. Standard rodent food pellets (Special Diet Services, Witham, Essex, United Kingdom) and water were freely available. Mice were maintained on a 12-hour light–dark cycle (lights on at 6 AM). All experiments were performed in accordance with the governmental guidelines for care and use of laboratory animals and approved by the Ethical Committee for Animal research of the Faculties of Sciences, Utrecht University, The Netherlands.

### 2.2 Drugs

Diazepam (base) and zolpidem (tartaric acid salt) were obtained from Sigma Aldrich. MPEP HCl (2-methyl-6-(phenylethynyl)pyridine) and MTEP (3-((2-Methyl-4-thiazolyl)ethynyl)pyridine) were obtained from Alexis Biochemicals. LY379268 (1*R*,4*R*,5*S*,6*R*)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate) was obtained from Tocris. SH-053-2'F-R-CH<sub>3</sub> (the (R) stereoisomer of 8-ethynyl-4-methyl-6-phenyl-4H-2,5,10b-triaza-benzo[e]azulene-3-carboxylic acid ethyl ester) was synthesized by the laboratory of Dr. J.M. Cook (University of Wisconsin-Milwaukee, USA). TP003 (4,2\_-difluoro-5\_-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-a']pyridin-3-yl]biphenyl-2-carbonitrile) was synthesized according to published methods (Dias et al 2005; Humphries et al 2006). CP154,526 HCl (butyl-ethyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo [2.3-d]pyrimidin-4-yl]amine) and DMP695 mesylate (N-(2-chloro-4,6-dimethylphenyl)-1-[1-methoxymethyl-(2-methoxyethyl)]-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine) were gifts from Institut de Recherche, Servier, Croissy/Seine France. An injection volume of 10 ml/kg was used for intraperitoneal injections of all drugs. All drugs were suspended in gelatin-mannitol 0.5% / 5%. Fresh solutions and suspensions were prepared each testing day.

### 2.3 The stress-induced hyperthermia (SIH) procedure

The SIH procedure was carried out according to standard procedures (Groenink et al 2009). Briefly, animals (n=10-13) were injected intraperitoneally with vehicle or drug 60 min before the first temperature measurement (T<sub>1</sub>). The temperature was again measured 10 min later (T<sub>2</sub>), representing the stress-induced body temperature. The stress-induced

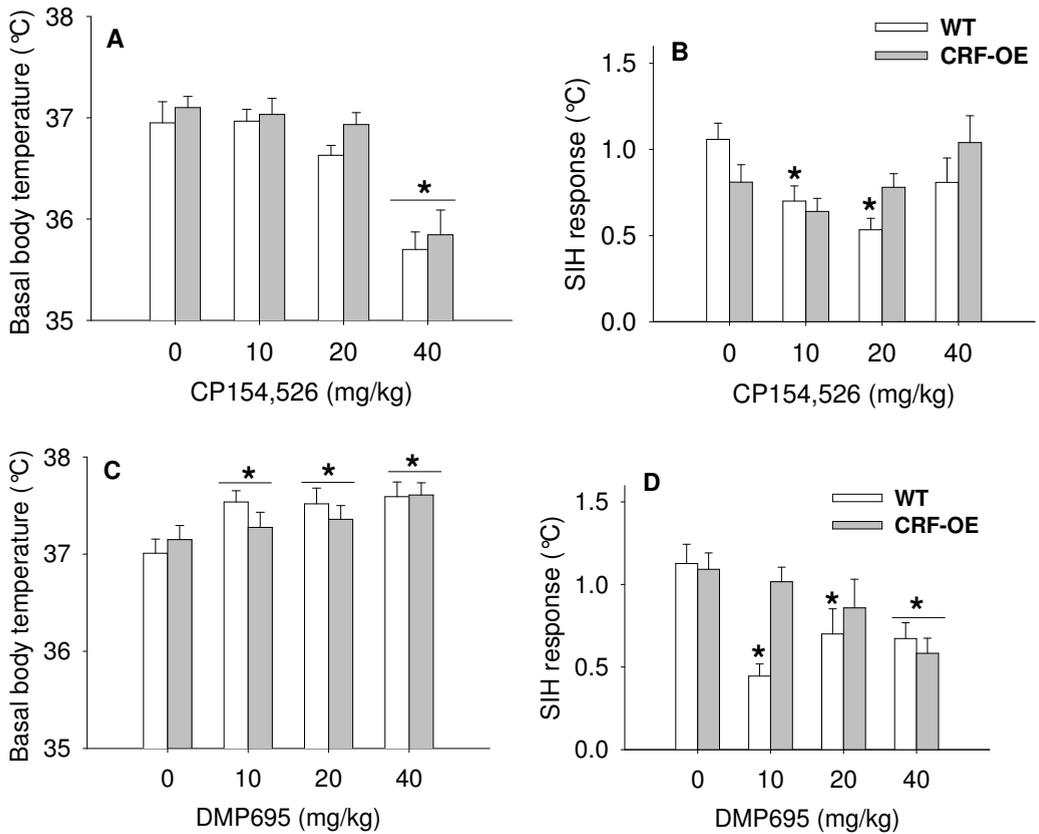
hyperthermia response was calculated by subtracting  $T_1$  from  $T_2$ . A within-subject design was used, and cages were randomly and evenly allocated over daytimes (morning–afternoon). The body temperature of mice was measured by rectally inserting a thermistor probe by a length of 2 cm. Digital temperature recordings were obtained with an accuracy of 0.1 °C using a Keithley 871A digital thermometer (NiCr– NiAl thermocouple). The probe, dipped into silicon oil before inserting, was held in the rectum until a stable rectal temperature had been obtained for 20 s.

## 2.4 Quantitative PCR analysis

GABA<sub>A</sub> receptor subunit levels as well as mGlu receptor mRNA levels were determined in drug-naïve wildtype and transgenic mice. Mice were decapitated, brains were removed and stored at -80°C until further use. Sections of 0.5 mm were made using a cryostat and were kept frozen while the hypothalamus and amygdala were dissected under a binocular microscope. Tissue samples were homogenized in Trizol reagent (Invitrogen, the Netherlands) followed by a clean up with NucleoSpin<sup>®</sup> RNA Clean-up XS (Machery Nagel, Germany). Reverse transcriptase was performed using the RevertAid™ kit (Fermentas, Germany) and an oligo(dT) primer. The following protocol was used for all PCR reactions, carried out in 20-µl reactions in a ABI PRISM 700 (Applied Biosystems, the Netherlands): 15 at min 95°C, followed by 40 cycles consisting of 15 s at 95°C and 60 s at 60°C. Each reaction mix contained 0.1 µM of each of the gene specific primers and ABsolute™ QPCR SYBR<sup>®</sup> Green mix (Abgene, Epsom, United Kingdom). The efficiency of a given PCR reaction was determined using five 2-fold dilutions of a mixture of the obtained cDNA samples. All the reactions had a efficiency close to 100%. Measured cDNA levels were normalized against the levels of GAPDH. The following primers were used (FP, RP): GAPDH (GCACCCTGCATTATTTGTCA, CTTCCAGGAGCGAGACCCCA), GABA<sub>A</sub> receptor  $\alpha_1$  subunit (CCACACCCCATCAATAGGTTCT, AATTCTCGGTGCAGAGGACTGAA), GABA<sub>A</sub> receptor  $\alpha_2$  subunit (GGGACGGGAAGAGTGTAGTCAA, CCGCATAGGCGTTGTTCTGT), GABA<sub>A</sub> receptor  $\alpha_3$  subunit (GCCCACTGAAGTTTGAAGCTAT, CATCCTGTGCTACTTCCACAGATT), GABA<sub>A</sub> receptor  $\alpha_5$  subunit (GCCCAGAGAGTCTCTGGAGCT,GGGCCACCTCTCCAAGTAAAC), mGlu2 receptor (AACCTTGGTCAAGGGTCTG, AAGCGACGATGTTGTTGAG), mGlu3 receptor (CCTGGATGGAAAGAAGTTG, TTGAATGGAGCTGTGAAG) and the mGlu5 receptor (TACTTCTGGGCAGTGATG, GACAGCTTCTCGCTGATAC).

## Data analysis

For SIH experiments, a basal temperature ( $T_1$ ), an end temperature ( $T_2$ ) and the difference (SIH response =  $T_2 - T_1$ ) was determined for each individual mouse. Treatment effects on the SIH response and basal body temperature ( $T_1$ ) were evaluated using a repeated-measures analysis of variance with 'drug' as within-subject factor and 'genotype' as between-subject factor. For post-hoc comparisons, t-tests with Bonferroni correction were used to determine genotype effects. Simple contrasts were used to compare drug effects with vehicle conditions. mRNA levels were analyzed using a univariate analysis of variance with genotype (WT/CRF<sub>OE</sub>) as a fixed factor. A probability level of  $p < 0.05$  was set as statistically significant, probability levels between  $p = 0.05$  and  $p = 0.1$  were regarded as trends.



**Figure 1:** Effects of the CRF<sub>1</sub> receptor antagonists CP154,526 (0-40 mg/kg, IP, **A-B**) and DMP 695 (0-40 mg/kg, IP, **C-D**) on basal body temperature and the SIH response in wildtype mice (WT) and CRF-overexpressing mice (CRF-OE). \*:  $p < 0.05$  drug effect relative to vehicle.

## 3. Results

### 3.1 CP154,526 (0-40 mg/kg, IP)

CP154,526 lowered basal body temperature regardless of genotype (CP154,526 effect  $F_{3,60}=38.57$ ,  $p < 0.01$ ; CP154,526 x genotype interaction,  $F_{3,60}=0.54$ ,  $p=0.66$ , NS; genotype effect  $F_{1,20}=0.66$ ,  $p=0.43$ , NS). Simple contrasts revealed that this effect was significant only at the highest dose of CP154,526 dose (simple contrasts: 10 mg/kg:  $F_{1,20}=0.01$ ,  $p=0.91$ , NS; 20 mg/kg:  $F_{1,20}=3.39$ ,  $p=0.08$ , NS; 40 mg/kg:  $F_{1,20}=70.17$ ,  $p < 0.001$ ).

CP154,526 affected the SIH response differently in WT animals compared to CRF-overexpressing mice (CP154,526 x genotype interaction  $F_{3,60}=3.60$ ,  $p < 0.05$ ). Post-hoc analysis revealed that this genotype difference was only significant at the 20 mg/kg dose (20 mg/kg:  $F_{1,20}=2.50$ ,  $p < 0.05$ ). Separate analysis of the genotypes further revealed that CP154,526 reduced the SIH response in WT animals ( $F_{3,33}=8.12$ ,  $p < 0.001$ ) but not in CRF-

overexpressing animals ( $F_{3,27}=2.59$ ,  $p=0.11$ ). In WT animals, CP154,526 reduced the SIH response at a dose of 10 mg/kg ( $F_{1,11}=7.81$ ,  $p<0.05$ ) and 20 mg/kg ( $F_{1,11}=19.53$ ,  $p<0.01$ ).

### 3.2 DMP695 (0-40 mg/kg, IP)

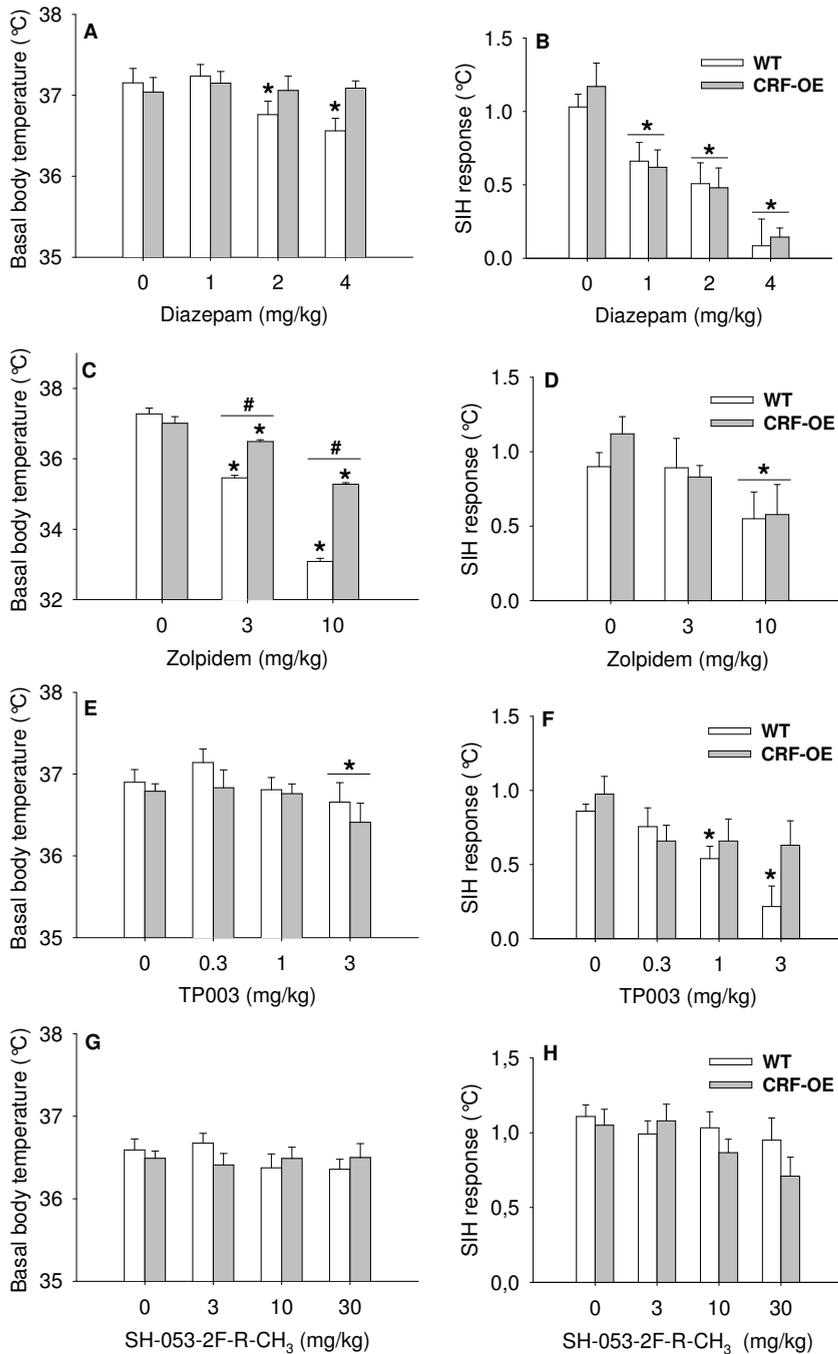
DMP695 increased body temperature regardless of genotype (DMP695 effect  $F_{3,63}=6.33$ ,  $p<0.01$ ; DMP695 x genotype interaction,  $F_{3,63}=1.07$ ,  $p=0.37$ , NS). Simple contrasts revealed that this difference was significant at all DMP695 doses (10 mg/kg:  $F_{1,21}=5.72$ ,  $p<0.05$ ; 20 mg/kg:  $F_{1,21}=6.56$ ,  $p<0.05$ ; 40 mg/kg:  $F_{1,21}=25.05$ ,  $p<0.001$ ).

DMP695 reduced the SIH response to a larger extent in WT animals compared to CRF-overexpressing mice (DMP695 x genotype interaction  $F_{3,63}=5.63$ ,  $p<0.01$ ). Post-hoc analysis further revealed that the genotype difference was significant at the 10 mg/kg dose (10 mg/kg:  $F_{1,21}=4.86$ ,  $p<0.001$ ). Separate analysis of the genotypes showed that DMP695 reduced the SIH response in WT animals ( $F_{3,30}=10.41$ ,  $p<0.001$ ) as well as CRF-overexpressing animals ( $F_{3,33}=6.26$ ,  $p<0.01$ ). In CRF-overexpressing animals, only the highest dose of 40 mg/kg reduced the SIH (simple contrasts:  $F_{1,11}=24.21$ ,  $p<0.001$ ), whereas in WT animals, all DMP doses reduced the SIH response (simple contrasts: 10 mg/kg:  $F_{1,10}=31.64$ ,  $p<0.001$ ; 20 mg/kg:  $F_{1,10}=9.04$ ,  $p=0.01$ ; 40 mg/kg:  $F_{1,10}=37.43$ ,  $p<0.001$ ).

### 3.3 Diazepam (0-4 mg/kg, IP)

The effect of diazepam on basal body temperature was dependent on genotype. (diazepam x genotype interaction,  $F_{3,63}=2.77$ ,  $p<0.05$ ). Post-hoc comparisons further revealed that genotypes significantly differed at the 4 mg/kg dose (4 mg/kg:  $F_{1,21}=2.71$ ,  $p<0.01$ ). Separate analysis of the genotypes indicated that diazepam reduced basal body temperature in WT animals ( $F_{3,36}=7.77$ ,  $p<0.001$ ) but not in CRF-overexpressing animals ( $F_{3,27}=0.10$ ,  $p=0.96$ , NS). In WT animals, only the highest doses reduced basal body temperature (simple contrasts: 2 mg/kg:  $F_{1,12}=6.51$ ,  $p<0.05$ ; 4 mg/kg:  $F_{1,12}=13.06$ ,  $p<0.01$ ).

Diazepam reduced the SIH response regardless of genotype (diazepam effect  $F_{3,63}=17.25$ ,  $p<0.001$ ; diazepam x genotype interaction  $F_{3,63}=0.17$ ,  $p=0.92$ , NS; genotype effect  $F_{1,21}=0.03$ ,  $p=0.86$ , NS). Simple contrasts revealed that diazepam significantly reduced the SIH response at all doses compared to vehicle (1 mg/kg:  $F_{1,21}=15.89$ ,  $p<0.01$ ; 2 mg/kg:  $F_{1,21}=17.07$ ,  $p<0.01$ ; 4 mg/kg:  $F_{1,21}=46.07$ ,  $p<0.01$ ).



**Figure 2:** Effects of the non subunit selective GABA<sub>A</sub> receptor agonist diazepam (0-4 mg/kg, IP, **A-B**), the preferential  $\alpha_1$  subunit GABA<sub>A</sub> receptor agonist zolpidem (0-10 mg/kg, IP, **C-D**), the GABA<sub>A</sub> receptor  $\alpha_3$  subunit agonist TP003 (0-3 mg/kg, IP, **E-F**) and the GABA<sub>A</sub> receptor  $\alpha_5$  subunit agonist SH-053-2F-R-CH<sub>3</sub> (0-30 mg/kg, IP, **G-H**) on basal body temperature and the SIH response in wildtype (WT) and CRF-overexpressing mice (CRF-OE). \*:  $p < 0.05$  drug effect relative to vehicle; #:  $p < 0.05$ : genotype difference.

### 3.4 Zolpidem (0-10 mg/kg, IP)

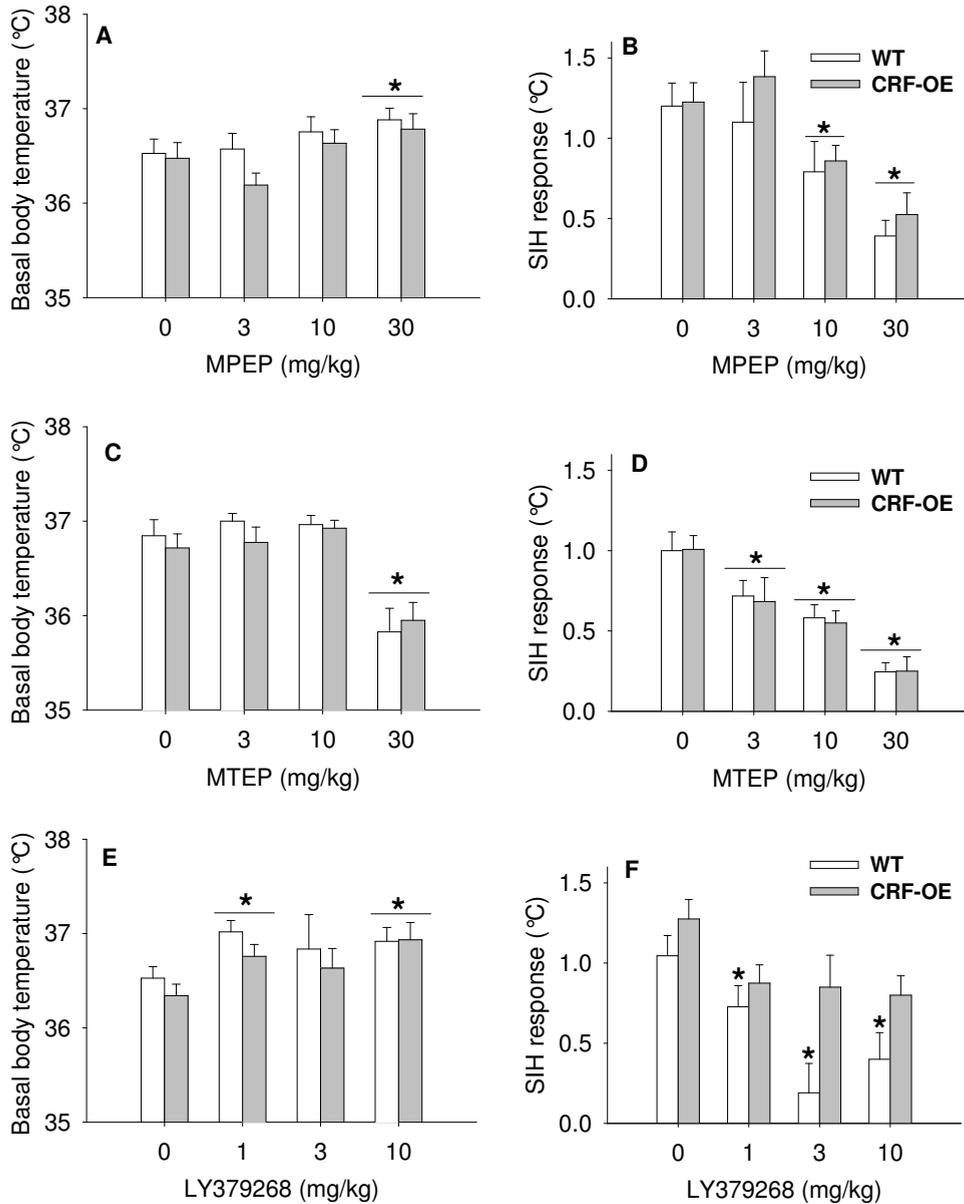
Zolpidem reduced basal body temperature more in WT animals than in CRF-overexpressing mice (zolpidem x genotype interaction,  $F_{2,40}=6.39$ ,  $p<0.01$ ). Post-hoc analysis t-tests revealed that this difference was significant at both zolpidem doses (3 mg/kg:  $F_{1,20}=2.11$ ,  $p<0.05$ ; 10 mg/kg:  $F_{1,20}=3.38$ ,  $p<0.01$ ). Separate analysis of the genotypes showed that zolpidem reduced basal body temperature in WT animals ( $F_{2,22}=27.86$ ,  $p<0.001$ ) as well as in CRF-overexpressing animals ( $F_{2,18}=21.93$ ,  $p<0.001$ ). Both doses reduced basal body temperature in both genotypes (simple contrasts: WT: 3 mg/kg:  $F_{1,11}=11.73$ ,  $p<0.01$  and 10 mg/kg:  $F_{1,11}=51.94$ ,  $p<0.001$ ; CRF-OE: 3mg/kg:  $F_{1,9}=11.84$ ,  $p<0.01$  and 10 mg/kg:  $F_{1,9}=26.80$ ,  $p<0.001$ ).

Zolpidem affected the SIH response regardless of genotype (zolpidem effect  $F_{2,40}=5.21$ ,  $p=0.01$ ; zolpidem x genotype interaction  $F_{2,40}=0.52$ ,  $p=0.60$ , NS; genotype effect  $F_{1,20}=0.17$ ,  $p=0.69$ , NS). Simple contrasts revealed that zolpidem significantly reduced the SIH response only at the highest dose compared to vehicle (3 mg/kg:  $F_{1,20}=1.17$ ,  $p=0.29$ , NS; 10 mg/kg:  $F_{1,20}=10.52$ ,  $p<0.01$ ).

### 3.5 TP003 (0-3 mg/kg, IP)

TP003 altered basal body temperature regardless of genotype (TP003 effect  $F_{3,60}=3.21$ ,  $p<0.05$ ; TP003 x genotype interaction,  $F_{3,60}=0.36$ ,  $p=0.78$ , NS; genotype effect  $F_{1,20}=1.41$ ,  $p=0.25$ , NS). Simple contrasts revealed that this difference was significant only at the highest TP003 dose (simple contrasts: 0.3 mg/kg:  $F_{1,20}=1.27$ ,  $p=0.27$ , NS; 1 mg/kg:  $F_{1,20}=0.26$ ,  $p=0.61$ , NS; 3 mg/kg:  $F_{1,20}=3.61$ ,  $p=0.05$ ).

TP003 reduced the SIH response, and a trend for a genotype effect was present (TP003 effect  $F_{3,60}=5.42$ ,  $p<0.01$ ; TP003 x genotype interaction  $F_{3,60}=1.99$ ,  $p=0.10$ , trend). Simple contrast showed that that this genotype trend was due to genotype difference at the 3 mg/kg dose (3 mg/kg:  $F_{1,20}=3.36$ ,  $p=0.05$ ). Overall, TP003 significantly reduced the SIH response at all doses compared to vehicle (0.3 mg/kg:  $F_{1,20}=4.94$ ,  $p<0.05$ ; 1 mg/kg:  $F_{1,20}=6.73$ ,  $p<0.05$ ; 3 mg/kg:  $F_{1,20}=11.75$ ,  $p<0.01$ ). Separate analysis of the genotypes showed that TP003 reduced the SIH in WT animals ( $F_{3,33}=5.16$ ,  $p<0.01$ ) but not in CRF-overexpressing animals ( $F_{3,27}=1.84$ ,  $p=0.16$ , NS). In WT animals, the higher TP003 doses reduced the SIH response (simple contrasts: 1 mg/kg:  $F_{1,11}=4.90$ ,  $p<0.05$ ; 3 mg/kg:  $F_{1,11}=11.88$ ,  $p<0.01$ ).



**Figure 3:** Effects of the mGlu<sub>rs</sub> antagonists MPEP (0-30 mg/kg, IP, **A-B**), MTEP (0-30 mg/kg, IP, **C-D**) and the mGlu<sub>2/3</sub> agonist LY379268 (0-10 mg/kg, IP, **E-F**), on basal body temperature and the SIH response in wildtype (WT) and CRF-overexpressing mice (CRF-OE). \*:  $p < 0.05$  drug effect relative to vehicle.

### 3.6 SH-053-2F-R-CH3 (0-30 mg/kg, IP)

SH-053-2F-R-CH3 did not affect body temperature (SH-053-2F-R-CH3 effect  $F_{3,60}=0.76$ ,  $p=0.52$ , NS; SH-053-2F-R-CH3 x genotype interaction,  $F_{3,60}=1.20$ ,  $p=0.32$ , NS; genotype effect  $F_{1,20}=0.08$ ,  $p=0.78$ , NS). SH-053-2F-R-CH3 did not affect the SIH response regardless of genotype (SH-053-2F-R-CH3 effect  $F_{3,60}=2.03$ ,  $p=0.12$ , NS; SH-053-2F-R-CH3 x genotype interaction  $F_{3,60}=0.67$ ,  $p=0.57$ , NS; genotype effect  $F_{1,20}=0.36$ ,  $p=0.56$ , NS).

### 3.7 MPEP (0-30 mg/kg, IP)

MPEP increased body temperature regardless of genotype (MPEP effect  $F_{3,63}=5.63$ ,  $p<0.01$ ; MPEP x genotype interaction,  $F_{3,63}=0.65$ ,  $p=0.58$ , NS; genotype effect  $F_{1,21}=1.66$ ,  $p=0.21$ , NS). Simple contrasts revealed that this difference was significant at the highest MPEP dose (simple contrasts: 3 mg/kg:  $F_{1,21}=0.78$ ,  $p=0.39$ , NS; 10 mg/kg:  $F_{1,21}=3.45$ ,  $p=0.08$ , NS; 30 mg/kg:  $F_{1,21}=8.02$ ,  $p<0.01$ ).

MPEP reduced the SIH response regardless of genotype (MPEP effect  $F_{3,63}=16.55$ ,  $p<0.001$ ; MPEP x genotype interaction  $F_{3,63}=0.31$ ,  $p=0.82$ , NS; genotype effect  $F_{1,21}=0.95$ ,  $p=0.34$ , NS). Simple contrasts revealed that MPEP significantly reduced the SIH response at the higher doses compared to vehicle-treated mice (10 mg/kg:  $F_{1,21}=10.30$ ,  $p<0.01$ ; 30 mg/kg:  $F_{1,21}=40.27$ ,  $p<0.001$ ).

### 3.8 MTEP (0-30 mg/kg, IP)

MTEP reduced body temperature regardless of genotype (MTEP effect  $F_{3,63}=19.04$ ,  $p<0.001$ ; MTEP x genotype interaction,  $F_{3,63}=0.42$ ,  $p=0.74$ , NS; genotype effect  $F_{1,21}=0.42$ ,  $p=0.53$ , NS). Simple contrasts revealed that this MTEP effect was significant at the highest dose (30 mg/kg:  $F_{1,21}=21.89$ ,  $p<0.001$ ).

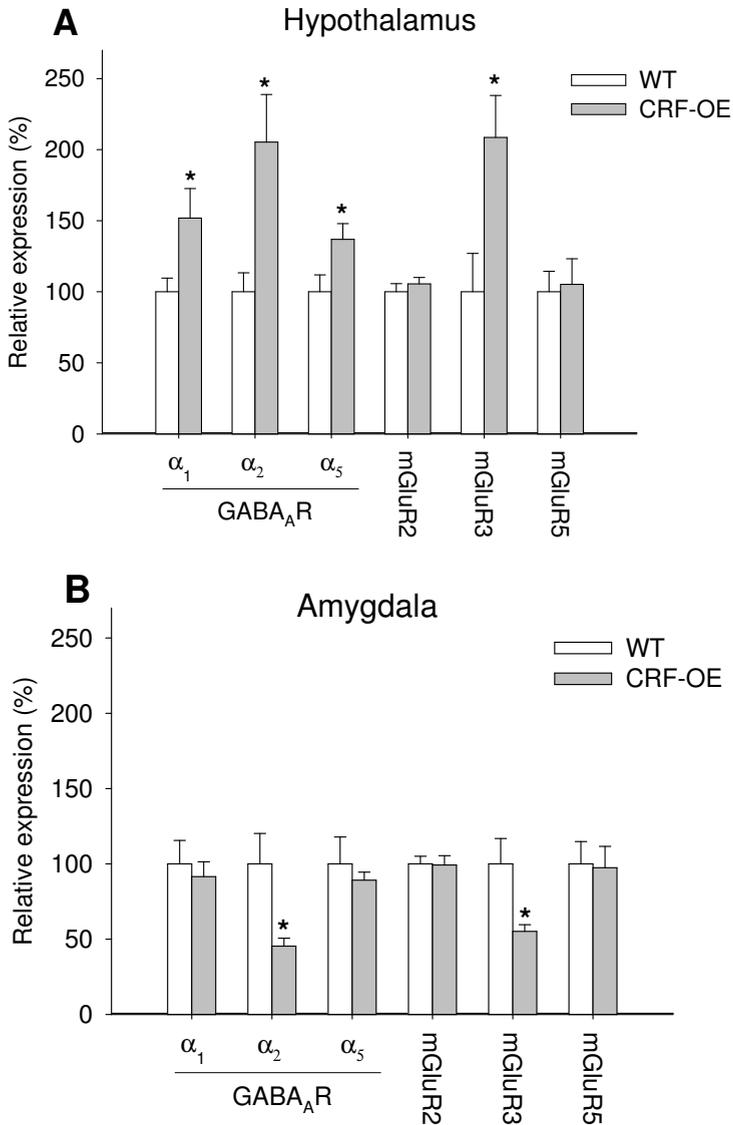
MTEP reduced the SIH response regardless of genotype (MTEP x genotype interaction  $F_{3,63}=0.03$ ,  $p=0.99$ , NS; MTEP effect  $F_{3,63}=21.87$ ,  $p<0.001$ ; genotype effect  $F_{1,21}=0.04$ ,  $p=0.85$ , NS). Simple contrasts revealed that MTEP significantly reduced the SIH response at all doses compared to vehicle-treated mice (3 mg/kg:  $F_{1,21}=6.79$ ,  $p<0.05$ ; 10 mg/kg:  $F_{1,21}=35.00$ ,  $p<0.001$ ; 30 mg/kg:  $F_{1,21}=53.69$ ,  $p<0.001$ ).

### 3.9 LY379268 (0-10 mg/kg, IP)

LY379268 increased body temperature regardless of genotype (LY379268 effect  $F_{3,60}=3.59$ ,  $p<0.05$ ; LY379268 x genotype interaction,  $F_{3,60}=0.22$ ,  $p=0.89$ , NS; genotype effect  $F_{1,21}=0.81$ ,  $p=0.38$ , NS). Simple contrasts revealed that this effect was significant at the lowest and highest LY379268 dose (simple contrasts: 1 mg/kg:  $F_{1,20}=25.01$ ,  $p<0.01$ ; 10 mg/kg:  $F_{1,20}=14.69$ ,  $p<0.01$ ).

Overall, the effect of LY379268 on the SIH response was dependent on the genotype in which it was tested (LY379268 x genotype interaction  $F_{3,60}=3.08$ ,  $p<0.05$ ). Post-hoc analysis t-tests revealed that the genotypes difference was significant at the 3

mg/kg LY379268 (3 mg/kg:  $F_{1,20}=4.1$ ,  $p<0.05$ ). Separate analysis of the genotypes showed that LY379268 reduced the SIH in WT animals ( $F_{3,27}=8.85$ ,  $p<0.001$ ) but not in CRF-overexpressing animals ( $F_{3,27}=2.30$ ,  $p=0.14$ , NS). In WT animals, all LY379268 doses reduced the SIH response (simple contrasts: 1 mg/kg:  $F_{1,9}=5.06$ ,  $p=0.05$ ; 3 mg/kg:  $F_{1,9}=14.51$ ,  $p<0.01$ ; 10 mg/kg:  $F_{1,9}=33.39$ ,  $p<0.01$ ).



**Figure 4:** mRNA levels of GABA<sub>A</sub> receptor subunits and mGluR receptors (mean  $\pm$  SEM) in the hypothalamus (A) and the amygdala (B) of wildtype (WT) and CRF-overexpressing mice (CRF-OE) mice. The mRNA expression was normalized against GAPDH level. \*:  $p<0.05$ .

### 3.10 Quantitative PCR analysis

Results of the PCR analysis showed increased GABA<sub>A</sub> receptor  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_5$  subunit and mGluR<sub>3</sub> mRNA levels in the hypothalamus in CRF-overexpressing group. In contrast, decreased GABA<sub>A</sub> receptor  $\alpha_2$  subunit and mGluR<sub>3</sub> mRNA levels were present in the amygdala of CRF-overexpressing mice compared to WT mice. GABA<sub>A</sub> receptor  $\alpha_3$  subunit mRNA levels were low. All mRNA levels were normalized against levels of GAPDH. Due to the low abundance of the  $\alpha_3$  subunit, technical difficulties (undetected sybregreen signal in several PCR reactions) were encountered, making the assessment of the change in  $\alpha_3$  mRNA levels unreliable.

## 4. Discussion

To study the potential effects of CRF on GABA and glutamate circuitry, we used a mouse model of central CRF overexpression (Dirks et al 2002; Groenink et al 2002; Groenink et al 2003a). In these CRF-overexpressing mice, we investigated CRF<sub>1</sub>, GABA<sub>A</sub> and mGlu receptor sensitivity using the stress-induced hyperthermia (SIH) paradigm. Moreover, we examined mRNA expression levels of GABA<sub>A</sub> receptor  $\alpha$  subunits as well as mGluRs in the amygdala and hypothalamus as these areas are both closely involved in stress- and anxiety-related behaviors and both areas express high concentrations of CRF<sub>1</sub> receptors (Reul and Holsboer 2002). Here, we report that chronic life-long CRF overexpression decreases GABA<sub>A</sub> and metabotropic glutamate receptor sensitivity at adult age. Specifically, CRF-overexpressing mice were less sensitive to the effects of the non-selective GABA<sub>A</sub> receptor agonist diazepam, the preferential  $\alpha_1$  subunit selective GABA<sub>A</sub> receptor agonist zolpidem (Petroski et al 2006), the  $\alpha_3$  subunit selective TP003 (Dias et al 2005) and the mGlu<sub>2/3</sub> receptor agonist LY379268. This blunted sensitivity was characterized by the inability of these compounds to decrease either the SIH response or basal body temperature which suggests that both circuitry involved in stress and thermoregulation may have adapted in these transgenic mice. No differences between CRF-overexpressing and WT mice were present after administration of the  $\alpha_5$  subunit selective compound SH-053-2F-R-CH<sub>3</sub> (Savic et al 2008) or the mGluR<sub>5</sub> antagonists MPEP and MTEP. A blunted GABA<sub>A</sub> and mGlu<sub>2/3</sub> receptor response in CRF-overexpressing mice was accompanied with an altered receptor expression in the amygdala and the hypothalamus. In the amygdala, GABA<sub>A</sub>  $\alpha_2$  subunit as well as mGluR<sub>3</sub> receptor mRNA levels were reduced, whereas no differences were found in other  $\alpha$  subunits and mGluR<sub>2</sub> and mGluR<sub>5</sub> expression. In contrast, CRF-overexpressing mice displayed increased hypothalamic mRNA levels of  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_5$  GABA<sub>A</sub> subunits as well as mGluR<sub>3</sub> receptors.

These functional and molecular changes in the GABA<sub>A</sub> and mGlu<sub>2/3</sub> receptor systems were accompanied by an impaired sensitivity to the CRF<sub>1</sub> receptor antagonists CP154,526 and DMP695 in CRF-overexpressing mice. In contrast to CRF-overexpressing mice, CRF<sub>1</sub> receptor antagonists CP154,526 and DMP695 reduced the SIH response in wildtype mice in a U-shaped dose-response curve which confirms the anxiolytic potential of these compounds (Millan et al 2001). Also, it extends the earlier finding that anxiolytic effects of CRF<sub>1</sub> receptor antagonists can be detected using the SIH paradigm (Griebel et al 2002). No differences in basal SIH response were observed between CRF-overexpressing and

wildtype littermates. Interestingly, acute central CRF administration increases body temperature, suggesting that the CRF system may directly affect thermoregulatory processes (Heinrichs et al 2001). In contrast to the acute effects, chronic exposure to CRF does not affect basal thermoregulation or the basal SIH response but rather modulates the CRF receptor system in a way which cannot be attributed to a rightward shift in responsivity to CRF<sub>1</sub> receptor antagonists.

The present data suggest a link between long-lasting CRF hyperactivity and functional and molecular alterations in GABA<sub>A</sub> and mGlu receptor circuitry in the amygdala and hypothalamus. A disruption of the balance between glutamatergic excitation and GABAergic inhibition could underlie CRF-induced anxiety-like behavior, as repeated administration of urocortin, a CRF-related ligand with higher affinity for CRF<sub>2</sub> receptors, into the basolateral amygdala induced long-lasting anxiety-like responses that were dependent on NMDA receptor activation but resulted in concomitant specific loss of GABA<sub>A</sub> receptor-mediated inhibition (Rainnie et al 2004). A number of studies support the notion that CRF may directly modulate GABA<sub>A</sub> and glutamate systems. Depressed neuronal excitability of GABAergic neurons was found in CRF<sub>1</sub> receptor knockout mice (Schierloh et al 2007). In contrast, CRF enhanced GABAergic neurotransmission in central amygdala neurons from wild-type and CRF<sub>2</sub> receptor knockout mice but not CRF<sub>1</sub> receptor knockout mice (Nie et al 2004). In this study, CRF<sub>1</sub> but not CRF<sub>2</sub> receptor antagonists blocked these effects in wildtype mice which was later confirmed using *in vitro* techniques (Bagosi et al 2008). CRF enhanced GABA<sub>A</sub>-mediated transmission via postsynaptic activation of CRF<sub>1</sub> receptors in the bed nucleus of the stria terminalis (Kash and Winder 2006). In serotonergic dorsal raphe neurons, CRF elevated presynaptic GABA levels and increased GABA<sub>A</sub> receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) that were mediated by both CRF<sub>1</sub> and CRF<sub>2</sub> receptors (Kirby et al 2008). To our knowledge, no GABA<sub>A</sub> receptor subunit levels have been assessed in transgenic CRF mice lines or in rodents that have repeatedly been exposed to CRF or urocortin infusions.

In addition to CRF effects on the GABA<sub>A</sub> neurotransmission, Liu and co-workers (Liu et al 2004) showed concentration-dependent and opposing effects of CRF on fast excitatory glutamatergic transmission in the central nucleus of the amygdala and the lateral septum/mediolateral nucleus. Moreover, CRF potentiated NMDA receptor-mediated excitation in the ventral tegmental area which was mediated by CRF<sub>2</sub> receptors (Ungless et al 2003). Although we found changes in mRNA expression levels that may suggest alterations at the receptor level associated with CRF overexpression, we cannot exclude the possibility that changes in response to chronic CRF overexpression may be the result of direct changes in intracellular signaling pathways. In support, CRF is implicated in protein kinase C and Ca<sup>2+</sup>/calmodulin-dependent kinase II-dependent long-term neuronal potentiation and depression (Blank et al 2003; Miyata et al 1999), and CRF increases calcium currents in central amygdala neurons (Yu and Shinnick-Gallagher 1998). Also, CRF-induced changes could be mediated through the serotonergic system (Lukkes et al 2008; Price et al 1998).

The finding that chronic CRF exposure exerts opposing effects on GABA<sub>A</sub> and mGluR<sub>3</sub> mRNA levels with increased hypothalamic and decreased amygdaloid levels may be the

result of differential effects of CRF<sub>1</sub> and CRF<sub>2</sub> receptors. In support, CRF<sub>1</sub> receptor activation in the amygdala decreased glutamate transmission, whereas CRF<sub>2</sub> receptor activation opposed these CRF<sub>1</sub> receptor-mediated effects on glutamate transmission (Liu et al 2004). Distinct CRF<sub>1</sub> and CRF<sub>2</sub> receptor distributions are present in the central nervous system, with high CRF<sub>1</sub> receptor mRNA levels in the cortex, the hypothalamic dorsomedial nucleus and basolateral amygdaloid nucleus, whereas abundant CRF<sub>2</sub> receptor immunoreactivity is observed in the ventromedial hypothalamic nucleus and cortical and medial amygdalar nuclei (Van Pett et al 2000). CRF-overexpressing mice do not display gross alterations in CRF<sub>1</sub> and CRF<sub>2</sub> receptor mRNA expression, suggesting that adaptations in the GABA<sub>A</sub> and glutamate receptor systems may not be directly related to concomitant changes in CRF receptor expression (Korosi et al 2006). Our results also indicate that an altered drug sensitivity between genotypes occur in either basal body temperature levels or differences in SIH response attenuation. Currently, we do not have a good explanation for these differences, but it may be speculated that an altered SIH attenuation without concomitant basal body temperature differences reflects specific dysfunction involving stress-related circuitry in CRF-overexpressing animals.

Recently, genetic and pharmacological evidence has indicated that different  $\alpha$  subunits may differentially contribute to the various classical benzodiazepines effects such as anxiolysis, dependence, anticonvulsant activity, sedation and amnesia (Crestani et al 2001; Rudolph et al 1999). More specifically, the  $\alpha_1$  subunit is thought to mediate the sedative and amnestic actions of benzodiazepines, whereas  $\alpha_2$  and/or  $\alpha_3$  subunits probably mediate the anxiolytic action of benzodiazepines (Dias et al 2005; Low et al 2000; McKernan et al 2000). The effects of the (non)selective GABA<sub>A</sub> compounds in the present study confirm and extend our earlier findings that hypothermia is associated with the activation of GABA<sub>A</sub> receptor  $\alpha_1$  subunit, whereas an anxiolytic reduction of the SIH response seems to be the result of GABA<sub>A</sub> receptor  $\alpha_3$  subunit activation without affecting basal body temperature levels (Olivier et al 2002; Vinkers et al 2009f; Vinkers et al 2008). The fact that the  $\alpha_5$ -subunit selective agonist SH-053-2'F-R-CH<sub>3</sub> did not affect basal body temperature nor the SIH response compared to vehicle-treated mice suggests that the  $\alpha_5$  subunit is not involved in the anxiolytic or hypothermic effects of benzodiazepines.

In conclusion, the present study shows that postnatal CRF overproduction reduced GABA<sub>A</sub> receptor and mGluR<sub>2/3</sub> sensitivity as well as altered mRNA receptor levels in the amygdala and hypothalamus. These data suggest that CRF may have a neuronal plasticity-like role on GABAergic and glutamatergic circuits in the central nervous system, including the amygdala and hypothalamus. Also, these data provide a putative link between an increased central CRF drive and the emergence of dysfunctional GABA<sub>A</sub> and mGlu<sub>2/3</sub> receptor pathways. As CRF dysfunction seems to be present in major depression and anxiety disorders, it may be speculated that CRF-induced changes in GABAergic and glutamatergic pathways may contribute to the development of stress-related disorders.

# Chapter 11

## **GABA<sub>A</sub> and serotonin receptor sensitivity in chromosome substitution strains of mice using stress-induced hyperthermia**

Christiaan H. Vinkers

Daniëlle Peterse

Hugo Oppelaar

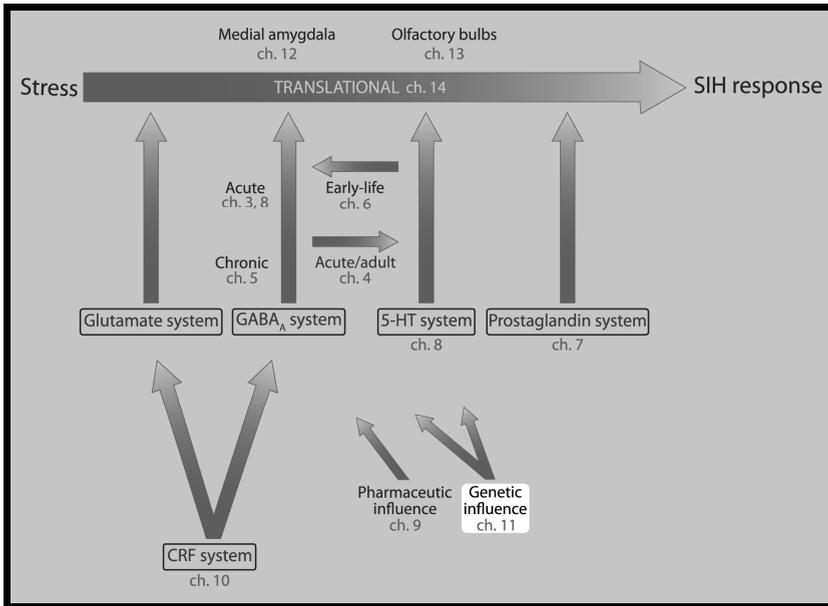
Berend Olivier

Martien J.H. Kas

# 11

*In progress*

## Abstract



**Background:** Abnormalities in the GABA<sub>A</sub> and serotonin system have been suggested to be present in stress-related disorders and clinically effective drugs include selective serotonin reuptake inhibitors, 5-HT<sub>1A</sub> receptor agonists and GABA<sub>A</sub> receptor agonists. The SIH paradigm uses the body temperature rise in response to stress which can be reduced using GABA<sub>A</sub> receptor agonists and 5-HT<sub>1A</sub> receptor agonists. Chromosome substitution strains (CS strains) have been proposed as a simple genetic screening strategy in behavioral research. Therefore, the present study aimed to screen male and female CS mice for basal SIH-related parameters and anxiolytic drug sensitivity. This way, the present study may aid in tracing genetic factors that affect the basal autonomic stress response as well as GABA<sub>A</sub> and 5-HT<sub>1A</sub> receptor drug sensitivity.

**Methods:** Eight different CS strains (CSS<sub>1</sub>, CSS<sub>2</sub>, CSS<sub>6</sub>, CSS<sub>7</sub>, CSS<sub>10</sub>, CSS<sub>12</sub>, CSS<sub>14</sub> and CSS<sub>15</sub>) on a C57Bl/6J (host) × A/J (donor) background were used to localize chromosomes involved in GABA<sub>A</sub> receptor (diazepam) and 5-HT<sub>1A</sub> receptor (flesionxan) sensitivity using the stress-induced hyperthermia (SIH) paradigm.

**Results:** Substitution of chromosome 10 resulted in decreased benzodiazepine sensitivity in male and female mice, whereas substitution of chromosome 1 results in increased benzodiazepine sensitivity in female mice. Moreover, substitution with A/J chromosome 7 led to an increased response to the 5-HT<sub>1A</sub> receptor agonist diazepam which may be indicative of an altered 5-HT<sub>1A</sub> receptor system.

**Conclusions:** The present results suggest that A/J chromosomes 1 or 10 in C57Bl/6J mice contribute to respective increased and decreased benzodiazepine sensitivity, whereas A/J chromosome 7 yields an increased response to the effects of a 5-HT<sub>1A</sub> receptor agonist. An important limitation of the present study is the fact that the SIH paradigm was used as an initial test using screening numbers based on behavioral genetics literature, following a 4.5:1 (27:6) ratio. Therefore, the obtained significant results in CSS<sub>1</sub>, CSS<sub>7</sub> and CSS<sub>10</sub> mice are only suggestive of putative changes in benzodiazepine and serotonergic sensitivity, and a follow up study has to be carried out in order to verify that an effect is indeed present.

## 1. Introduction

Stress-related disorders such as anxiety disorders and major depressive disorder are common psychiatric disorders (Kessler et al 2005; Merikangas et al 2007; Murray and Lopez 1997). However, the frequency with which stressful life events result in psychopathology is variable, suggesting that interindividual differences exist in stress vulnerability and resilience. There is compelling evidence that genetic factors may contribute to the etiology of stress-related disorders (Gillespie et al 2004; Levinson 2006; Villafuerte and Burmeister 2003). So far, a number of different preclinical genetic strategies have been employed to further unravel the neurobiology of stress-related disorders, including the use of knock-out, knock-in, point-mutated and transgenic mice (Cryan and Holmes 2005). These approaches have resulted in increased knowledge on the genetic factors that are associated with stress-related disorders,

Chromosome substitution strains (CS strains) have been proposed as a simple genetic screening strategy in behavioral research (Nadeau et al 2000). In each CS mouse, a single chromosome from one inbred strain (donor) has been substituted from another inbred strain (donor). This way, 21 different strains can be generated with the substitution of either autosomal or sex chromosomes, allowing to test the contribution of single donor chromosomes in the controlled genetic background of the host strain. So far, this panel has successfully identified quantitative trait loci (QTLs) for complex psychiatric traits such as anxiety (Kas et al 2009b; Laarakker et al 2008; Singer et al 2004), prepulse inhibition (Petryshen et al 2005), locomotor activity (Kas et al 2009a), alcohol preference (Boyle and Gill 2008; Lesscher et al 2009) and nicotine psychostimulant effects (Boyle and Gill 2009). Thus, the use of CS strains constitutes a hypothesis-free preclinical approach that enables the quick genetic screening for stress-related parameters.

Abnormalities in the GABA<sub>A</sub> and serotonin system have been suggested to be present in stress-related disorders (Akimova et al 2009; Kalueff and Nutt 2007; Nemeroff 2003), and clinically effective drugs include selective serotonin reuptake inhibitors, 5-HT<sub>1A</sub> receptor agonists and GABA<sub>A</sub> receptor agonists (Nutt 2005; Zohar and Westenberg 2000). In the present study, we screened eight different CS strains (CSS<sub>1</sub>, CSS<sub>2</sub>, CSS<sub>6</sub>, CSS<sub>7</sub>, CSS<sub>10</sub>, CSS<sub>12</sub>, CSS<sub>14</sub> and CSS<sub>15</sub>) on a C57Bl6/J (host) × A/J (donor) background to localize chromosomes involved in GABA<sub>A</sub> receptor and 5-HT<sub>1A</sub> receptor sensitivity using the stress-induced hyperthermia (SIH) paradigm. The SIH paradigm uses the body temperature rise in response to stress which can be reduced using GABA<sub>A</sub> receptor agonists and 5-HT<sub>1A</sub> receptor agonists (Vinkers et al 2008). Although these anxiolytic drug classes robustly block the SIH response, large strain differences exist in anxiolytic drug responsiveness (Bouwknicht and Paylor 2002; Van Bogaert et al 2006a; van Bogaert et al 2006b; Vinkers et al 2008). Therefore, the present study aimed to screen male and female chromosome substitution mice for basal SIH-related parameters and anxiolytic drug sensitivity. This way, the present study may aid in tracing genetic factors that affect the basal autonomic stress response as well as GABA<sub>A</sub> and 5-HT<sub>1A</sub> receptor drug sensitivity.

## 2. Materials and methods

### 2.1 Animals

A part of the complete CS strain panel (C57BL/6J-Chr 1A/NaJ, C57BL/6J-Chr 2A/NaJ, C57BL/6J-Chr 6A/NaJ, C57BL/6J-Chr 7A/NaJ, C57BL/6J-Chr 10A/NaJ, C57BL/6J-Chr 12A/NaJ, C57BL/6J-Chr 14A/NaJ, C57BL/6J-Chr 15A/NaJ: referred to as CS strains or CSS) (Singer et al 2004) were screened in the SIH paradigm. Original CS strain breeding pairs were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used in an internal breeding program. Males and females of the C57BL/6J and A/J strains ( $n=27$ ) and male and female CS strains ( $n=6$  per strain, except for CSS<sub>7</sub>, female mice:  $n=3$ ) were generated and tested. Animal screening numbers were based on behavioral genetics literature, following a 4.5:1 (27:6) ratio (Belknap 2003; Laarakker et al 2006). All mice were housed in Macrolon type 3 cages enriched with bedding and nesting material under a 12-h light/12-h dark cycle (lights on from 0600 to 1800 h) at controlled temperature ( $22\pm 2$  °C) and relative humidity (40–60%) with free access to standard food pellets and tap water. Experiments were carried out with approval of the ethical committee on animal experiments of the Faculties of Sciences, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki.

### 2.2 Drugs

Diazepam (base) was obtained from Sigma Aldrich. Flesinoxan (hydrochloride) was synthesized by Solvay (Solvay Pharmaceuticals, Weesp, The Netherlands). An injection volume of 10 ml/kg was used for intraperitoneal injections of all drugs. Diazepam was suspended in gelatin-mannitol 0.5% / 5%, and flesinoxan was dissolved in saline. Fresh solutions and suspensions were prepared each testing day.

### 2.3 The stress-induced hyperthermia (SIH) procedure

The SIH procedure was carried out according to standard procedures (Groenink et al 2009). Briefly, animals were injected intraperitoneally with vehicle or drug 60 min before the first temperature measurement ( $T_1$ ). The temperature was again measured 10 min later ( $T_2$ ), representing the stress-induced body temperature. The stress-induced hyperthermia response was calculated by subtracting  $T_1$  from  $T_2$ . A within-subject design was used, and cages were randomly and evenly allocated over daytimes (morning–afternoon). The body temperature of mice was measured by rectally inserting a thermistor probe by a length of 2 cm. Digital temperature recordings were obtained with an accuracy of 0.1 °C using a Keithley 871A digital thermometer (NiCr– NiAl thermocouple). The probe, dipped into silicon oil before inserting, was held in the rectum until a stable rectal temperature was obtained.

### 2.4 Data analysis

For SIH experiments, a basal temperature ( $T_1$ ), an end temperature ( $T_2$ ) and the difference (SIH response =  $T_2 - T_1$ ) was determined for each individual mouse. Treatment effects on the SIH response and basal body temperature ( $T_1$ ) were evaluated using a repeated-measures analysis of variance with explanatory factors drug as within-subject factor and genotype as between-subject factor. CS strains were separately compared to the C57Bl6/J host strain. If a certain consomic strain displayed a significant difference compared to the

host strain, this warrants further investigation with a higher number of animals (the number depending on the amount of CS strains tested) (Belknap 2003; Laarakker et al 2006). This way, a maximal reduction in the number of laboratory animals is established. A probability level of  $p < 0.05$  was set as statistically significant, probability levels between  $p = 0.05$  and  $p = 0.1$  were regarded as trends.

### 3. Results

#### 3.1 Diazepam sensitivity in CSS strains compared to C57Bl/6J mice.

**Donor strain:** Diazepam had comparable effects on the SIH response in male C57Bl/6J and A/J mice (diazepam x genotype interaction  $F_{3,147} = 2.52$ ,  $p = 0.06$ ). In female mice, again, diazepam reduced the SIH response in both C57Bl/6J and A/J mice (diazepam x genotype interaction  $F_{2,104} = 2.28$ ,  $p = 0.11$ ).

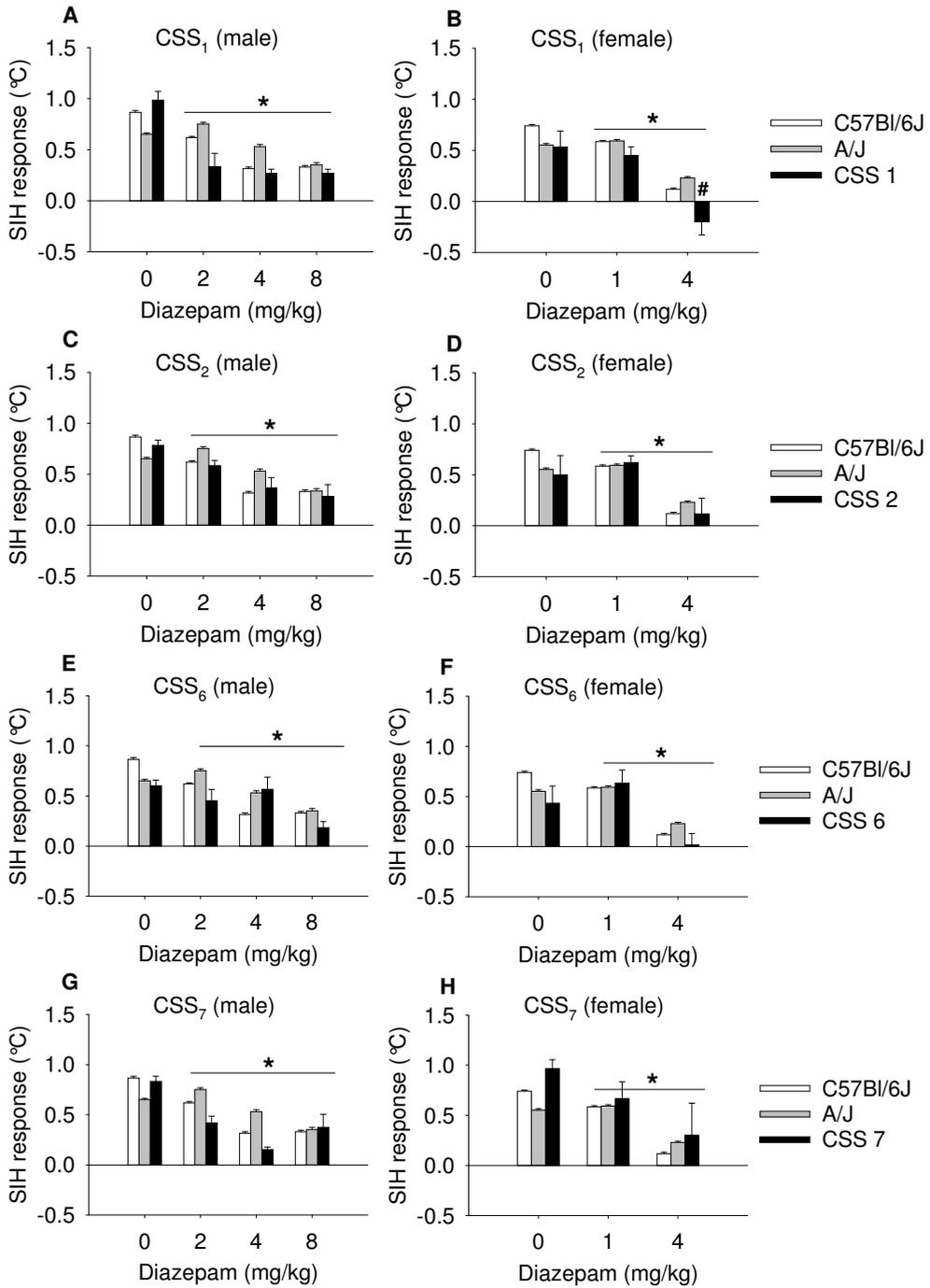
**CSS 1:** In male CSS<sub>1</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90} = 9.34$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{3,90} = 0.82$ ,  $p = 0.49$ ). In female mice, diazepam also reduced the SIH response independent of genotype (diazepam effect  $F_{2,62} = 21.32$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{2,62} = 0.35$ ,  $p = 0.71$ ). However, in female CSS<sub>1</sub> mice, an overall genotype effect was present ( $F_{1,31} = 8.83$ ,  $p < 0.01$ ).

**CSS 2:** In male CSS<sub>2</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90} = 6.82$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{3,90} = 0.93$ ,  $p = 0.66$ ). In female mice, diazepam also reduced the SIH response independent of genotype (diazepam effect  $F_{2,62} = 12.90$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{2,62} = 0.88$ ,  $p = 0.42$ ).

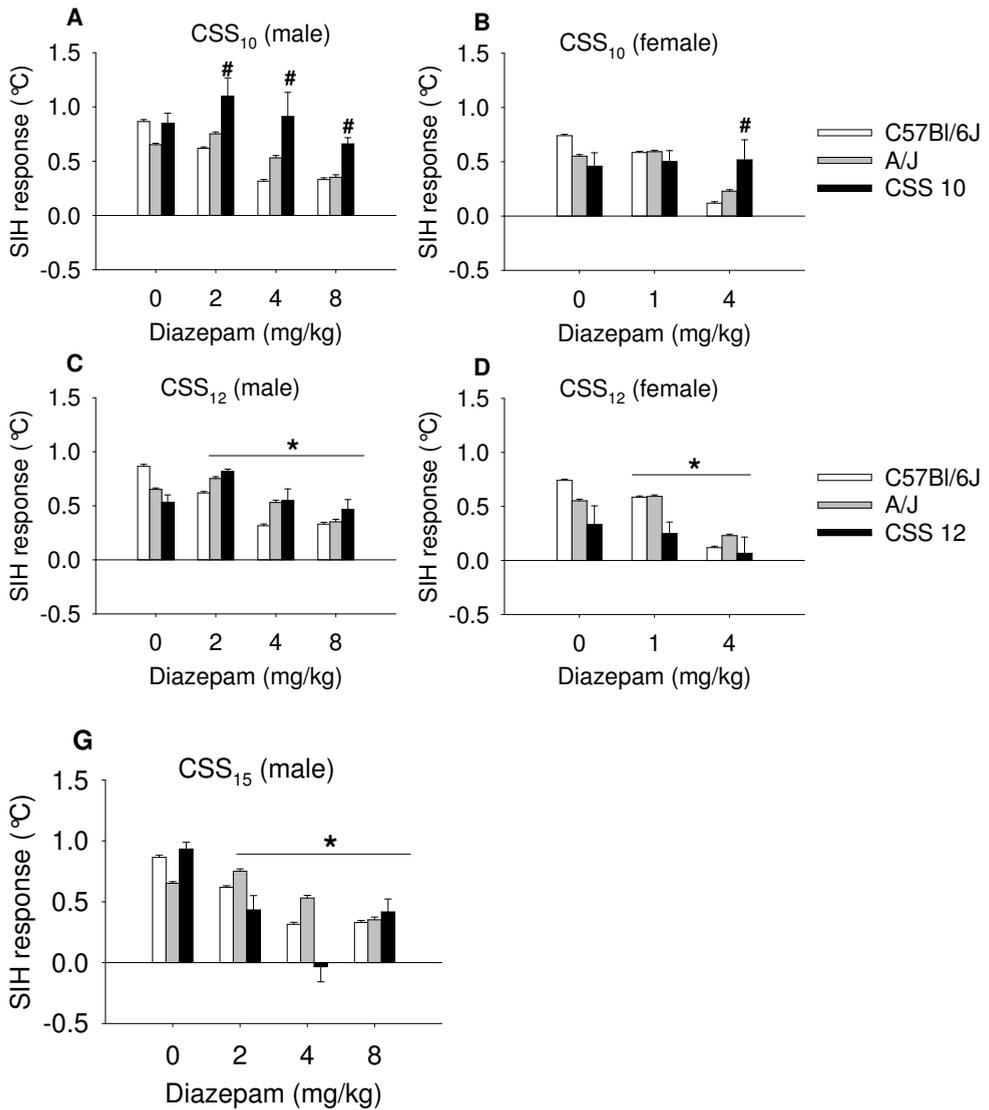
**CSS 6 :** In male CSS<sub>6</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90} = 3.93$ ,  $p < 0.05$ ; diazepam x genotype interaction  $F_{3,90} = 0.68$ ,  $p = 0.42$ ). In female mice, diazepam also reduced the SIH response independent of genotype (diazepam effect  $F_{2,62} = 16.32$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{2,62} = 1.38$ ,  $p = 0.26$ ).

**CSS 7:** In male CSS<sub>7</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90} = 10.47$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{3,90} = 0.61$ ,  $p = 0.61$ ). In female mice, diazepam also reduced the SIH response independent of genotype (diazepam effect  $F_{2,56} = 9.99$ ,  $p < 0.001$ ; diazepam x genotype interaction  $F_{2,56} = 0.13$ ,  $p = 0.88$ ).

**CSS 10:** In male CSS<sub>10</sub> mice, genotype influenced the diazepam effects on the SIH response (diazepam x genotype interaction  $F_{3,90} = 3.89$ ,  $p < 0.05$ ; diazepam effect  $F_{3,90} = 1.53$ ,  $p = 0.21$ ). In female mice, a similar diazepam insensitivity in CSS<sub>10</sub> mice was seen (diazepam x genotype interaction  $F_{2,62} = 7.53$ ,  $p < 0.01$ ; diazepam effect  $F_{2,56} = 4.16$ ,  $p < 0.05$ ).



**Figure 1:** Effects of diazepam (0-8 mg/kg, IP) on the stress-induced hyperthermia (SIH) response in CSS mice (CSS<sub>1-7</sub>, n=6) and the host strain (C57Bl/6J, n=26) and donor strain (A/J, n=26). \*: p<0.05: diazepam effect. #: genotype effect compared to the host strain.



**Figure 2:** Effects of diazepam (0-8 mg/kg, IP) on the stress-induced hyperthermia (SIH) response in CSS mice (CSS<sub>10-15</sub>, n=6) and the host strain (C57Bl/6J, n=26) and donor strain (A/J, n=26). \*: p<0.05: diazepam effect. #: genotype effect compared to the host strain.

**CSS 12:** In male CSS<sub>12</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90}=3.29$ ,  $p<0.05$ ; diazepam x genotype interaction  $F_{3,90}=1.98$ ,  $p=0.12$ ). In female mice, diazepam also reduced the SIH response independent of genotype (diazepam effect  $F_{2,62}=9.67$ ,  $p<0.001$ ; diazepam x genotype interaction  $F_{2,62}=1.61$ ,  $p=0.21$ ).

**CSS 14:** In male CSS<sub>14</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90}=9.16$ ,  $p<0.001$ ; diazepam x genotype interaction  $F_{3,90}=0.72$ ,  $p=0.54$ ). In female mice, diazepam also reduced the SIH response independent of genotype (diazepam effect  $F_{2,62}=20.63$ ,  $p<0.001$ ; diazepam x genotype interaction  $F_{2,62}=0.66$ ,  $p=0.49$ ).

**CSS 15:** In male CSS<sub>15</sub> mice, diazepam reduced the SIH response independent of genotype (diazepam effect  $F_{3,90}=10.02$ ,  $p<0.001$ ; diazepam x genotype interaction  $F_{3,90}=1.09$ ,  $p=0.36$ ).

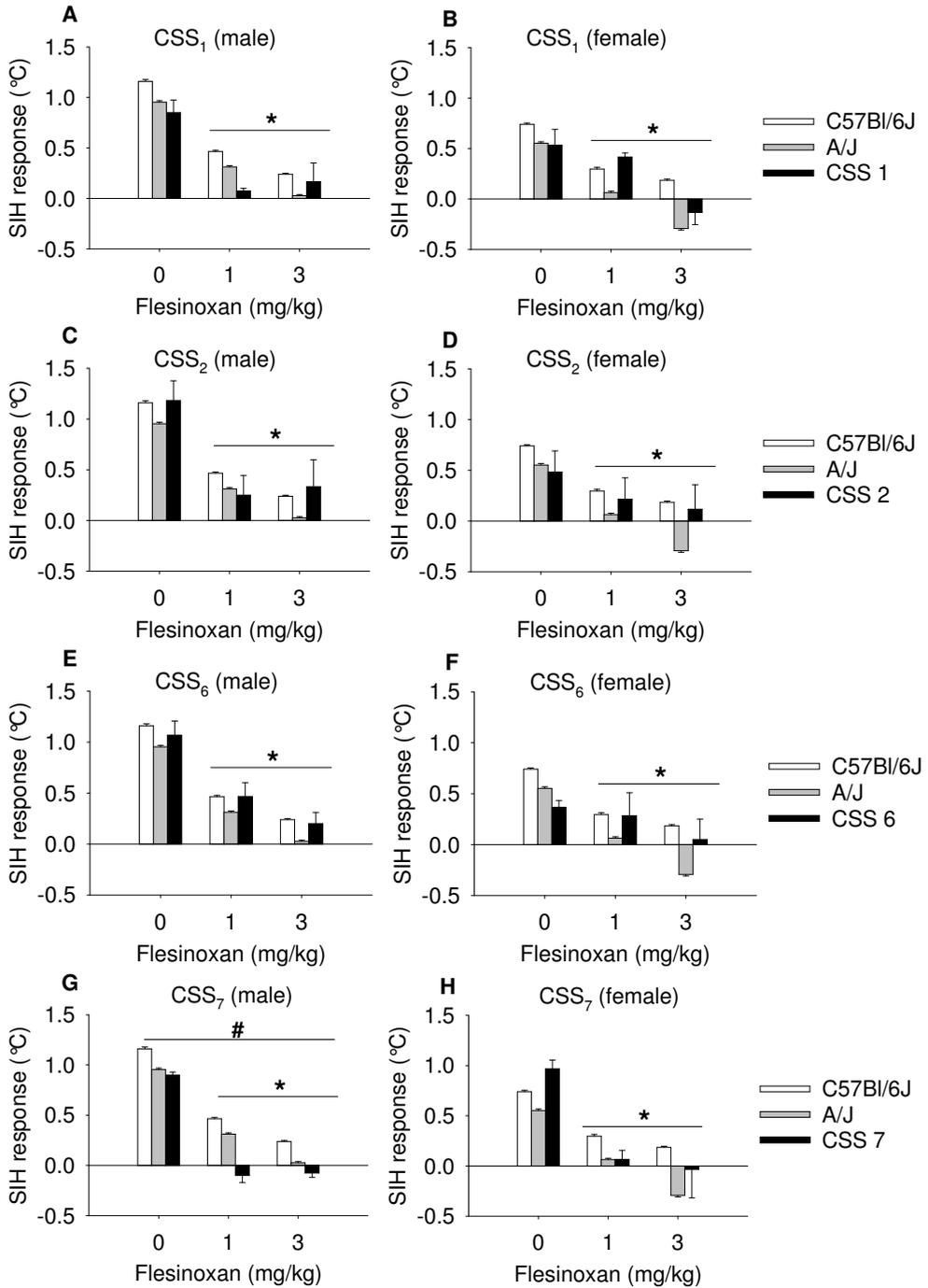
### 3.2 Flesinoxan sensitivity in CSS strains compared to C57Bl/6, mice.

**Donor strain:** Male A/J mice were more sensitive to the effects of flesinoxan on the SIH response compared to male C57Bl/6J mice (genotype effect  $F_{1,50}=7.64$ ,  $p<0.01$ ). Also, female A/J mice were more sensitive to the acute flesinoxan effects (genotype interaction  $F_{1,52}=28.79$ ,  $p<0.001$ ).

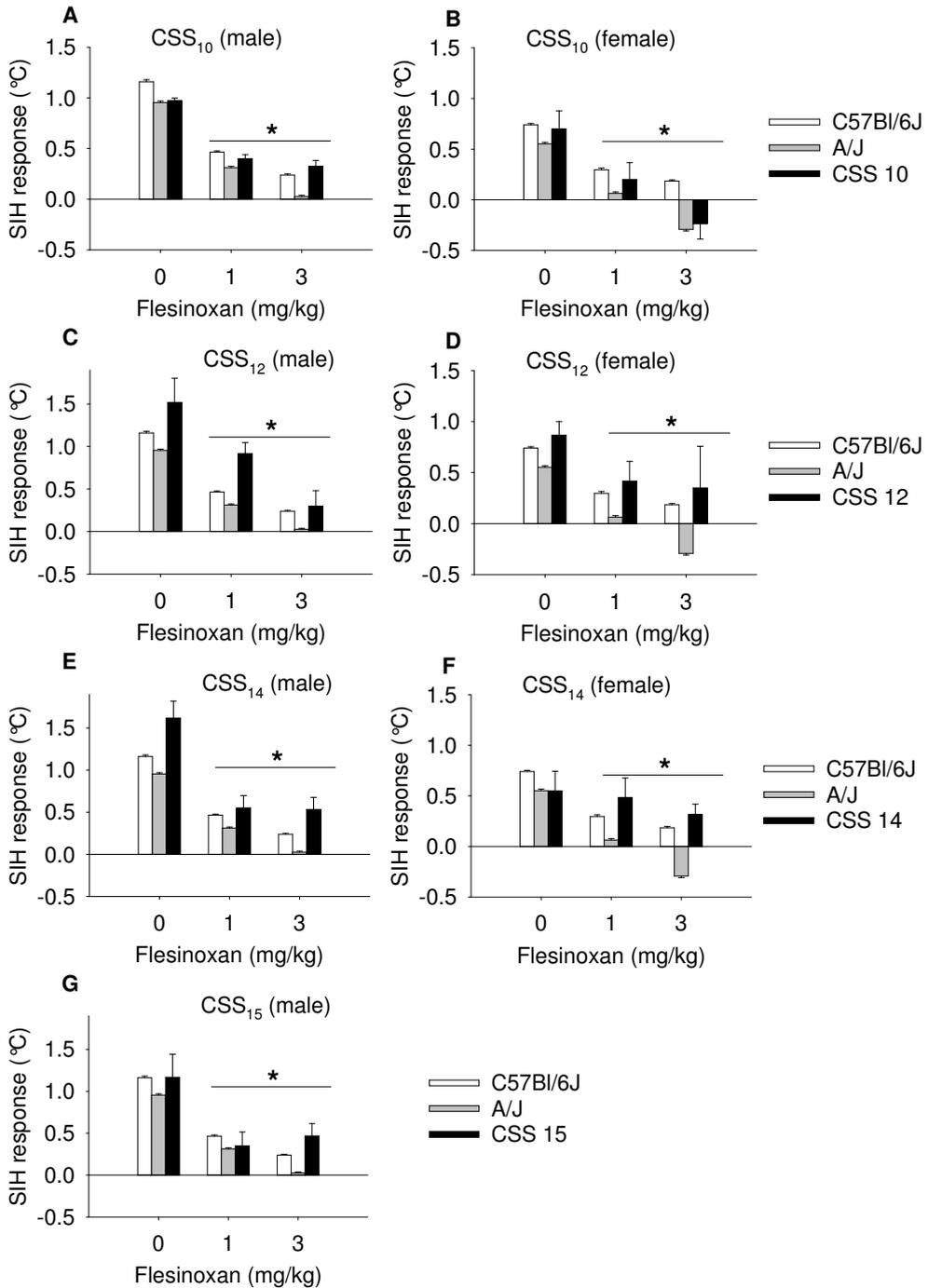
**CSS 1:** In male CSS<sub>1</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=24.57$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=0.97$ ,  $p=0.37$ ). In female mice, flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,62}=13.55$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,62}=1.89$ ,  $p=0.16$ ).

**CSS 2:** In male CSS<sub>2</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=29.75$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=0.42$ ,  $p=0.42$ ). In female mice, flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,62}=7.96$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,62}=0.38$ ,  $p=0.68$ ).

**CSS 6:** In male CSS<sub>6</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=32.02$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=0.95$ ,  $p=0.95$ ). In female mice, flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,62}=6.26$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,62}=1.09$ ,  $p=0.34$ ).



**Figure 3:** Effects of the 5-HT<sub>1A</sub> receptor agonist flesinoxan (0-8 mg/kg, IP) on the stress-induced hyperthermia (SIH) response in CSS mice (CSS<sub>1-7</sub>, n=6) and the host strain (C57Bl/6J, n=26) and donor strain (A/J, n=26). \*: p<0.05: diazepam effect. #: genotype effect compared to the host strain.



**Figure 4:** Effects of the 5-HT<sub>1A</sub> receptor agonist flestinoxan (0-8 mg/kg, IP) on the stress-induced hyperthermia (SIH) response in CSS mice (CSS<sub>10-15</sub>, n=6) and the host strain (C57Bl/6J, n=26) and donor strain (A/J, n=26). \*: p<0.05: diazepam effect. #: genotype effect compared to the host strain.

**CSS 7:** In male CSS<sub>7</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=15.71$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=1.90$ ,  $p=0.17$ ). However, a main genotype effect was present ( $F_{1,30}=5.22$ ,  $p<0.05$ ). In female mice ( $n=3$ ), flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,56}=13.55$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,56}=1.29$ ,  $p=0.29$ ).

**CSS 10:** In male CSS<sub>10</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=26.88$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=0.38$ ,  $p=0.69$ ). In female mice, flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,62}=13.66$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,62}=0.07$ ,  $p=0.94$ ).

**CSS 12:** In male CSS<sub>12</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=38.09$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=1.44$ ,  $p=0.25$ ). In female mice, flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,62}=8.64$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,62}=0.02$ ,  $p=0.99$ ).

**CSS 14:** In male CSS<sub>14</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=41.54$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=1.38$ ,  $p=0.26$ ). In female mice, flesinoxan again reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,62}=5.35$ ,  $p<0.05$ ; flesinoxan x genotype interaction  $F_{2,62}=0.37$ ,  $p=0.37$ ).

**CSS 15:** In male CSS<sub>14</sub> mice, flesinoxan reduced the SIH response independent of genotype (flesinoxan effect  $F_{2,60}=25.43$ ,  $p<0.001$ ; flesinoxan x genotype interaction  $F_{2,60}=0.39$ ,  $p=0.39$ ).

## 4. Discussion

Variation in individual clinical response to anxiolytic drug treatment may at least be partially explained by genetic factors (Malhotra et al 2004). To study the potential contribution of chromosomes on the basal autonomic stress response as well as GABAergic and serotonergic drug sensitivity, we tested eight different chromosome substitution strains (CSS) in the stress-induced hyperthermia (SIH) paradigm. In these mice, a single chromosome from one inbred strain (donor, A/J mice) has been substituted in the genetic background of the host inbred strain (here, C57Bl/6J mice). In this way, the contribution of single A/J chromosomes can be systematically investigated in relation to SIH phenotypes. Here, we report that the non-subunit selective GABA<sub>A</sub> receptor agonist diazepam did not reduce the SIH response in male and female CSS<sub>10</sub> mice compared to the host strain, suggesting that a certain degree of benzodiazepine insensitivity is present in these mice. Moreover, female but not male CSS<sub>1</sub> mice displayed increased sensitivity to the effects of diazepam. In the other CS strains tested (CSS<sub>2</sub>, CSS<sub>6</sub>, CSS<sub>7</sub>, CSS<sub>12</sub>, CSS<sub>14</sub> and CSS<sub>15</sub>), diazepam reduced the SIH response to a similar degree compared to the host strain, indicating that no gross altered benzodiazepine sensitivity is present in these CS strains. Thus, substitution of chromosome 10 yields decreased benzodiazepine sensitivity in male and female mice, whereas substitution of chromosome 1 results in increased benzodiazepine sensitivity in female mice. In support, an earlier study that used recombinant inbred strains derived from C57Bl/6J and A/J mice found evidence that the response to diazepam in the open field and the light-dark box in these strains had the best probabilities of linkage on loci on chromosomes 1 (*Xmv-41*) and 10 (*D10Mit2*) (Mathis et al 1995). The differences in benzodiazepine sensitivity may be attributed to adaptation of the GABA<sub>A</sub> receptor. GABA<sub>A</sub> receptors are composed of five subunits with various possibilities per subunit ( $\alpha_{1-6}$ - $\beta_{1-3}$ - $\gamma_{1-3}$ - $\delta$ - $\epsilon$ - $\theta$ - $\pi$ ) that assemble to form a pentameric ligand-gated chloride channel (Rudolph and Mohler 2006). GABA<sub>A</sub> receptors most commonly consist of  $\alpha$ ,  $\beta$  and  $\gamma$  subunits. So far, highly preserved  $\alpha$ - $\beta$ - $\gamma$  gene clusters have been localized on human chromosomes 4 (p12-13), 5 (q34-35) and 15 (q11-13) (Russek 1999). Thus, altered benzodiazepine sensitivity in the CS strains is not the direct result of altered GABA<sub>A</sub> receptor expression.

In addition to diazepam, the 5-HT<sub>1A</sub> receptor agonist flesinoxan was used to screen for altered serotonergic sensitivity. Flesinoxan has received ample attention as an anxiolytic drug that attenuates the SIH response (Groenink et al 1996b; Olivier et al 2003; Zethof et al 1995). In male CSS<sub>7</sub> mice, an increased flesinoxan effect was found. In female CSS<sub>7</sub> mice, the absence of this effect may be ascribed due to a small power (n=3). In the other CS strain tested, no altered flesinoxan sensitivity was found. Thus, substitution with A/J chromosome 7 leads to an increased flesinoxan response which may be indicative of an altered 5-HT<sub>1A</sub> receptor system. The gene encoding for the 5-HT<sub>1A</sub> receptor in mice is located on chromosome 13. Again, the changed flesinoxan response cannot directly be related to 5-HT<sub>1A</sub> receptor expression.

Overall, no differences in basal SIH response were found between the host strain and the CS strains, suggesting that the SIH response is preserved. This is supported by other studies that showed that an altered SIH response is not a common finding in genetically

modified animals with increased anxiety levels, such as 5-HT<sub>1A</sub> receptor knockout mice (Pattij et al 2001; Van Bogaert et al 2006a). However, we cannot exclude the possibility that the basal SIH response may be altered in other CS strains. Importantly, no large differences in basal SIH response and diazepam sensitivity were found between the host and donor strain. In contrast, A/J mice were more sensitive to the SIH-reducing effects of flesinoxan. A study in 9 different mouse strains revealed a SIH response between 0.6 and 1.9 °C (Bouwknicht and Paylor 2002), and locomotor activity and body weight levels alone cannot account for these differences (Pardon et al 2004; Van Bogaert et al 2006a). C57BL/6J (B6) mice showed the largest autonomic response compared to Swiss-Webster (SW) and 129Sv/Ev (129Sv) mice (Bouwknicht and Paylor 2002; van Bogaert et al 2006b). However, between-strain SIH variance was smaller compared to light-dark box test variance (Bouwknicht and Paylor 2002). Generally, 5-HT<sub>1A</sub> receptor responses are dependent on genetic background as well as the anxiety model used (Bouwknicht et al 2004a).

Together, the present results suggest that A/J chromosomes 1 or 10 in C57Bl/6J mice contribute to respective increased and decreased benzodiazepine sensitivity, whereas A/J chromosome 7 yields an increased response to the effects of a 5-HT<sub>1A</sub> receptor agonist. An important limitation of the present study is the fact that the SIH paradigm was used as an initial test using screening numbers based on behavioral genetics literature, following a 4.5:1 (27:6) ratio (Belknap 2003; Laarakker et al 2006). Therefore, the obtained significant results in CSS1, CSS7 and CSS10 mice are only suggestive of putative changes in benzodiazepine and serotonergic sensitivity, and a follow up study has to be carried out in order to verify that an effect is indeed present. If effects of chromosome substitution remain present, the exact genetic background could be examined using a F<sub>2</sub> genetic strategy.



# Chapter 12

## **Medial amygdala lesions differentially influence stress responsivity and sensorimotor gating in rats**

Christiaan H. Vinkers

Elisabeth Y. Bijlsma

Lotte C. Houtepen

Koen G.C. Westphal

Jan G. Veening

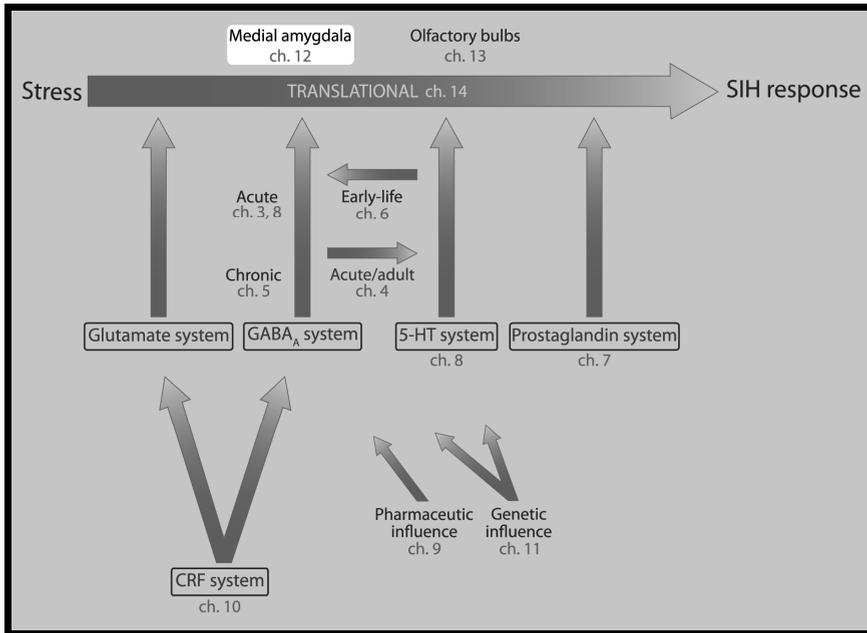
Lucianne Groenink

Berend Olivier

# 12

*Submitted*

## Abstract



**Background:** The amygdala is involved in the coordination of the stress response but is also an important gatekeeper involved in the regulation of vigilance. The amygdala is structurally complex, consisting of several nuclei with specific functions in the affective response to environmental stimuli. There are indications that the medial amygdaloid nucleus may be a pivotal player in acute responses to emotional environmental stimuli.

**Methods:** The present study therefore aimed to study the effects of bilateral electrolytic lesions of the medial amygdala on unconditioned anxiety-related behavior as well as a sensorimotor gating parameter (prepulse inhibition, PPI) in rats. Anxiety-related behavior was assessed with the use of stress-induced hyperthermia (SIH), light-enhanced startle (LES) and open-field behavior.

**Results:** Bilateral electrolytic lesions of the medial amygdala decreased the SIH response and anxiety-related open field behavior. In contrast, lesioned animals displayed augmented LES and disrupted PPI. No changes in basal locomotor activity, body temperature and acoustic startle were found between lesioned and sham animals.

**Conclusions:** The present study suggests that the medial amygdala is an important player in response to acute environmental stimuli. Decreased unconditioned psychological stress responses were found, whereas LES was enhanced and sensorimotor processing was disrupted. However, considering the existing data on basolateral amygdala involvement in PPI and bed nucleus of the stria terminalis involvement in LES, local infusion studies into the MeA should be performed to further substantiate these findings.

## 1. Introduction

The amygdala plays a complex but essential role in response to actual or potential environmental disturbances, ranging from the emotional processing of anxiety (Blanchard and Blanchard 1972; Joels and Baram 2009; Roozendaal et al 2009) to the orchestration of sensorimotor gating (the processing and transferring of incoming sensory information) (Decker et al 1995; Wan and Swerdlow 1997). In line with a pivotal function, the amygdala receives input from sites involved in sensory information, attention and arousal (including the olfactory system, thalamus, cortex, locus coeruleus and raphe nuclei), and projects to structures throughout the central nervous system, including the prefrontal cortex, hippocampus and hypothalamic nuclei that control endocrine and autonomic stress responses (Herman et al 2005; LeDoux 2000). The structurally complex amygdala is divided into separate nuclei (including the medial, central, lateral, basolateral nucleus) that specifically modulate stress-related behavior (Swanson 2003). Moreover, these amygdaloid subdivisions directly interact to fine-tune neuronal signaling in response to stress (Joels and Baram 2009).

While generating a coordinated output, the amygdala mediates stress and arousal in a site-specific and complex manner. There is evidence that the medial amygdaloid nucleus is pivotal in unconditioned anxiety-related responses (Cullinan et al 1995; Dayas et al 2001; Dayas and Day 2002; Li et al 2004). In support, lesions of the medial amygdala result in disruption of neuroendocrine responses (Masini et al 2009), and injection of GABA<sub>A</sub> receptor agonist muscimol reversed the stress-induced activation of autonomic nervous system (Kubo et al 2004). In contrast, the medial amygdala is not involved in conditioned fear response, although it may serve as a gateway for sensory information (Markham and Huhman 2008; Roozendaal et al 1991; Walker et al 2005). A paucity of data exists on the role of the medial amygdala in sensorimotor processing including prepulse inhibition of the acoustic startle response (PPI). PPI is the reduction in startle reflex magnitude when a startling stimulus is preceded by a weak prestimulus. Complete amygdala lesions result in disturbed PPI (Decker et al 1995), which is proposed to be mediated via the basolateral amygdala as both specific lesioning and local antagonism of the dopamine 2/3 receptor disrupted PPI (Shoemaker et al 2003; Stevenson and Gratton 2004). However, in view of a close integration within the amygdaloid complex, the medial amygdala may at least to a certain extent be involved in sensorimotor gating processes (Sah et al 2003).

Therefore, the present study aimed to study the effects of bilateral electrolytic lesions of the medial amygdala on unconditioned anxiety-related behavior as well as sensorimotor gating parameters. Anxiety-related behavior was assessed with the use of stress-induced hyperthermia (SIH), light-enhanced startle (LES) and open-field behavior. SIH uses the stress-induced rise in body temperature mediated by the autonomic nervous system (Vinkers et al 2008; Zethof et al 1995), whereas LES measures the unconditioned increase in the acoustic startle reflex in response to bright light (de Jongh et al 2002; Walker and Davis 1997). Sensorimotor gating was assessed using the PPI paradigm. As anxiety-related behavior and sensorimotor gating both constitute unconditioned responses that integrate different aspects of an organism's response to external stimuli, the present study tested the hypothesis that both paradigms may rely on activation of the medial amygdala.

## 2. Materials and methods

### 2.1 Animals

Twenty male Wistar rats weighing around 300 grams (Harlan Zeist, the Netherlands) were housed socially in a controlled environment with a non-reversed 12 hour light/dark cycle (white lights on from 7am-7pm). Animals had unlimited access to standard lab chow and water. One week after arrival, telemetry transmitters were implanted in the abdominal cavity and ID microchips (UNO Micro ID 12/13mm transponder, UNO BV, Zevenaar, The Netherlands) were injected subcutaneously as described earlier (Pattij et al 2002a). After surgery, rats were group-housed in type II Macrolon® cages with a plastic tube as cage enrichment. Food (standard lab chow) and tap water were available ad libitum. Two weeks later, bilateral stereotactic lesions were placed in the medial amygdala. All experiments were carried out with approval of the ethical committee on animal experiments of the Academic Biomedical Center, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki.

### 2.2 Surgeries

#### *Telemetry transmitter procedure*

Shortly, telemetric devices (type ETA-F20, Data Sciences International, St Paul, MN, USA) were implanted in the abdominal cavity as described earlier (Pattij et al 2001). Prior to surgery, rats received a subcutaneous injection (2 ml/kg) of the antibiotic Baytril® (2.5% enrofloxacin). Carprofen (5 mg/kg, s.c.) was given as an analgetic immediately after surgery and twice daily for two days after surgery. After surgery, body weight was measured to monitor recovery from surgery. Wound recovery was regularly checked.

#### *Lesion procedure*

Animals were anesthetized and placed into a stereotaxic apparatus (Kopf) with the incisor bar at -3.3 mm. Blunted ear bars were used to protect the tympanic membranes. After the incision was made, lidocaine 5% was applied as a local anesthetic. Flat skull coordinates are given in millimeters, obtained from Paxinos and Watson, 1986 (Paxinos and Watson 1986). The anteroposterior (AP), mediolateral (ML), and dorsoventral (DV) coordinates were referenced from bregma. The electrode was aimed at the medial amygdala AP -2,6 mm, ML +/- 3,3 mm and DV -9,4 mm. Bilateral lesions were made with a custom made monopolar stainless steel electrode with a diameter of 500 µm and an uninsulated tip of 900 µm (FHC, Bowdoinham, ME, USA) connected to an electrolytic lesion machine (Ugo Basile Lesion Maker, Italy) using the following procedure: a small incision was made in the dura; the electrode was lowered to the selected coordinate and a positive current (2 mA) was passed for 13s. Current amplitude and duration were determined in a separate experiment (data not shown). Sham groups were treated identically to the lesion groups, except the electrode tips were placed 1.0 mm dorsal (i.e. -8,4 mm DV) to the lesion coordinates and no current was passed. Carprofen (5 mg/kg, s.c.) was given as an analgetic immediately after surgery and twice daily for two days after surgery. Recovery from surgery was closely monitored.

### 2.3 Radiotelemetry system

The radio telemetry system consisted of an implanted transmitter (DSI, type ETA-F20), a receiver placed under the cage (DSI model RPC-1) and a data exchange matrix collecting receiver signals and subsequently sending them to a computer where all raw data was stored. Data were collected using Dataquest Gold A.R.T. software (DSI, version 2.2). Raw data consisted of locomotor activity and body temperature responses and was collected for 10 seconds every 3 minutes.

### 2.4 Experimental Procedures

#### *Home cage stress responsivity and circadian rhythm of locomotor activity and body temperature*

In the third week after surgery, circadian rhythm data (temperature and locomotor activity) were collected over 24 hours during which animals were left undisturbed. In the fourth week after lesion surgery the effect of two different stressors on body temperature and locomotor activity was determined in the home cage. To induce stress in the home cage, rats first received an intraperitoneal saline injection (injection stress), followed by placement in a clean cage with fresh bedding 60 minutes later (novel cage stress) after which rats were left undisturbed.

#### *Open field test*

All animals were tested in an open field in the fourth week after lesion surgery. Each animal was placed in the center of the open field and allowed to explore for 30 min. The open field was dimly lit (20 lux) at the bottom of the box (Ø 48 cm). Animals were tracked using an automatic tracking system (TSE ActiMot V7.01 (TSE systems GmbH, Bad Homburg, Germany)).

#### *Prepulse inhibition and light-enhanced startle*

All startle, PPI and LES experiments were carried out in the fifth and sixth week after lesion surgery. Eight startle devices were used simultaneously (SR-lab, San Diego instruments, San Diego CA, USA). The startle devices consisted of a Plexiglas cylinder (9 cm in diameter and 20 cm in length). Each startle device was placed in a ventilated sound attenuated cubicle. Cage movements were measured with a piezoelectric film attached to the Plexiglas base of the startle device. A calibration system (San Diego Instruments) was used to ensure comparable startle magnitudes across the eight devices. Startle stimuli, consisting of 50 ms white-noise bursts, were presented through a piezoelectric tweeter situated 15 cm from the top of the cylinder. Background noise was 70 dB for all tests. Sound intensities were measured using a microphone which was placed on top of the Plexiglas cylinder and fitted to a Bruel and Kjaer sound level meter (Type 2226). Startle amplitudes were sampled each ms during a period of 65 ms beginning at the onset of the startle stimulus. There was no background illumination in any of the experiments.

#### *Baseline startle*

Animals were placed in the startle chamber and, after a 5 min acclimation period, presented with 30 startle stimuli (10x 100, 105 and 115 dB). The inter stimulus interval was set at 30s.

### *Prepulse inhibition*

Differential sensitivity to startle stimulus intensity may influence the level of inhibition by a preceding prepulse. As complete amygdala ablation has been reported to result in altered sensitivity to specific stimulus intensities (Decker et al 1995), the level of prepulse inhibition was assessed at different startle stimulus intensities. Animals were placed in the startle chamber and, after a 5 min acclimation period, presented with startle stimuli (110 and 115dB, 50ms) that were presented alone or preceded by noise prepulses (20ms), with 100ms between onsets of the prepulse and startle stimulus. The test session was built up in 4 blocks. Block 1 and block 4 consisted of 10 startle stimulus trials (5x 110dB, 5x 115dB). Block 2 and 3 both consisted of 5 presentations each of 110dB and 115dB startle stimulus trials and 5 presentations each of 6 different prepulse trials (4, 8 and 16 above background preceding either at 110dB or 115dB pulse). In addition, no-stimulus trials were included as a measure of general activity. All trials were presented in a pseudorandom order and the inter trial intervals ranged from 10 to 20 seconds. Within-session habituation was analysed from the 110dB and 115dB stimulus trials during block 1 to 4. Percentage prepulse inhibition was calculated for each prepulse intensity as percent change compared to the mean startle reflex in response to the 115dB startle stimulus.

### *Light-enhanced startle*

For light-enhanced startle measurements, each startle device was equipped with a white fluorescent bulb on the back wall of the sound attenuated cubicle, which produced an illumination level of approximately 2000 lux measured from inside the Plexiglas cylinder using a Gossen luxmeter (MAVOLUX 5032C). Animals were placed in the startle chamber and, after a 5 min acclimation period, presented with 30 startle stimuli (10x 100, 105 and 115 dB) under dark control conditions and with 30 startle stimuli (10x 100, 105 and 115dB) under brightly lit conditions. The inter stimulus interval was set at 30s.

## **2.5 Histology**

After behavioral testing was completed, lesioned animals were anesthetized and decapitated. Brains were removed, placed in buffered 10% formalin for 2 days and subsequently immersed in sucrose solution (30% v/v) for at least 2 weeks. Then, brains were frozen and sectioned (60  $\mu$ m) in the frontal plane through the relevant brain areas using a cryostat. Relevant sections were mounted onto gelatin-coated slides and stained with cresyl violet. Electrolytic lesions were evaluated according to the location and extent of the tissue damage using a three-dimensional damage reconstruction on a series of stereotaxic atlas planes (Paxinos and Watson 1986). Data were only included for lesioned animals with bilateral damage to the medial amygdala with minimal damage on neighboring nuclei (see Figure 1).

## **2.6 Data analysis**

Statistical analysis was performed using SPSS for Windows version 16.0 (SPSS, Chicago, IL, USA). All data are displayed as mean  $\pm$  SEM. A probability level of  $p < 0.05$  was set as statistically significant, probability levels between  $p = 0.05$  and  $p = 0.1$  were regarded as trends. All reported results were corrected by the Greenhouse Geisser procedure where appropriate.

#### *Home cage stress responsivity*

All data were collected in 3-minute blocks and are displayed as mean  $\pm$  SEM. All experiments were carried out with a within-subject design. Lesion effects on body temperature and locomotor activity were analyzed for 120 minutes following injection using a repeated measures analysis of variance (ANOVA) with time as within-subject factor and lesion as between-subject factor.

#### *Circadian rhythm of locomotor activity and body temperature*

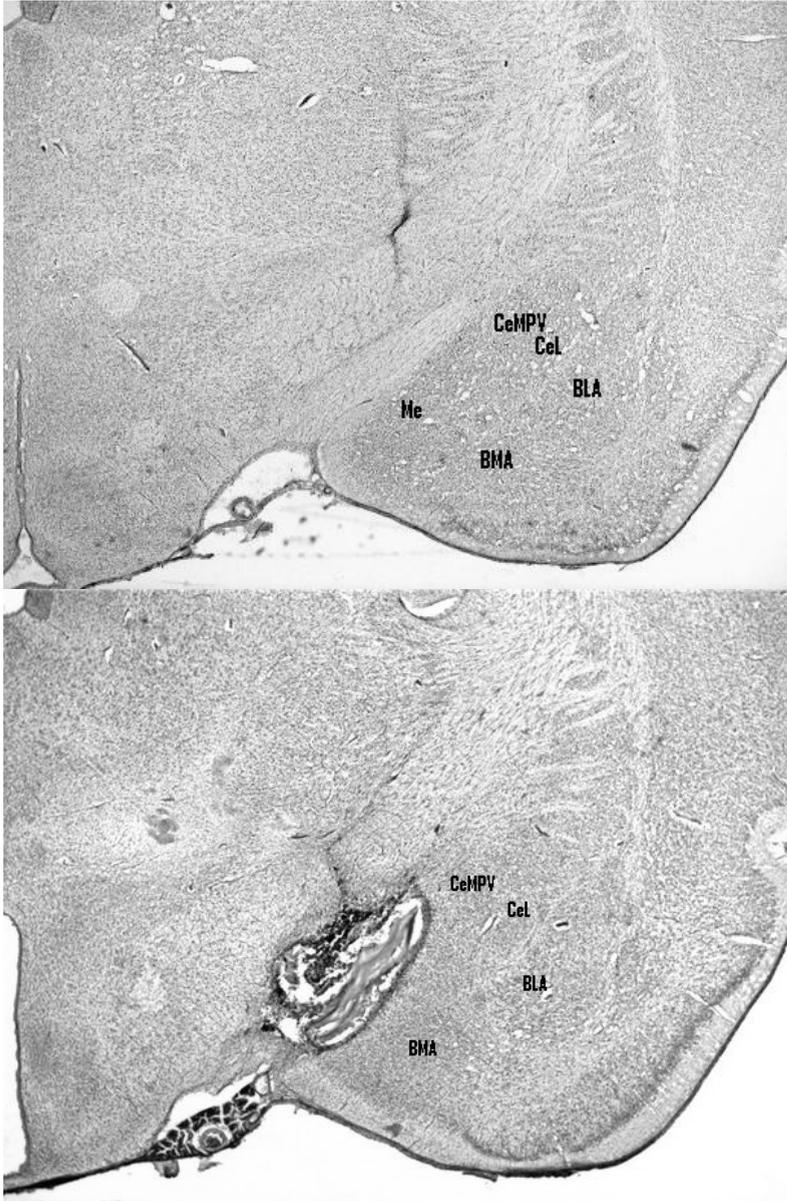
All data were analyzed using a complete undisturbed day. All body temperature and locomotor activity data were averaged to twenty-four 1-h blocks for each rat. All data were analyzed by means of repeated measures analysis of variance (ANOVA) with surgery (lesion or sham) as between-subject factor and the 1-h time blocks of body temperature and locomotor activity as within-subject factor.

#### *Open field test*

Data were averaged (% time in center) or cumulated (total distance travelled). Analysis of the open field data used a one-way ANOVA of the total distance travelled or average percentage of the time spent in center with surgery (lesion and sham) as the between subject factor.

#### *Prepulse inhibition and light-enhanced startle*

Baseline startle responding was analysed with the use of a repeated measures ANOVA with stimulus intensity (100, 105 and 115dB) as within-subject factor and surgery (lesion and sham) as between-subject factor. Percentage prepulse inhibition was analysed with the use of a repeated measures ANOVA with prepulse intensity (4, 8 and 16dB) as within-subject factor and surgery (lesion and sham) as between-subject factor. Analyses of surgery-effects at the specific prepulse intensities were done with the use of independent samples t-tests. Within-session habituation was analysed with the use of a repeated measures ANOVA with block (block 1 to 4) as within-subject factor and surgery (lesion and sham) as between-subject factor. Light-enhanced startle was analysed with the use of repeated measures ANOVA with condition (dark and light) and intensity (100, 105 and 115dB) as within-subject factors and surgery (lesion and sham) as between-subject factor.

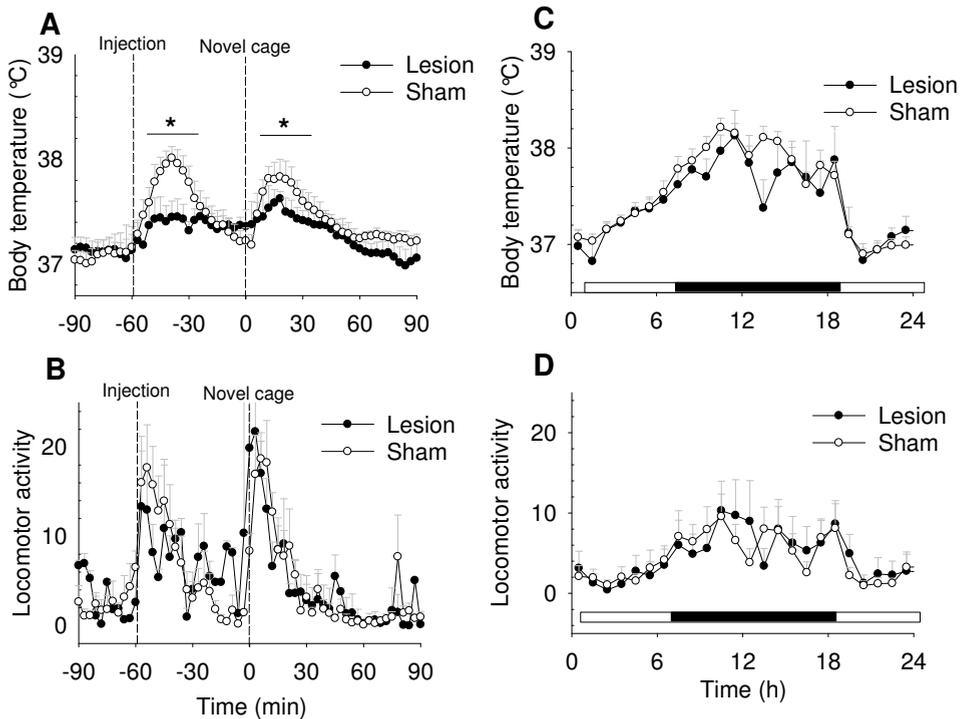


**Figure 1:** A representative photomicrograph (50 x enhanced) of sections through the medial amygdala (MeA) with cresyl-violet staining. A typical sham lesion and a typical MeA lesion are shown. Abbreviations: CeMPV: Central amygdaloid nucleus Medial Posteroventral division; Cel : Central amygdaloid nucleus, Lateral division; BLA: Basolateral amygdaloid nucleus, Anterior part, BMA: Basomedial amygdaloid nucleus, Anterior part.

### 3. Results

#### 3.1 Histology

Based on the histological results, 4 of the 10 rats were excluded from the analysis resulting in 6 lesion animals and 10 sham animals. The extent of a typical medial amygdala lesion is shown in Figure 1. Lesions were confined to the medial amygdala, and typically included the majority of the medial amygdala (including the anteroventral, anterodorsal, posteroventral and posterodorsal divisions) with minimal damage to the medial division of the central nucleus and the basomedial nuclei. All lesions extended into the nucleus of the optic tract and a few lesions extended into the internal capsula.



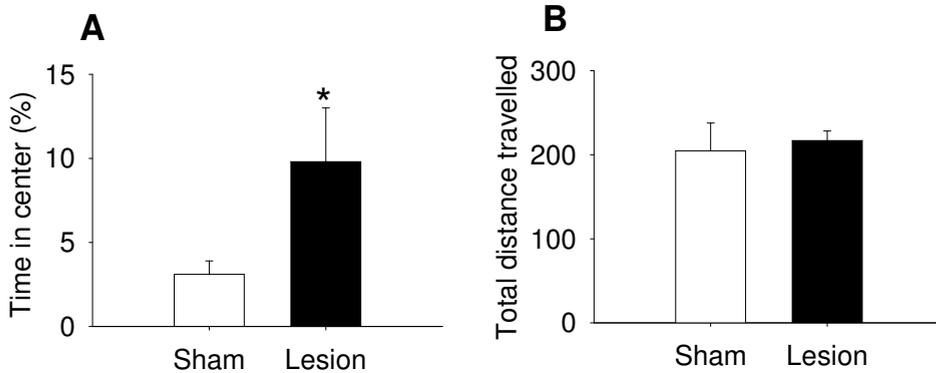
**Figure 2:** Stress-induced hyperthermia (A) and stress-induced locomotor (B) responses in sham ( $n=10$ ) and lesion animals ( $n=6$ ). Circadian rhythm of body temperature (C) and locomotor activity (D) levels over 24 hours. \*:  $p<0.05$

#### 3.2 Home cage stress responsivity (Figure 2A-B)

The SIH response was significantly reduced in lesioned animals in response to both injection and novel cage stress (lesion  $\times$  time interaction  $F_{40,560}=2.02$ ,  $p<0.001$ ). Stress-induced locomotor levels were not different between lesion and sham animals (lesion  $\times$  time interaction  $F_{40,520}=0.92$ ,  $p=0.62$ ; lesion effect  $F_{1,13}=0.05$ ,  $p=0.83$ , NS). Before lesion surgery, no differences were present between the future sham and future lesion groups in their body temperature and locomotor activity responses to injection and novel cage stress (data not shown).

### 3.3 Circadian rhythm of locomotor activity and body temperature (Figure 2C-D)

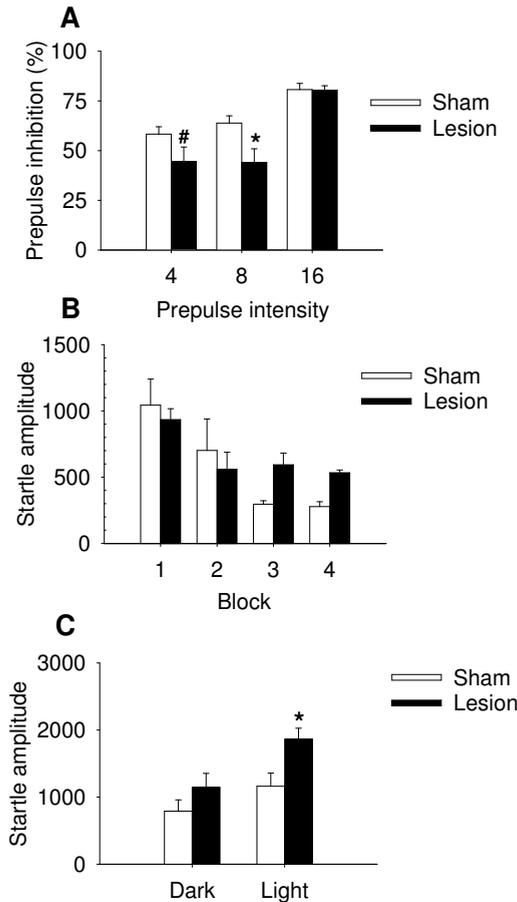
No significant differences were present between lesioned and sham animals in the circadian rhythm of either body temperature ( $F_{1,14}=0.11$ ,  $p=0.74$ , NS) or locomotor activity ( $F_{1,13}=0.39$ ,  $p=0.54$ , NS).



**Figure 3:** Percentage of time spent in centre of the open field (A) and total distance travelled in the open field (B). Lesion animals ( $n=6$ ) and sham animals ( $n=10$ ). \* indicates lesion effect ( $p<0.05$ ).

### 3.4 Open field (Figure 3)

Lesioned rats spent significantly more time in the centre of the open field compared to shams (lesion effect  $F_{1,15}=6.73$ ,  $p<0.05$ ). In contrast, total distance travelled did not significantly differ between the lesion and sham groups (lesion effect  $F_{1,15}=0.67$ ,  $p=0.43$ ).



**Figure 4:** Prepulse inhibition with a 115 dB stimulus and 4, 8 and 16 dB prepulse (A), startle habituation to a 100 + 115 dB startle session (B) and light-enhanced startle. \*:  $p < 0.05$ , #:  $p = 0.07$ , trend.

### 3.5 Prepulse inhibition and light-enhanced startle (Figure 4)

Overall, medial amygdala lesions did neither affect basal startle responding (lesion effect:  $F_{1,14} = 0.08$ ,  $p = 0.79$ , NS) nor startle responding at specific startle intensities (intensity  $\times$  lesion interaction:  $F_{1,14} = 2.28$ ,  $p = 0.71$ , NS).

Lesioned animals displayed significantly decreased percentage prepulse inhibition of the 115dB startle stimulus (prepulse  $\times$  surgery interaction:  $F_{2,28} = 5.63$ ,  $p < 0.01$ ). Independent samples t-tests revealed that the level of inhibition in response to the 8dB prepulse was most strongly decreased (4dB:  $t = 1.87$ ,  $p = 0.08$ ; 8dB:  $t = 2.84$ ,  $p = 0.01$ ; 16dB:  $t = 0.07$ ,  $p = 0.95$ ). In contrast, the lesion group did not significantly affect within-session startle habituation (block  $\times$  lesion interaction:  $F_{3,42} = 1.76$ ,  $p = 0.17$ , NS).

Significant light-enhanced startle was induced in both groups (lesion:  $F_{1,8} = 9.24$ ,  $p = 0.016$ , sham:  $F_{1,4} = 28.14$ ,  $p = 0.006$ ). Lesion animals showed a trend towards increased light-enhanced startle (condition  $\times$  lesion interaction  $F_{1,13} = 3.81$ ,  $p = 0.07$ ). Separate analysis of the conditions showed that lesion animals showed a significantly stronger response during the brightly lit condition (lesion effect:  $F_{1,13} = 5.33$ ,  $p < 0.05$ ), whereas both groups responded similarly during control conditions (lesion effect:  $F_{1,13} = 1.56$ ,  $p = 0.23$ , NS).

## 4. Discussion

The present study investigated the role of the medial amygdala in stress responsivity and sensorimotor gating in rats. Bilateral electrolytic lesions of the medial amygdala decreased acute anxiety-related autonomic and open field stress responses, whereas augmented light-enhanced startle and disrupted prepulse inhibition were found. Thus, the medial amygdala seems to be involved in the responses to environmental stimuli as well as in sensorimotor gating. Electrolytic lesions were confined to the medial amygdala and size and placement were confirmed histologically. However, the current study design has intrinsic limitations as electrolytic lesions are not specific to cell bodies in the medial amygdala but also disrupt fibers of passage.

The amygdala is involved in the coordination of behavioral, autonomic and endocrine responses to stress (Joels and Baram 2009; Roozendaal et al 2009), but is also an important gatekeeper involved in the regulation of vigilance (Anderson and Phelps 2001; Phelps and LeDoux 2005; van Marle et al 2009). This vigilance comprises an evaluation of emotional environmental stimuli resulting in an enhanced processing of sensory information (Davis and Whalen 2001). The amygdala is structurally complex, consisting of several nuclei with specific functions in the affective response to environmental stimuli (Swanson and Petrovich 1998). However, the exact contribution of each nucleus of the amygdala is unknown. The medial amygdala (MeA) has been proposed to mediate the unconditioned response to stress (Cullinan et al 1995; Dayas et al 1999; Masini et al 2009). So far, the medial amygdala has been shown to be involved in responses to restraint and acute swim stress (Cullinan et al 1995; Dayas et al 2001; Dayas et al 1999; Emmert and Herman 1999; Pezzone et al 1992), the neuroendocrine stress response (Masini et al 2009), the autonomic blood pressure stress response (Kubo et al 2004), condition taste aversion (Rollins et al 2001) and aggressive as well as sexual behavior (Veening et al 2005; Vochteloo and Koolhaas 1987). Structurally, the medial amygdala is primarily composed of GABAergic neurons that provide tonic inhibitory input to various brain structures including the posterior and medial divisions of the bed nucleus of the stria terminalis (BNST), the central and basolateral amygdala, periaqueductal grey, hippocampus, ventromedial hypothalamus and medial preoptic area (Cullinan et al 2008; Dong et al 2001; Herman et al 2003; Herman et al 2004; Herman et al 2002; Ikegaya et al 1995; Sah et al 2003). Therefore, irreversible interruption of inhibitory medial amygdala activity may result in neuronal disinhibition of these central structures.

Medial amygdala lesions reduced the autonomic SIH response without affecting locomotor activity levels (Figure 2). The neuronal circuitry underlying the SIH response has not been elucidated (Veening et al 2004), but it has been shown that the dorsomedial hypothalamus (DMH) plays an important role in general thermoregulation as well as autonomic stress responsivity (Dimicco and Zaretsky 2007; Ulrich-Lai and Herman 2009). However, direct connections between the medial amygdala and the DMH have not been observed (Thompson and Swanson 1998), and direct medial amygdala projections to other autonomic output areas are scarce (Ulrich-Lai and Herman 2009). Therefore, connections between the medial amygdala and DMH may be indirect via other amygdaloid structures including the central, lateral, basal nuclei (Sah et al 2003), or,

alternatively, via the ventromedial hypothalamus or medial preoptic area. This may also explain attenuated stress-induced blood pressure responses after local administration of muscimol into the medial amygdala (Kubo et al 2004). However, medial amygdala lesions do not completely abolish the SIH response, and other brain areas including the thermoregulatory preoptic area may also be involved in autonomic stress responses (Cullinan et al 2008; Herman et al 2004). In addition to a decreased autonomic response, medial amygdala lesions increased the time spent in the center of an open field without affecting overall locomotor activity levels. Limbic projections to special somatic motor neurons have been proposed to be involved in emotional responsivity, being located in the brainstem ventromedial medulla, periaqueductal gray, periventricular nucleus and the lateral hypothalamus with direct spinal projections to somatomotor efferents (Kerman 2008). Also, amygdaloid innervations to the basal ganglia (including the nucleus accumbens) are proposed to modulate emotional locomotor behavior (Mogenson et al 1980; Takakusaki et al 2004). The medial amygdala receives important and direct input from the olfactory bulbs (Kang et al 2009; Kevetter and Winans 1981; Scalia and Winans 1975) and mediates freezing to olfactory cues (Chen et al 2006; Dielenberg et al 2001). Therefore, medial amygdala lesions may at least partially prevent olfactory information to be processed by the amygdala, which may directly explain the altered stress-related behavior. Along these lines, altered autonomic stress responses after olfactory bulbectomy suggest the involvement of the medial amygdala (Vinkers et al 2009b). Other sensory information (e.g. auditory, visual, gustatory) reaches the amygdala through projections from the medial thalamus to the amygdaloid lateral and basal nuclei (Sah et al 2003).

Interestingly, the circadian rhythm of body temperature and locomotor activity remained unaffected, indicating that basal thermoregulatory and locomotor processes are unaltered in the lesioned animals. Also, basal and stress-induced locomotor activity levels in the home cage were not affected. Thus, the overall locomotor capacity in lesioned animals seems to be intact. These data are supported by a study in which local injection of the GABA<sub>A</sub> receptor agonist muscimol into the medial amygdala impaired the escape response in the elevated T-maze without affecting overall locomotor levels in the open field (Herdade et al 2006). Also, complete amygdala lesions did not affect the total distance travelled in the open field (Decker et al 1995; Werka et al 1978). This indicates that different brain circuitry is involved in non-specific stress-induced locomotor activity levels in contrast to specific anxiety-related behavior.

In lesioned animals, baseline acoustic startle responses were unchanged, which is in line with the primary neurocircuitry (Koch 1999). In contrast, affective startle modulation changed, resulting in an increased light-enhanced startle (LES) response. The MeA is connected to other structures that are known to be closely involved in LES including the BNST (Dong et al 2001) and the hippocampus (Veening et al 2009). The BNST is important in the expression of light-enhanced startle as both pharmacological and electrolytical lesions of the BNST block light-enhanced startle (Walker and Davis 1997; Walker et al 2009), whereas the septohippocampal system also plays a role in LES (Veening et al 2009). Our data suggest that lesions of the medial amygdala result in an augmented LES which may be the direct effect of the lesion or which may be due to disrupted fibers of passage.

These results contrast with a decreased SIH response in the lesion group, indicating that the SIH and LES anxiety paradigms probably measure either different anxiety-related behavior, or anxiety-related behavior mediated by different brain regions. Thus, although both the SIH and LES response measure direct responses to environmental disturbances and both are sensitive to the effects of benzodiazepines and 5-HT<sub>1A</sub> receptor agonists (de Jongh et al 2002; Olivier et al 2002), MeA lesions differentially affect both paradigms.

In the lesioned rats, prepulse inhibition was disrupted, indicating that the medial amygdala may be important in sensorimotor processing. To our knowledge, this is the first study to show involvement of the medial amygdaloid nucleus in sensorimotor gating. However, the neuronal mechanisms underlying these effects are unknown. Previous studies have shown that lesions of the whole amygdala result in disturbed PPI (Decker et al 1995). Also, specific lesions of the basolateral amygdala (BLA) disrupt PPI, which implicates a role for the BLA in sensory motor processing (Shoemaker et al 2003; Stevenson and Gratton 2004). As the medial amygdala has direct connections with the BLA, altered BLA activity may be responsible for the lesion-induced disruption of PPI. Alternatively, direct projections from the medial amygdala to other brain regions that are involved in the regulation of prepulse inhibition such as the hippocampus may be involved (Swerdlow et al 2001).

In conclusion, the results of the present study suggest a differential role for the medial amygdala in the regulation of unconditioned psychological stress responses as well as sensory motor processing in rats. After bilateral lesion of the medial amygdala, reduced autonomic and open field anxiety-related behavior was present, whereas these lesions resulted in an augmented LES and a decreased PPI. In contrast, basal locomotor and thermoregulatory capacity and basal acoustic startle were unaffected. Altogether, our data suggest that the medial amygdala is an important player in response to acute environmental stimuli. However, considering the existing data on BLA involvement in PPI and BNST involvement in LES, local infusion studies into the MeA should be performed to further substantiate these findings.

### **Acknowledgements**

The authors would like to thank Noëlle de Jong and Marianne Klanker for their assistance during the experiments.

## Chapter 13

# **Olfactory bulbectomy induces rapid and stable changes in basal and stress-induced locomotor activity, heart rate and body temperature responses in the home cage**

Christiaan H. Vinkers

Megan E. Breuer

Koen G.C. Westphal

S. Mechiel Korte

Ronald S. Oosting

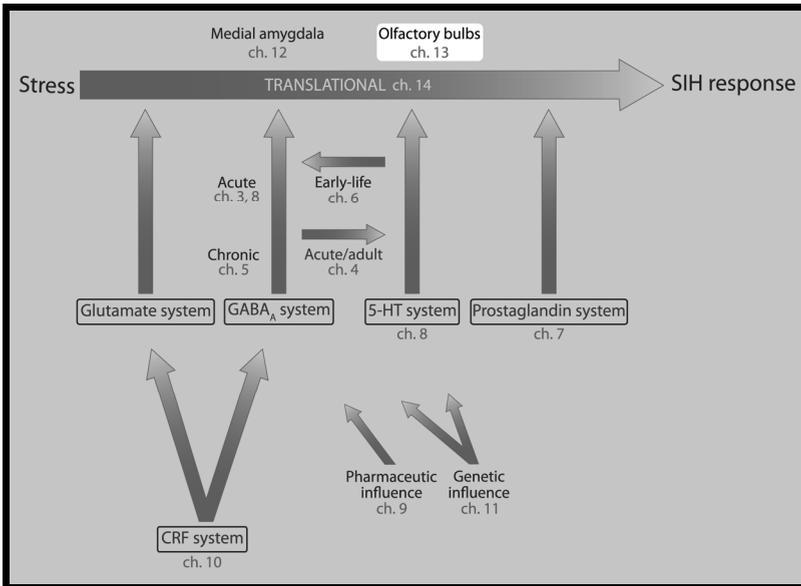
Berend Olivier

Lucianne Groenink

# 13

*Neuroscience (2009) 159(1):39-46*

## Abstract



**Background:** Olfactory bulbectomy (OBX) in rats causes several behavioral and neurochemical changes. However, the extent and onset of physiological and behavioral changes induced after bulbectomy have been little examined.

**Methods:** Male Sprague-Dawley rats received telemetric implants. Before and immediately after OBX surgery, basal and stress-induced heart rate, body temperature, and locomotor activity were measured in the home cage in sham (n=9) and OBX animals (n=11). Stress was induced using novel cage stress or witness stress.

**Results:** Bulbectomized animals differed physiologically and behaviorally from shams. Nocturnally, OBX animals were significantly more active compared to shams and had a higher core body temperature and displayed decreased heart rate variability. During the light period, OBX animals had a significantly lower basal heart rate and reduced heart rate variability. These effects became apparent after 2-3 days after OBX surgery, and were stable over time. After witness stress, OBX animals showed smaller autonomic (body temperature and heart rate) responses compared to shams, but showed no difference in locomotor responses. In contrast, novel cage stress led to increased locomotor responses in OBX rats compared to sham rats, while no differences were found in autonomic responses.

**Conclusions:** Removal of the olfactory bulbs results in rapid, stable and persistent changes in basal locomotor activity, body temperature, heart rate and heart rate variability. Although the sleep-wake cycle of these parameters is not altered, increases in circadian amplitude are apparent within three days after surgery. This indicates that physiological changes in the OBX rat are the immediate result of olfactory bulb removal. Further, stress responsivity in OBX rats depends on stressor intensity. Bulbectomized rats display smaller temperature and heart rate responses to less intense witness stress compared to sham rats. Increased locomotor responses to more intense novel cage stress are present in the home cage as well as the open field. The present study shows that olfactory bulbectomy has a rapid and persistent influence on basal and stress-induced physiological parameters.

## 1. Introduction

The olfactory bulbectomy (OBX) rat model is an animal model of depression where removal of the olfactory bulbs leads to a variety of behavioral and neurochemical alterations (Song and Leonard 2005). Besides the overwhelming data on neurochemical and behavioral changes in the OBX rat (Song and Leonard 2005), studies on OBX-induced physiological parameters as heart rate, locomotor activity and body temperature are not abundant. As depressed patients are known to possess reduced heart rate variability and altered autonomic tone (Davydov et al 2007; Owens and Nemeroff 1998), it is interesting to study these parameters in an animal model.

After bulbectomy, locomotor circadian rhythmicity is changed with increased nocturnal locomotor activity in rats (Giardina and Radek 1991) and mice (Possidente et al 1996), with lengthened free-running activity periods with delayed acrophase (Pieper and Lobocki 1991; Possidente et al 1996; Possidente et al 1990). Also, bulbectomy leads to altered circadian rhythmicity of body temperatures and thermoregulation (Forster et al 1980; Marcilhac et al 1997), as well as to decreased basal heart rate and blood pressure (Song and Leonard 2005). These behavioral changes are not just due to anosmia (van Riezen et al 1977), but rather the result of neuronal reorganization in various brain regions which is the result of retrograde neuronal degeneration after bulbectomy (Kelly et al 1997). Surprisingly, data on the onset of long-lasting and persisting bulbectomy-induced changes are scarce. One study reports that nocturnal hyperactivity occurs at 10 days postsurgery (Giardina and Radek 1991). Therefore, our first aim was to study the onset and extent of putatively altered circadian rhythmicity and amplitude of basal locomotor activity, heart rate and body temperature after OBX surgery by continuously monitoring these parameters before and immediately after OBX surgery.

In light of autonomic dysfunction in depression (Carney et al 2005), an important aspect of the OBX rat seems the inability to adapt to stress (Song and Leonard 2005). Because the olfactory bulbs project to the (medial) amygdala and the bed nucleus of the stria terminalis, their removal leads to subsequent amygdaloid disinhibition (Kelly et al 1997; Wrynn et al 2000). Generally, OBX rats display increased responses to stressful stimuli such as hyperactivity in the open field (van Riezen and Leonard 1990) and increased startle reflexes (McNish and Davis 1997). However, to our knowledge there are no studies assessing the stress responses in the home cage. Assessing home cage behavior yields advantages, including the possibilities to monitor circadian rhythms, habituation rates and baseline responses. Therefore, our second aim was to study stress responses of heart rate, body temperature and locomotor activity in the home cage of OBX animals. In the present study, we used novel cage stress and witness stress to elicit a stress response. Novel cage stress is a moderate psychological stressor with increases in heart rate, temperature and locomotor activity (van den Buuse et al 2001). Witness stress is induced by entering the animal room and is a mild psychological stressor, eliciting significant but smaller autonomic and locomotor stress responses (Bouwknicht et al 2001). As a positive control, a locomotor test was run in a separate open field to establish OBX hyperactivity.

In short, the present study examined the onset of bulbectomy-induced changes in circadian rhythmicity and amplitude of heart rate, heart rate variability, locomotor activity

and body temperature. Also, we examined whether OBX surgery would lead to altered acute stress responses after stress exposure in the home cage using two stressors of different intensity.

## **2. Experimental procedures**

### **2.1 Animals**

20 male albino Sprague Dawley rats (Harlan, Zeist, The Netherlands), aged 9 weeks were housed under 12h light/12h dark cycle (light on at 6:00 AM) at a temperature of  $21 \pm 2^\circ\text{C}$  with a relative humidity of  $60 \pm 15\%$ . Rats were housed two per cage. One rat was equipped with a telemetric device, whereas the other functioned as a social companion. All animals were housed in the same room and were tested at the same time. Food and water were available ad lib. All experiments were carried out with approval of the ethical committee on animal experiments of the Faculties of Sciences, Utrecht University, the Netherlands, and in accordance with the Declaration of Helsinki. After all experiments were carried out, all animals were euthanized via decapitation, and the brains were examined for verification of complete olfactory bulb ablation. No animals were excluded from analysis due to partial bulbectomies or damaged prefrontal cortices.

### **2.2 Surgeries**

#### *Telemetry transmitter surgery*

Radio telemetry transmitters (Data Sciences International (DSI), type ETA-F20, St. Paul, MN, USA) were implanted in the abdominal cavity as earlier described (Pattij et al 2002a). Animals received Rimadyl, (5 mg/kg, subcutaneously) post-surgically twice a day for 2 days, as well as a solid energy drink (Triple A Trading, Otterlo, The Netherlands) and soaked food pellets. Animals were allowed to recover during a period of 2 weeks.

#### *Bulbectomy (OBX) surgery*

After transmitter implantation, OBX surgery was carried out as earlier described (Vinkers et al 2009b). In short, two burr holes overlying the olfactory bulbs were drilled. For the bulbectomized animals, the tissue was removed with a blunt hypodermic needle and a vacuum pump, for sham animals the olfactory bulbs were not removed. Animals received Rimadyl, (5 mg/kg, subcutaneously) post-surgically twice a day for 2 days and were monitored for signs of discomfort or infection. All subjects were allowed to recover for 2 weeks.

### **2.3 Radiotelemetry system**

The radio telemetry system consisted of an implanted transmitter with two flexible leads (DSI, type ETA-F20), a receiver placed under the cage (DSI model RPC-1) and a data exchange matrix collecting receiver signals and subsequently sending them to a computer where all raw data was stored. Data were collected using Dataquest Gold A.R.T. software (DSI, version 2.2). Raw data consisted of locomotor activity, temperature and heart rate responses collected for 10 seconds every 5 minutes. All animals were sampled together.

## 2.4 Procedures

### *Circadian rhythm*

Circadian rhythm data (temperature, heart rate and locomotor activity) of undisturbed days were collected before OBX surgery. Starting immediately after OBX surgery (as soon as animals were recovering from the surgery), circadian rhythm data were again collected.

### *Acute stress exposure*

Rats were exposed to either novel cage stress or witness stress before and after OBX surgery. Before OBX surgery, all animals were subjected to both stressors to confirm the intensity relation between the two stressors. Subjects were put either into a novel cage (Macrolon type II) ("novel cage stress") or exposed to witness stress in the fifth week after surgery, which consisted of an investigator entering the animal room without any actual cage disturbance. The investigator entered the room in order to give half of the animals a novel cage, while taking care not to touch, move or otherwise directly disturb the other half of the animals. The cage change took less than 5 minutes, and immediately afterwards, the investigators left the room. The witness stress procedure resembles the procedure used by Bouwknecht in our lab (Bouwknrecht et al 2001). Acute stress experiments were thus performed over 2 consecutive days at the same time of day. The first day, half of the animals were subjected to novel cage stress, automatically exposing the other half of the animals to witness stress. The second day, the same procedure was applied to the other half of the rats. After two days, all animals had been exposed to both novel cage and witness stress.

### *Open field procedure*

All animals were tested in the open field in the third week after OBX surgery in the same week as the home cage stress exposure. Each animal was placed in the center of the open field and allowed to explore for 15 minutes. The open field was lit at normal room illumination (420 lux at floor level), and the boxes measured 72x72 cm. Each box was painted a light gray color for ease of observation. Animals were tracked with Noldus EthoVision (Noldus Information Technology, Leesburg, Virginia).

## 2.5 Data analysis

### *Circadian rhythmicity*

Presurgery data were analyzed using 4 undisturbed days, whereas postsurgically, data were analyzed during the first 14 days immediately after surgery. All temperature, heart rate and locomotor activity data were averaged to a single 24-h period for each rat, after which group values were averaged to time periods of 8 3-h blocks. For heart rate, also heart rate variability (HRV), a parameter for autonomic control of heart rate (Stockmeier et al 2009) was computed as the mean standard deviation of heart rate as described earlier (Pattij et al 2002a). All data were analyzed by means of repeated measures ANOVA with surgery (OBX or sham) as 'between subject' factor and the 3-h blocks of respectively temperature, heart rate and locomotor activity as 'within subject' factor. All reported results were corrected by the Greenhouse Geisser procedure where appropriate, which is indicated by an adjustment of the degree of freedom. As planned, light and dark periods were also separately analyzed.

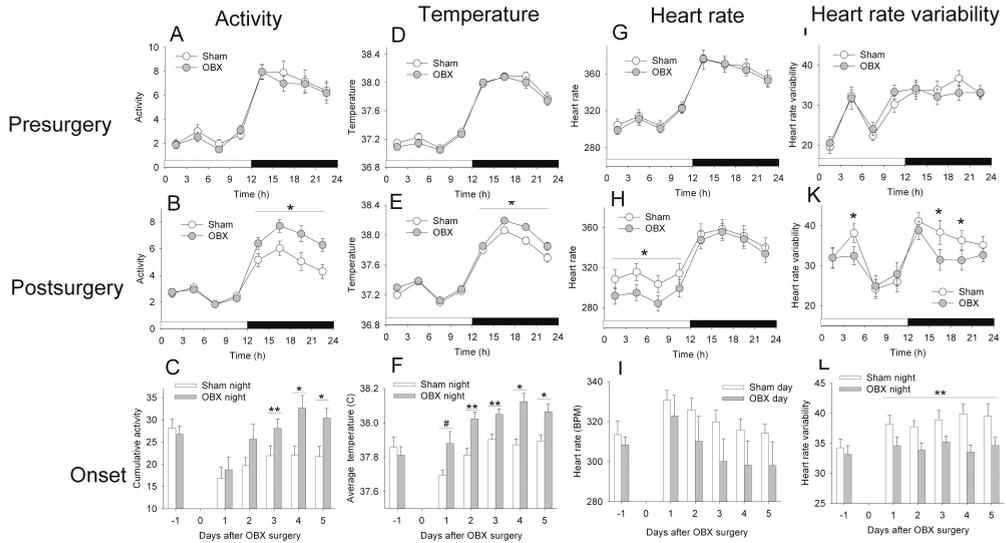
The onset of changes in circadian rhythmicity and amplitude was studied by cumulating (locomotor activity) or averaging (body temperature, heart rate and heart rate variability) daily values (dark and light separately) the day before OBX surgery until the first week after OBX surgery. The onset of changes in amplitude were analyzed using either the dark phase or the light phase depending when OBX-increased changes were visible using repeated measures ANOVA with surgery (OBX or sham) as 'between subject' factor and the daily average of respectively temperature, heart rate and locomotor activity as 'within subject' factor. The diurnal and nocturnal period were separately analyzed. Simple contrast tests were used to compare days postsurgery with the presurgery day whenever a significant main effect for surgery or a significant day x surgery interaction effect was observed. The level of significance was set at  $p < 0.05$ . Statistical analysis was performed using SPSS for Windows version 14.0 (SPSS, Chicago, Ill, USA). All data are expressed in mean  $\pm$  SEM.

#### *Acute stress*

Every 5 minutes, locomotor activity, body temperature and heart rate were measured for 10 seconds. Before surgery, witness and novel cage stress were compared by means of repeated measures ANOVA with time and witness/novel cage stress as within subject factors. All postsurgery data were analyzed by means of repeated measures ANOVA with surgery (OBX or sham) as 'between subject' factor and 5 min-blocks of respectively temperature, heart rate and locomotor activity as 'within subject' factor. Temperature and heart rate data were analyzed up to 120 minutes after the onset of the stressor, whereas shorter-lasting locomotor activity data were analyzed up to 60 minutes after the start of the stressor. In addition, locomotor activity responses after stress were summed and analyzed using a univariate ANOVA with surgery (OBX and sham) as the main factor. Novel cage and witness stress were analyzed separately. All data are expressed as mean  $\pm$  SEM.

#### *Open field test*

Analysis of the open field data used univariate analysis of variance (ANOVA) of the mean distance traveled, with surgery (OBX and sham) as the main factor.



**Figure 1:** Circadian rhythmicity of locomotor activity (A and B), body temperature (D and E) heart rate (G and H) and heart rate variability (J and K) before and after OBX surgery. Postsurgical data reflect the average over 14 days, starting immediately after surgery. The onset of changes induced by bulbectomy are within the first week for locomotor activity during the dark phase(C), body temperature during the dark phase (F), heart rate during the light phase (I) and heart rate variability during the dark phase (L) . \*:  $p < 0.01$ ; \*\*:  $p < 0.05$ ; #:  $p = 0.06$ .

### 3. Results

#### 3.1 Circadian rhythms

##### General

As shown in Figure 1, circadian rhythms were present before and after OBX surgery in both the sham and OBX rats for all parameters tested. During the inactive, lights-on period lower values were observed for locomotor activity (block effect  $F_{7,126} = 89.33$ ,  $p < 0.001$ ,  $\epsilon = 0.47$ ), body temperature (block effect  $F_{7,126} = 266.76$ ,  $p < 0.001$ ,  $\epsilon = 0.48$ ) and heart rate (block effect  $F_{7,126} = 198.79$ ,  $p < 0.001$ ,  $\epsilon = 0.51$ ). OBX surgery led to amplitude changes of all locomotor activity, body temperature and heart rate. However, no apparent changes or shifts were found in the circadian rhythms of these parameters.

##### Locomotor activity

Before OBX surgery, there was no difference in locomotor activity between animals (block\*surgery interaction  $F_{7,126} = 0.45$ ,  $p = 0.87$ , NS; surgery effect  $F_{1,18} = 0.47$ ,  $p = 0.50$ , NS) (Figure 1A). After OBX surgery, OBX animals were more active during the nocturnal period (block\*surgery interaction  $F_{7,126} = 5.78$ ,  $p < 0.001$ ,  $\epsilon = 0.36$ ; surgery effect  $F_{1,18} = 3.96$ ,  $p = 0.062$ , trend) (Figure 1B). Separate analysis of the nocturnal and diurnal period showed OBX animals were more active during the nocturnal period (surgery effect  $F_{1,18} = 6.68$ ,  $p < 0.05$ ), but not during the diurnal period (surgery effect  $F_{1,18} = 0.06$ ,  $p = 0.80$ , NS). Nocturnal hyperactivity (figure 1C) (day x surgery interaction  $F_{7,126} = 4.23$ ,  $p < 0.01$ ,  $\epsilon = 0.57$ ; surgery effect  $F_{1,18} = 5.91$ ,  $p < 0.05$ ) was present from day 3 after OBX surgery (simple contrasts day x

surgery interaction: day 1 vs day -1:  $F_{1,18}=0.34$ ,  $p=0.58$ , NS; day 2 vs day -1:  $F_{1,18}=2.44$ ,  $p=0.14$ , NS; day 3 vs day -1  $F_{1,18}=4.97$ ,  $p<0.05$ ; day 4 and further  $F_{1,18}=9.97$ ,  $p<0.01$ ).

#### *Body temperature*

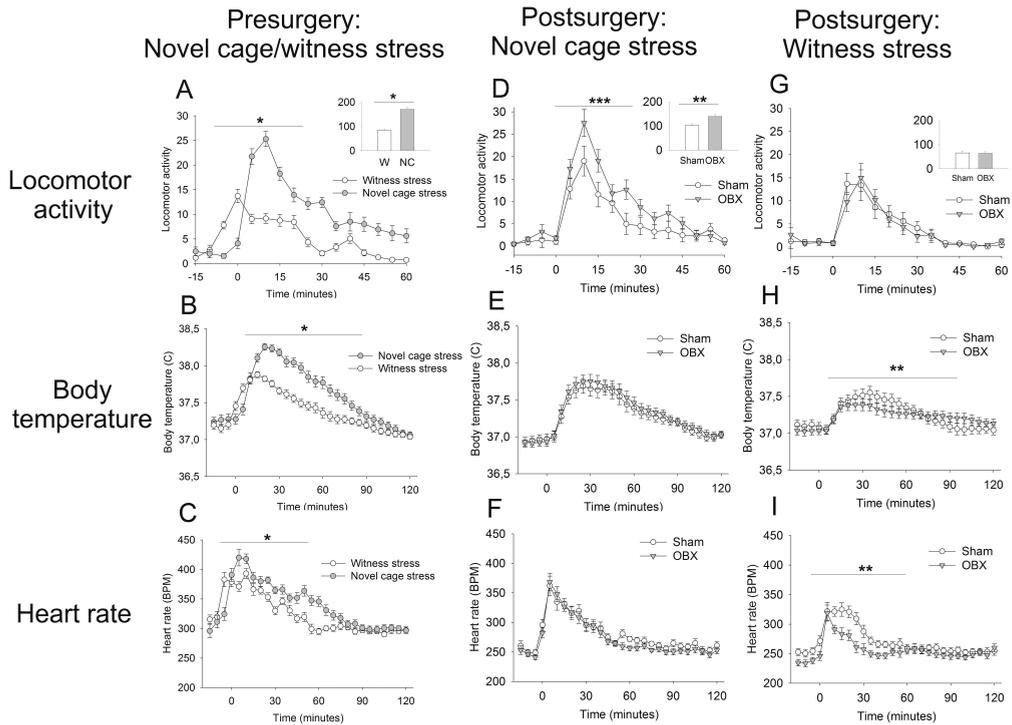
Before OBX surgery, there was no difference in body temperature between animals (block\*surgery interaction  $F_{7,126}=0.60$ ,  $p=0.75$ , NS; surgery effect  $F_{1,18}=0.78$ ,  $p=0.39$ , NS) (Figure 1D). After OBX surgery, OBX animals had an overall higher body temperature (surgery effect  $F_{1,18}=6.01$ ,  $p<0.05$ ) (figure 1E). Separate analysis of the nocturnal and daily period showed OBX animals had a higher body temperature during the nocturnal period (surgery effect  $F_{1,18}=8.56$ ,  $p<0.01$ ), but not during the light period (surgery effect  $F_{1,18}=1.40$ ,  $p=0.25$ , NS). Nocturnal body temperature increases (figure 1F) (day x surgery interaction  $F_{7,126}=3.21$ ,  $p<0.05$ ,  $\epsilon=0.52$ ; surgery effect  $F_{1,18}=14.08$ ,  $p=0.001$ ) were significant from day 2 after OBX surgery (simple contrasts: day x surgery interaction: day 1 vs day -1:  $F_{1,18}=3.78$ ,  $p=0.07$ , trend; day 2 vs day -1:  $F_{1,18}=7.93$ ,  $p<0.05$ ; day 3 vs day -1  $F_{1,18}=6.24$ ,  $p<0.05$ ; day 4 and further  $F_{1,18}=12.44$ ,  $p<0.01$ ).

#### *Heart rate*

Before OBX surgery, there was no difference in heart rate between animals (block\*surgery interaction  $F_{7,126}=0.22$ ,  $p=0.98$ , NS; surgery effect  $F_{1,18}=0.09$ ,  $p=0.77$ , NS) (Figure 1G). After OBX surgery, OBX animals had lower resting heart rate during the light period (block\*surgery interaction  $F_{7,126}=4.00$ ,  $p=0.01$ ,  $\epsilon=0.43$ ) (figure 1H). Separate analysis of the nocturnal and day period did not show significant heart rate differences between OBX and sham animals during the nocturnal (surgery effect  $F_{1,18}=0.14$ ,  $p=0.72$ , NS), but also not during the light period (surgery effect  $F_{1,18}=2.31$ ,  $p=0.15$ , NS). After averaging total diurnal heart rates (figure 1I), there was no impact of surgery (day x surgery interaction  $F_{7,126}=0.68$ ,  $p=0.50$ , NS,  $\epsilon=0.25$ ; main surgery effect  $F_{1,18}=1.51$ ,  $p=0.24$ , NS).

#### *Heart rate variability*

Before OBX surgery, there was no difference in heart rate variability (HRV) between animals (block\*surgery interaction  $F_{7,126}=0.69$ ,  $p=0.68$ , NS; surgery effect  $F_{1,18}=0.01$ ,  $p=0.95$ , NS) (Figure 1J). After OBX surgery, OBX animals had an overall lower HRV (block\*surgery effect  $F_{7,126}=6.52$ ,  $p<0.01$ ,  $\epsilon=0.56$ ) (figure 1K). Separate analysis of the nocturnal and day period showed significant HRV differences between OBX and sham animals during the nocturnal (block\*surgery interaction  $F_{3,54}=4.27$ ,  $p<0.01$ ; surgery effect  $F_{1,18}=4.01$ ,  $p=0.06$ , trend), but also during the day period (block\*surgery interaction  $F_{3,54}=6.35$ ,  $p<0.01$ ). Post hoc analysis using a one-way ANOVA showed that HRV was reduced in OBX animals in one 3h-block during the light period (9-12AM:  $F_{1,19}=3.08$ ,  $p<0.05$ ), and during two 3h-blocks during the dark period (9-12 PM:  $F_{1,19}=5.25$ ,  $p<0.05$ ; 12-3 AM:  $F_{1,19}=9.63$ ,  $p<0.01$ ). After averaging total nocturnal HRV (figure 1L), there was an overall impact of surgery (day x surgery interaction  $F_{7,126}=0.84$ ,  $p=0.56$ , NS; surgery effect  $F_{1,18}=10.47$ ,  $p<0.01$ ).



**Figure 2:** The novel cage and witness stress response before and after OBX surgery in OBX (n=11) and sham (n=9) rats. \*:  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.05$ . Inset locomotor figures: cumulative locomotor activity.

### 3.2 Acute stress responsivity

#### *Locomotor activity*

Witness stress elicited smaller locomotor responses compared to novel cage stress (stress effect  $F_{1,19}=89.83$ ,  $p < 0.001$ ; stress\*time interaction  $F_{15,285}=18.87$ ,  $p < 0.001$ ) (figure 2A). Cumulative activity levels after novel cage stress were larger than those after witness stress (stress effect  $F_{1,19}=83.36$ ,  $p < 0.001$ ). After surgery, OBX animals showed larger locomotor activity responses to novel cage stress (time x surgery interaction  $F_{15,270}=1.72$ ,  $p < 0.05$ ; surgery effect  $F_{1,18}=7.35$ ,  $p=0.01$ ) (figure 2D). Simple contrasts revealed a significant time x surgery interaction only at  $t=10$  minutes (simple contrasts, time x surgery,  $F_{1,18}=4.29$ ,  $p=0.05$ ). Cumulative activity levels after novel cage stress in OBX rats were also increased compared to shams (surgery effect  $F_{1,19}=9.82$ ,  $p < 0.01$ ) (figure 2D, inset), OBX animals and sham animals had similar locomotor activity responses to witness stress (time x surgery interaction  $F_{15,270}=0.51$ ,  $p=0.93$ , NS; surgery effect  $F_{1,18}=0.08$ ,  $p=0.78$ , NS) (figure 2G, and cumulative activity levels after witness stress in OBX rats were not different compared to shams (figures 2G, inset).

#### *Body temperature*

Witness stress elicited smaller stress-induced hyperthermia responses compared to novel cage stress (stress effect  $F_{1,19}=24.54$ ,  $p < 0.001$ ; stress\*time interaction ( $F_{27,513}=10.92$ ,  $p < 0.0001$ ) (Figure 2B). After surgery, OBX and sham animals showed similar



stress-induced hyperthermia responses to novel cage stress (time x surgery interaction  $F_{27,486}=0.28$ ,  $p=1.00$ , NS; surgery effect  $F_{1,18}=0.40$ ,  $p=0.54$ , NS) (figure 2E). OBX and sham animals were different in their stress-induced hyperthermia responses to witness stress (time x surgery interaction  $F_{27,486}=1.94$ ,  $p<0.01$ ) (figure 2H). Simple contrasts revealed a significant differences between OBX and sham animals from  $t=30$  until  $t=70$  minutes (simple contrasts, time x surgery,  $p\leq 0.01$ ).

#### Heart rate

Witness stress elicited smaller heart rate responses compared to novel cage stress (stress effect  $F_{1,19}=17.78$ ,  $p<0.001$ ; stress\*time interaction ( $F_{27,513}=6.61$ ,  $p<0.0001$ ) (Figure 2C). After surgery, OBX and sham animals showed similar heart rate responses to novel cage stress (time x surgery interaction  $F_{27,486}=0.55$   $p=0.97$ , NS; surgery effect  $F_{1,18}=1.48$ ,  $p=0.24$ , NS) (figure 2F). In contrast, OBX animals had smaller heart rate responses to witness stress compared to shams (time x surgery interaction  $F_{27,486}=2.08$ ,  $p<0.01$ ; surgery effect  $F_{1,18}=9.14$ ,  $p<0.01$ ) (figure 2I). Simple contrasts revealed no significant time x surgery interactions.

**Table 1:** Postsurgical locomotor responses in sham (n=9) and OBX rats (n=11) in an open field test. Data are mean  $\pm$  S.E.M..

Sham	OBX	
Distance Travelled (cm)	Distance Travelled (cm)	Significance (P)
2728 $\pm$ 497	5861 $\pm$ 769	0.004

#### Open field test

In the open field test OBX animals were significantly more active compared to shams (Table 1).

## 4. Discussion

The present study shows that olfactory bulbectomy results in an almost immediate onset of several changes in the basal circadian amplitude in the home cage, including increased nocturnal activity, increased nocturnal body temperature, and decreased daily heart rate (Figure 1). The OBX nocturnal hyperactivity and bradycardia during the light period are in agreement with earlier studies (Giardina and Radek 1991; van Riezen and Leonard 1990). The fast onset of these changes within a few days after bulbectomy demonstrated in this study indicates that these changes in circadian amplitude are not the result of a slowly developing neuronal reorganization over weeks, but rather an immediate emerging phenomenon. We found that the nocturnal body temperature of the OBX animals was elevated, perhaps due to the elevated activity levels during this time period. However, an earlier study showed that despite close synchrony, temperature rhythm is not just a byproduct of the activity rhythm, and nocturnal temperature increases may therefore be an independent result of bulbectomy (Refinetti 1999). Due to a large variation in heart rate values, it was not possible to significantly assess the onset of decreased heart rate after OBX surgery, although a decreased heart rate is visible from the third day after OBX

surgery (Figure 1I). Our postsurgical circadian data are an average of 14 days, starting immediately after surgery. We therefore explored the possibility of postsurgical interference with OBX-induced changes. Separate analysis of the second week after surgery resulted in identical significant changes in circadian rhythmicity, whereas additional analysis of the circadian rhythm in the third and fifth week after surgery resulted in similar OBX-induced changes in circadian rhythmicity (data not shown). Thus, although surgery might have generally influenced our parameters, the presented changes are stable, long lasting and reflect true OBX-induced changes.

The present study also demonstrates that olfactory bulbectomy leads to altered stress responses in the home cage. Using two psychological stressors with different intensity (Figure 2A-C), we show that these two stressors differentially affect home cage stress responses in OBX animals. Novel cage stress like the open field test led to an increased locomotor response in OBX rats with no impaired temperature and heart rate responses (Figure 2D-F). In contrast, witness stress exposure in OBX rats resulted in smaller autonomic (body temperature and heart rate) responses, and a faster recovery to prestress baseline levels also seems to be present (Figure 2H-I). However, OBX rats show comparable locomotor responses compared to the sham animals (Figure 2G). Witness stress (entering the animal room) and novel cage stress are both psychological stressors with different intensity (van den Buuse et al 2001; Zethof et al 1995). Locomotor activity and heart rate levels are heightened shortly before the stress procedures (figure 2,  $t=0$  min) because data between the first and last animal that were put into a novel cage were averaged without correcting for the time lag between them.

We confirmed the different intensity of these stressors in the present study, where witness stress leads to less pronounced reactions in temperature, locomotor activity and heart rate (Figure 2A-C). The present study shows that more intense stressors like novel cage stress or an open field test induce exaggerated locomotor responses in bulbectomized rats, whereas less intense stressors such as witness stress induce smaller autonomic responses without affecting locomotor responses. Hence, autonomic activation in OBX rats seems blunted to less intense stressors, whereas above a certain stress level threshold (novel cage stress), arousal is apparently such that autonomic stress responses of OBX rats become comparable to those of sham animals. Our data are in agreement with a study that established attenuated sympathoexcitatory responses after OBX surgery (Moffitt et al 2002), lower heart rate responses (Kawasaki et al 1980). Another study found diminished temperature and heart rate responses in OBX animals in the home cage after open field stress, although this was not associated with increased locomotor responses (Roche et al 2007). The smaller temperature and heart rate responses to witness stress on one hand and increased locomotor responses to novel cage stress on the other hand suggest that locomotor and autonomic responses are mediated by independent central pathways. Body temperature and heart rate activation are thought to be caused by activation of amygdala-dorsomedial hypothalamus connections that mediate sympathetic activation via the rostral raphe pallidus and sympathetic preganglionic neurons (DiMicco et al 2006; Dimicco and Zaretsky 2007). As locomotor activity is a result of both exploration and anxiety, it is difficult to locate the exact neural circuitry, although a role for the amygdala in stress-induced locomotion is plausible (Daniels et al 2004).

The underlying mechanisms of increased behavioral stress reactivity in OBX animals are unknown, but are postulated to be the result of either enhanced stress reactivity, decreased habituation (McKernan et al 2000), or a decrease in competing behaviors (Primeaux and Holmes 1999). The amygdala nuclei are involved in the acute stress reaction (Davis 1997) and as the olfactory bulbs project to the (medial) amygdala and the bed nucleus of the stria terminalis, their removal putatively leads to subsequent amygdaloid disinhibition (Kelly et al 1997; Wrynn et al 2000). OBX stress sensitivity can therefore be caused by hyperactive amygdaloid functioning leading to altered stress-induced sensory input integration (Moffitt et al 2002; Song and Leonard 2005). In support, *c-fos* expression in the OBX rat after the open field test is increased in the central and basolateral amygdala, but decreased in the bed nucleus of the stria terminalis (Roche et al 2007). Stress may therefore elicit a distinct pattern of amygdala activation in the OBX rats depending on, among others, stressor intensity. In fact, animals lesioned in the amygdala display decreased open field habituation and a decrease in immobility in the forced swim test, and display enhanced general activity (Daenen et al 2003). Besides the amygdala, both the locus coeruleus and raphe nuclei could be involved in the modulation of acute stress response in OBX rats, both displaying structural changes after bullectomy (Morimoto et al 1991). So far, OBX animals have shown impaired stress adaptation by showing open field test hyperactivity (Vinkers et al 2009b), sensitization of the acute startle reflex (McNish and Davis 1997), hyperactive bite, startle, struggle and fight responses (Takakusaki et al 2004), increased serotonin stress responses (Connor et al 1999) and larger and longer lasting corticosterone responses to stress (Cairncross et al 1977). It is interesting that our animals were hyperactive not only in an unstressed state, but also in a relatively stressful environment (open field and novel cage). Since these animals were housed with a "buddy" animal, their home cage environment was not particularly stressful. Therefore, increases in basal nocturnal activity may have been due to some other factor, such as increased striatal glutamate (Ho et al 2000) or altered dopamine levels (Bertaina-Anglade et al 2006).

Some of these basal and stress-induced changes are reminiscent of changes that have been found in depressed patients. Depressed patients have been found to display circadian rhythm disturbances (Yeragani et al 1990), among which an increased core temperature and altered day-night amplitudes (Avery et al 1982; Souetre et al 1989). Also, heart rate variability (Stockmeier et al 2009) is decreased and autonomic tone in depressed patients is altered (Davydov et al 2007; Stein et al 2000). However, in contrast to our results, an elevated resting heart rate is present in patients with major depression (Carney et al 1999), indicating that OBX-induced changes in basal and stress-induced physiological parameters cannot be directly extrapolated to autonomic deficits in depressed patients. However, further studies must elucidate whether bullectomy-induced changes respond to chronic antidepressant treatment. Also, during anxiety and stress, respiration rates are increased (Boiten et al 1994; Masaoka and Homma 2004) and a decreased respiratory variability might well be expected in anxiety disorders (Van Diest et al 2006). Since the amygdala is involved in respiratory reactivity after stress (Harper et al 1984; Masaoka and Homma 2004) and since perception of odors directly influences respiration, altered respiratory stress reactivity in OBX animals is expected. However, in

the present study, no respiratory parameters were measured, and there are no reports on respiration and stress in the OBX model.

In conclusion, the present study compared basal and stress-induced responses in the home cage of OBX rats. We conclude that changes in circadian amplitude of locomotor activity, body temperature and heart rate emerge quickly after OBX surgery with increased nocturnal locomotor activity and body temperature levels and decreased daily heart rate levels. Further, OBX animals persistently show increased home cage and open field locomotor stress responses, with an impaired autonomic stress response after less intense witness stress. Removal of the olfactory bulbs thus permanently alters the physiology of these animals rather than only altering the animal's ability to cope with a stressor. Evidence for impaired and dysfunctional physiological responses in rest and after stress stems from research in animals and humans, and the OBX model of depression constitutes a useful model to study basal and stress-induced levels in rodents.

### **Acknowledgements**

We would like to thank Meg van Bogaert and Sanne Claessens for their excellent technical assistance.



# Chapter 14

## **Exposure to stress differentially affects central and peripheral body temperature in human subjects**

Christiaan H. Vinkers

Renske Penning

Marieke M. Ebbens

Juliane Hellhammer

Joris C. Verster

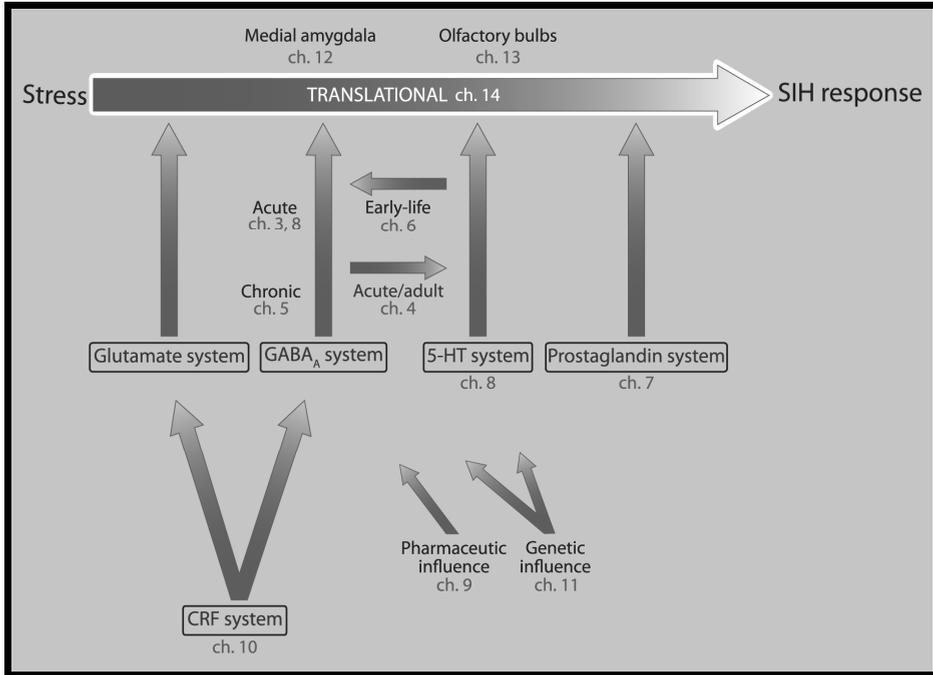
Berend Olivier

Cor J. Kalkman

14

*Submitted*

## Abstract



Stress-induced hyperthermia (SIH) is a consistent transient rise in body temperature in response to stress that is used as a preclinical anxiety paradigm. There are indications that stress may also alter body temperature in human subjects. Therefore, the present study aimed to investigate the effects of stress (using the Trier Social Stress Test, a standardized laboratory stress task) on central (intestinal) core temperature and peripheral (skin) temperature in male and female healthy subjects. In addition, we measured subjective stress levels, blood pressure, heart rate and respiration rate.

Here, we report that exposure to the Trier Social Stress Test increased skin temperature on the upper arm in male and female subjects, whereas it resulted in a small but statistically significant decrease in intestinal core temperature in male subjects only. Interestingly, the core temperature decrease correlated significantly ( $R=-0.59$ ) with the subjective stress increase. Moreover, increases in heart rate, respiration rate and blood pressure levels were found during the TSST, accompanied by higher subjective stress levels. Our results also indicate that a direct translation of the preclinical SIH paradigm to a human version is not without difficulty as the direction of stress-induced temperature changes depends on the site of temperature measurement. However, our finding that stress-induced changes in body temperature are present in humans indicates that body temperature changes possess translational potential and may constitute a novel approach to study stress responsivity in humans. If the TSST consistently induces a SIH response in human subjects, this opens up possibilities for a combined preclinical and clinical approach to study the genetic and pharmacological background of both functional and dysfunctional stress responses.

## 1. Introduction

Any physical or emotional trigger may induce a stress response, comprising several endocrine, autonomic and behavioral responses that enable an organism to adapt to a changing environment (Korte et al 2005). Several psychiatric disorders are associated with inadequate or excessive stress responses, among which anxiety disorders, major depressive disorder and schizophrenia (de Kloet et al 2005; Roozendaal et al 2009). In stress research, the role of the autonomic nervous system (ANS) has received ample attention. Stress activates the ANS in animals as well as human subjects, yielding comparable increases in heart rate and blood pressure (Ulrich-Lai and Herman 2009). In addition to heart rate and blood pressure, ANS activation also affects body temperature. Stress-induced hyperthermia (SIH) is a consistent short-lasting rise in body temperature (up to 2 °C) in experimental animals in response to stress (Vinkers et al 2008). This response has been found in a variety of species, including rodents, pigs, squirrels, baboons, and chimpanzees (Bouwknicht et al 2007; Nakayama et al 2005; Parr and Hopkins 2000b). There are indications that exposure to stress (e.g. exams, prior to a sport contest) can lead to body temperature changes in human subjects as well (Briese 1995; Kleitman and Jackson 1950; Marazziti et al 1992; Renbourn 1960). If body temperature responses to stress are robust and consistent across species, stress-induced temperature changes possess translational potential. Since anxiolytic drugs - including benzodiazepines and 5-HT<sub>1A</sub> receptor agonists - consistently reduce the SIH response in rodents, the SIH response could be used as a translational approach to study stress and anxiety at a pharmacological level (Bouwknicht et al 2007; Vinkers et al 2008).

To our knowledge, no standardized study has investigated whether stress-induced central and peripheral body temperature changes are present in healthy volunteers. Human thermoregulation is a continuous but complex process regulated by the central nervous system, and the central and peripheral temperatures may respond differently under stressful conditions. The Trier Social Stress Test (TSST) has been extensively validated as a laboratory-stress task in humans, including changes in subjective as well as in physiological parameters (heart rate, blood pressure and stress hormones) (Dickerson and Kemeny 2004). Therefore, the present study aimed to investigate differential effects of the Trier Social Stress Test on central (intestinal) core temperature and peripheral skin temperature in male and female healthy subjects. In addition, we measured subjective stress levels, blood pressure, heart rate, respiration rate and saliva cortisol. If a standard laboratory stress task consistently induces a SIH response in human subjects, this would open up possibilities in which a combined preclinical and clinical approach could be used to study both functional and dysfunctional stress responses.

## 2. Materials and methods

### 2.1 Participants

Twenty-four adult healthy volunteers (18–26 years old) with an equal representation of both genders (i.e., 12 men, 12 women) were recruited. The study was approved by the

Utrecht University Hospital medical ethics committee and performed according to the ICH guidelines for Good Clinical Practice and the Declaration of Helsinki and its latest amendments. All subjects gave their written informed consent prior to their inclusion in the study. Subjects were not eligible to participate in case of current drug use, use of psychoactive medication, physical or mental illness, smoking, any previous intestinal surgery, any chronic intestinal condition, any speech, language or swallowing disorder, not being fluent in Dutch or a MRI-appointment shortly after participation. Subjects were also excluded on the day of participation if they felt ill, had fever, consumed any caffeine, carried out heavy exercise or had eaten a heavy meal. Additionally, a commercial urine multi-drug screening was taken to exclude any recent intake of psychoactive substances by testing (InstantView) for the presence of amphetamines (including MDMA), barbiturates, cannabinoids, benzodiazepines, cocaine, and opiates. If no exclusion criteria were met, participants were allowed to stay and entered the study protocol.

**Table 1:** Trier Social Stress Test study protocol

Time (min)	Action
-90	Start protocol STAI-trait, STAI-state (1) and VAS-1
-60	Blood pressure and saliva sample (1)
-5	Standing up Blood pressure and saliva sample (2)
0	Start TSST
7.5	VAS-2
15	End TSST Blood pressure and saliva sample (3) STAI-state (2) and effort scale
20	Sitting position
25	Blood pressure and saliva sample (4)
35	Blood pressure and saliva sample (5)
45	Blood pressure and saliva sample (6)
60	Blood pressure and saliva sample (7)
90	Blood pressure and saliva sample (8)
120	Blood pressure and saliva sample (9) End protocol

## 2.2 General procedure

Participants were seated in a room with no windows and under controlled temperature ( $20 \pm 2$  °C) to measure physiological baseline values before and after the Trier Social Stress Test. Throughout the session, heart rate and respiration rate (Suunto T<sub>6</sub>, Vantaa, Finland), core temperature (ingestible radiotelemetric capsule, Respironics, Bend, OR, USA) and skin temperature (radiotelemetric skin patch attached on upper arm, Respironics) were constantly measured. Core and skin body temperature values were stored using a Vitalsense Physiological Monitor (Respironics, Bend, OR, USA) that participants wore on their belt. Also, at nine distinct time points, blood pressure (Microlife BPA 100, Widnau, Switzerland) was measured. The participant filled in one questionnaire assessing trait anxiety before the TSST and a questionnaire assessing state anxiety before and after the TSST (STAI-S and STAI-T, (Spielberger 1989)). Also, before, during and after the TSST,

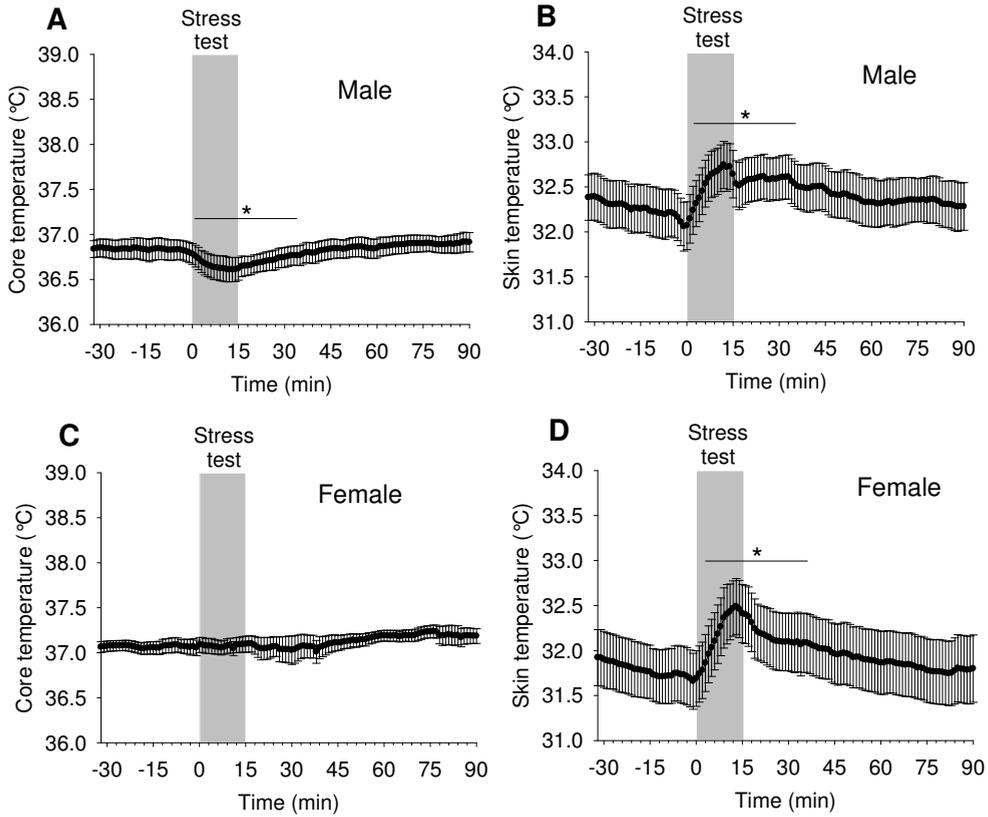
participants additionally rated their perceived stress, anxiety, insecurity, sweatiness and warmth levels on a 100 mm visual analog scale (VAS). Because subjects had been sitting before the start of the experiment, they were asked to stand up 5 min prior to the TSST to exclude the interference of an increase in heart rate in response to the change of position (orthostatic reaction) with the increase in heart rate in response to the TSST. Experimental procedures were always carried out between 8 and 12 AM, as larger cortisol increases are generally established during the morning (Kudielka et al 2004). At 7 AM, all participants received a wake-up call from the investigator to ensure that sufficient time elapsed between the cortisol awakening peak and the time of actual participation.

### 2.3 The Trier Social Stress Test

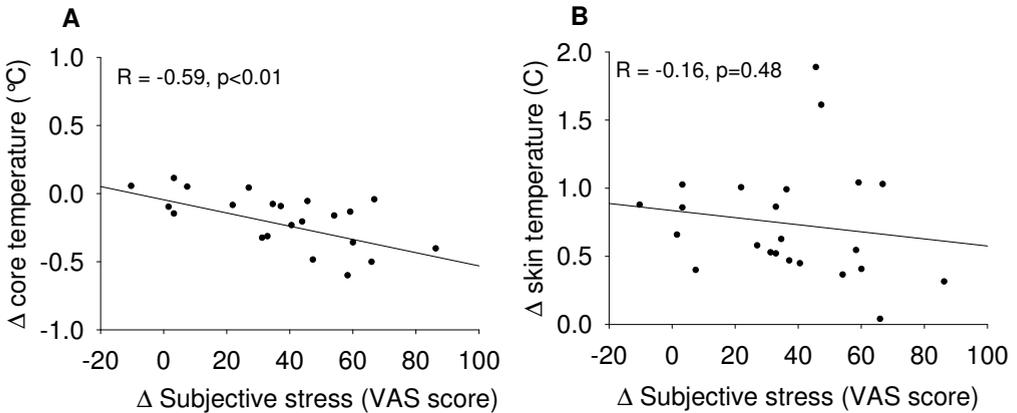
The Trier Social Stress Test was carried out as described earlier (Fries et al 2006). Briefly, a participant was led to the TSST room, where he/she was instructed to stand behind a microphone in front of a committee, consisting of one man and one woman. Subjects were then instructed to prepare (3 min) and deliver (5 min) a speech for a job interview, in which they were only allowed to talk about their personal positive and negative points. Before starting the speech, the subject was informed that the whole session would be video- and voice-recorded and that the committee was trained in behavioral observation. After the 5 minute free speech, the participant was asked to fill in a second visual analog scale. After that, the subject was asked to solve a mental arithmetic task (5 min) which consisted of continuously subtracting 17 from 2023 as quickly and correctly as possible. If any miscalculation occurred, the subject was asked to start again at 2023. Saliva samples were stored at -20°C. Free cortisol saliva levels were determined employing a radioactive immunoassay (RIA).

### 2.4 Statistics

Student t tests were applied to test for group differences in demographics (age) and endocrine pre-stressor baselines. Repeated-measures ANOVAs were used to analyze temperature, heart rate, respiration rate and blood pressure responses to the stressor with the between-subject factor gender and the repeated factor time. Where appropriate, results were corrected by Greenhouse-Geisser procedure (indicated by  $\epsilon$  values). Simple contrast tests were used to compare time points whenever a significant main effect for a parameter or a significant parameter x time interaction effect was observed. Post-hoc planned comparisons were conducted. VAS scales (before and during the TSST) were analyzed using a repeated-measures ANOVA. Stress-induced changes in all physiological parameters except heart rate were calculated by subtracting the baseline value (averaged over 15 to 5 minutes before the TSST) from the maximum value reached during the TSST. For heart rate, the baseline value was obtained by averaging the heart rate in the upright position prior to the TSST. Subsequently, these physiological changes were analyzed using a one-way ANOVA and were also correlated with subjective stress level changes. Correlations were calculated following the Pearson product moment procedure (two-tailed). The significance level was set at  $p=0.05$ . All results shown are the mean  $\pm$  standard error of mean (SEM).



**Figure 1:** Effects of the TSST on core and peripheral body temperature in male and female healthy subjects. \*:  $p < 0.05$  compared to baseline.



**Figure 2:** Correlation between increase in subjective stress level (measured via VAS scales) and core and skin body temperature change.

## 3. Results

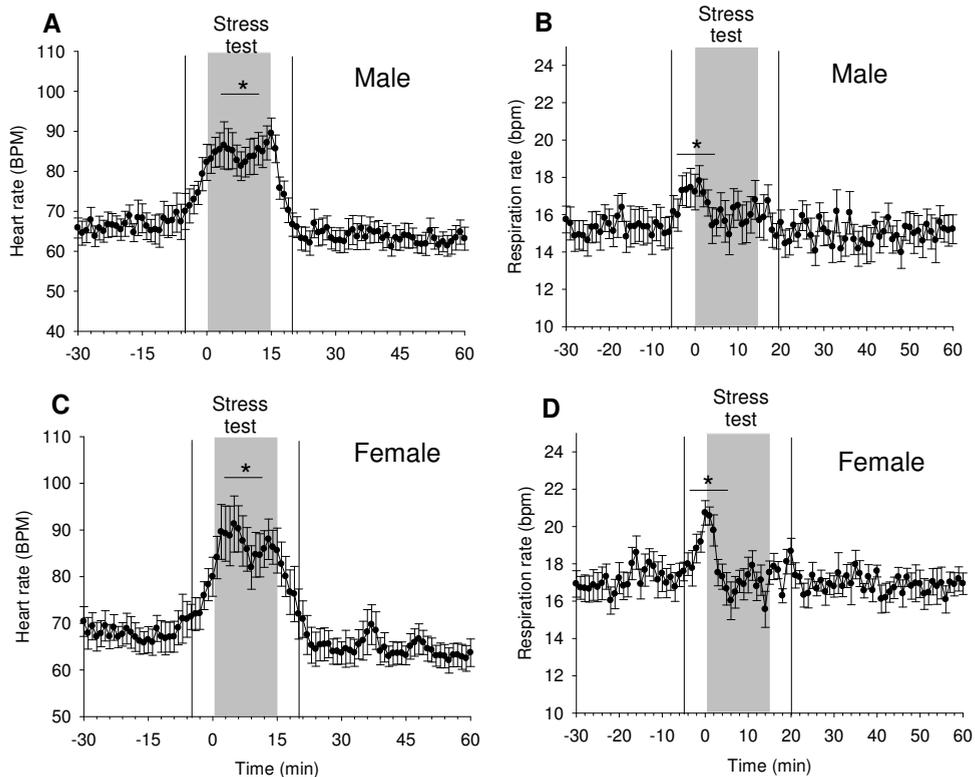
### 3.1 General

Due to technical problems, core and body temperature of one male subject and heart rate, respiration rate and cortisol levels of one male and one female subject were excluded from analysis.

### 3.2 Core and skin body temperature (t=0 until 60 min) (Figure 1)

Changes in core temperature in response to stress were dependent on gender (time x gender interaction  $F_{59,1239}=1.82$ ,  $p<0.001$ ). Post-hoc analysis revealed that female core temperature did not change in response to stress (time effect  $F_{59,649}=0.93$ ,  $p=0.63$ , NS) (Figure 1C), whereas male core temperature decreased slightly in response to stress (time effect  $F_{59,590}=8.23$ ,  $p<0.001$ ) (Figure 1A). Simple contrasts revealed that male core temperature decreased from t=5 to t=25 compared to t=0 (simple contrasts compared to t=0,  $p<0.05$ ). The average change was  $0.07\pm 0.04$  °C (male:  $-0.01\pm 0.05$  °C; female:  $0.14\pm 0.05$  °C). The core temperature decrease in response to the TSST significantly correlated with the subjective stress increase (Figure 2A) (Pearson correlation  $R=-0.59$ ,  $p=0.003$ ). Separate gender correlations indicated that the correlation was present in males ( $R=-0.71$ ,  $p=0.01$ ) but not in females ( $R=-0.44$ ,  $p=0.17$ , NS).

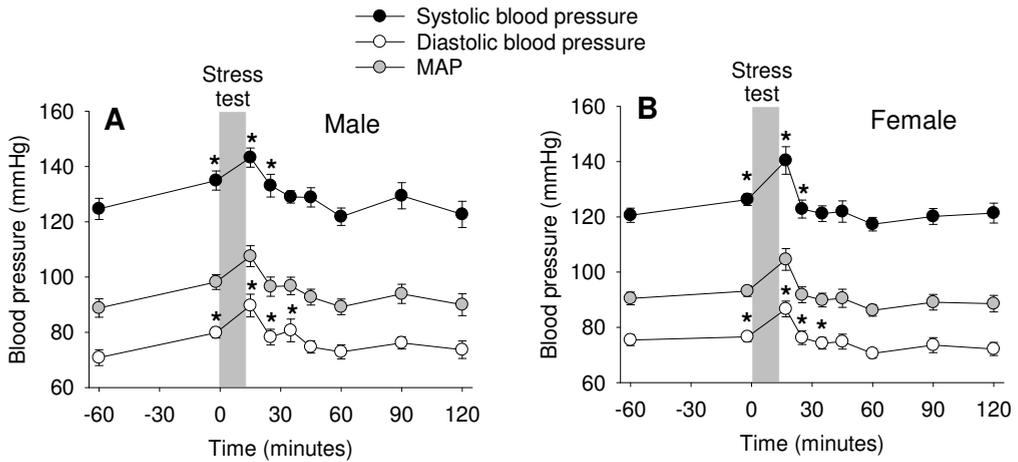
Skin temperature increased in response to stress (time effect,  $F_{59,1239}=9.94$ ,  $p<0.001$ ). This effect was not dependent on gender (time x gender interaction  $F_{59,1239}=1.01$ ,  $p=0.46$ , NS; gender effect  $F_{1,21}=1.08$ ,  $p=0.31$ , NS) (Figure 1 B and D). Compared to t=0, skin temperature increased during all 59 subsequent minutes (simple contrasts,  $p<0.05$ ). The average maximum increase was  $0.74\pm 0.09$  °C (male:  $0.62\pm 0.12$  °C; female:  $0.84\pm 0.13$  °C). The skin temperature increase in response to the TSST did not correlate with the subjective stress increase (Figure 2B) (Pearson correlation  $-0.16$ ,  $p=0.48$ , NS).



**Figure 3:** Effects of the TSST on heart rate and respiration rate levels in male and female healthy subjects. \*:  $p < 0.05$  compared to baseline.

### 3.3 Heart rate and respiration rate (t=-30 until +30 min) (Figure 2)

Average heart rate changed over time (time effect  $F_{59,1121}=25.46$ ,  $p < 0.001$ ) which was independent of gender (time x gender interaction  $F_{59,1121}=0.49$ ,  $p=0.81$ ). Simple contrasts revealed that, compared to baseline, heart rate increased from 7 minutes prior to the TSST until 21 minutes after the start. In comparison to the 5 minutes before the TSST when subjects were in standing position, TSST increased heart rate above the basal level while standing (time effect  $F_{24,456}=10.18$ ,  $p < 0.001$ ). The average maximum was  $23 \pm 2$  beats per minute (bpm) (male:  $21 \pm 3$  bpm; female:  $24 \pm 3$  bpm). Average respiration rate increased over time (time effect  $F_{59,1121}=2.59$ ,  $p < 0.001$ ) which was independent of gender (time x gender interaction  $F_{59,1121}=0.81$ ,  $p=0.85$ ). Simple contrasts revealed that, compared to baseline, respiration rate increased from 4 minutes prior to the TSST until 3 minutes after the start. Respiration rate levels increased with  $5 \pm 1$  bpm (male:  $4 \pm 1$  bpm; female:  $6 \pm 1$  bpm).



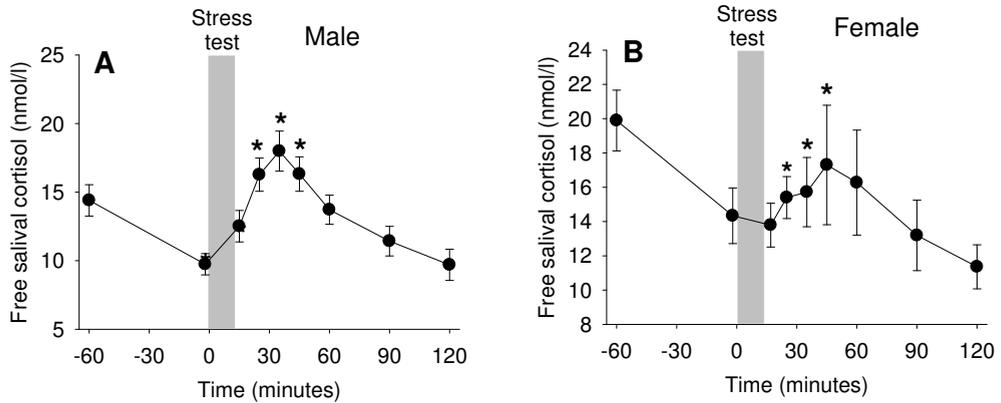
**Figure 4:** Effects of the TSST on blood pressure (mmHg). \*:  $p < 0.05$  compared to baseline

### 3.4 Blood pressure

Both systolic and diastolic blood pressure increased in response to stress (diastolic:  $F_{8,176}=14.07$ ,  $p < 0.001$ ; systolic:  $F_{8,176}=15.92$ ,  $p < 0.001$ ). Simple contrasts revealed that, compared to the first measurement at  $t = -60$  min, the blood pressure was elevated between  $t = -2$  min (diastolic:  $F_{1,22}=13.98$ ,  $p < 0.001$ ; systolic:  $F_{1,22}=16.07$ ,  $p < 0.001$ ),  $t = 15$  (diastolic:  $F_{1,22}=71.97$ ,  $p < 0.001$ ; systolic:  $F_{1,22}=49.46$ ,  $p < 0.001$ ),  $t = 25$  (diastolic:  $F_{1,22}=4.33$ ,  $p < 0.05$ ; systolic:  $F_{1,22}=5.54$ ,  $p < 0.05$ ) and  $t = 35$  (diastolic:  $F_{1,22}=4.76$ ,  $p < 0.05$ ; systolic:  $F_{1,22}=1.47$ ,  $p = 0.24$ , NS). No gender effects were present in either the response of diastolic or systolic blood pressure (diastolic: gender effect  $F_{1,22}=0.46$ ,  $p = 0.50$ , NS; gender x time interaction  $F_{8,176}=1.32$ ,  $p = 0.24$ , NS; systolic: gender effect  $F_{1,22}=3.17$ ,  $p = 0.09$ , NS; gender x time interaction  $F_{8,176}=0.89$ ,  $p = 0.53$ , NS).

### 3.5 Cortisol levels

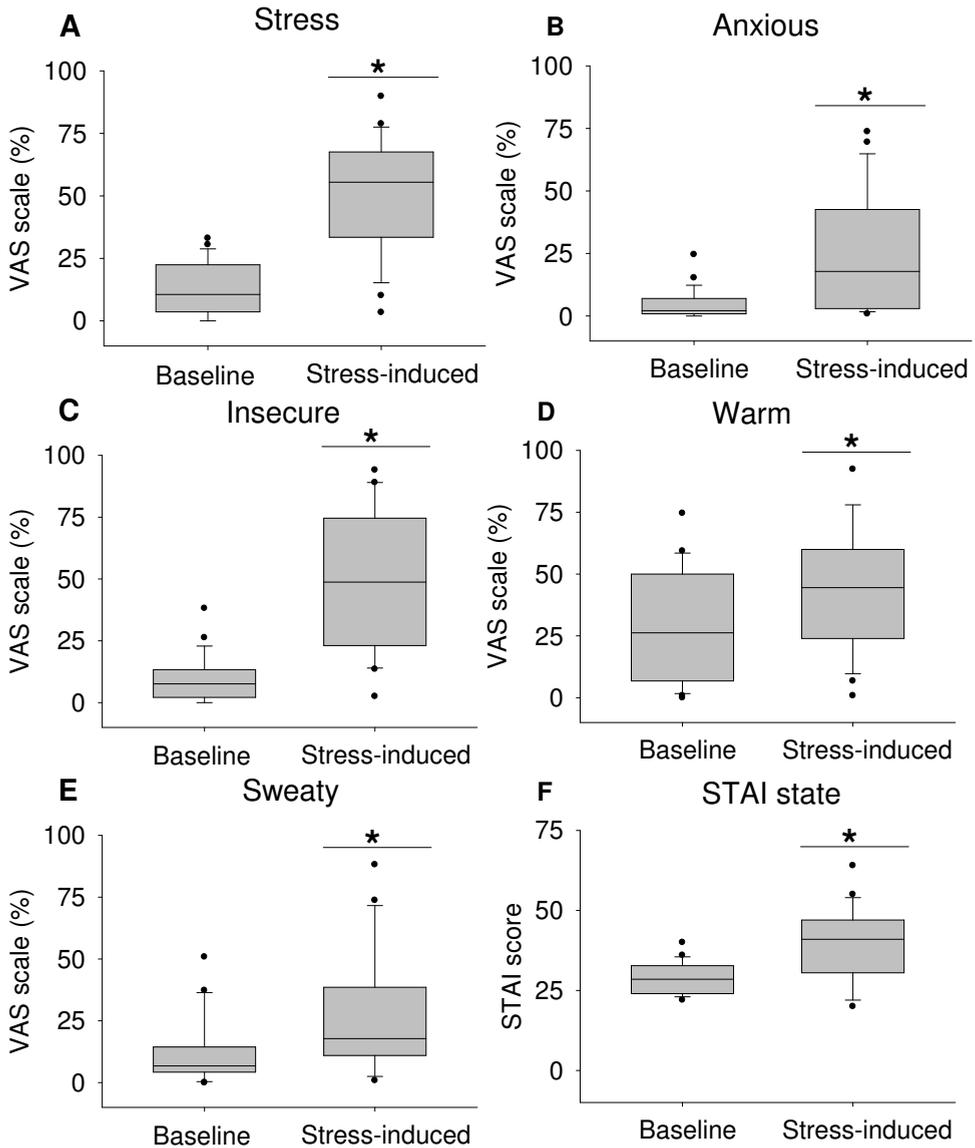
Cortisol levels increased over time (cortisol effect  $F_{8,160}=7.83$ ,  $p<0.001$ ) independent of gender (cortisol x gender interaction  $F_{8,160}=1.76$ ,  $p=0.20$ , NS; gender effect  $F_{1,20}=0.83$ ,  $p=0.37$ , NS). Simple contrast revealed that compared to just before the TSST ( $t=-2$  min), cortisol levels increased at  $t=15$  ( $p<0.1$ , trend),  $t=25$ ,  $t=35$  and  $t=45$  min ( $p<0.05$ ).



**Figure 4:** Effects of the TSST on free saliva cortisol (nmol/l). \*:  $p<0.05$  compared to baseline ( $t=-2$  min). Overall, no gender differences were present.

### 3.6 Subjective stress levels

Anxiety levels increased during the TSST independent of gender as measured by the STAI state inventory (before:  $5\pm 1$ ; after:  $11\pm 2$ ) (STAI effect  $F_{1,22}=32.77$ ,  $p<0.001$ ; STAI x gender  $F_{1,22}=2.67$ ,  $p=0.12$ , NS) and VAS responses in levels of stress ( $37\pm 5\%$ ) ( $VAS_{\text{stress}}$  effect  $F_{1,22}=56.01$ ,  $p<0.001$ ;  $VAS_{\text{stress}}$  x gender  $F_{1,22}=0.38$ ,  $p=0.54$ , NS), anxiety ( $21\pm 4\%$ ) ( $VAS_{\text{anxiety}}$  effect  $F_{1,22}=22.04$ ,  $p<0.001$ ;  $VAS_{\text{anxiety}}$  x gender  $F_{1,22}=0.64$ ,  $p=0.43$ , NS), insecurity ( $26\pm 5\%$ ) ( $VAS_{\text{insecure}}$  effect  $F_{1,22}=52.23$ ,  $p<0.001$ ;  $VAS_{\text{insecure}}$  x gender  $F_{1,22}=0.07$ ,  $p=0.79$ , NS), warmth ( $25\pm 5\%$ ) ( $VAS_{\text{warm}}$  effect  $F_{1,22}=6.40$ ,  $p<0.05$ ;  $VAS_{\text{warm}}$  x gender  $F_{1,22}=0.53$ ,  $p=0.48$ , NS) and sweatiness ( $20\pm 4\%$ ) ( $VAS_{\text{sweatiness}}$  effect  $F_{1,22}=13.21$ ,  $p<0.01$ ;  $VAS_{\text{sweatiness}}$  x gender  $F_{1,22}=0.06$ ,  $p=0.80$ , NS). Also, the level of effort was comparable between male and female subjects ( $F_{1,23}=0.87$ ,  $p=0.36$ , NS).



**Figure 6:** Effects of the TSST on subjective stress levels in male and female healthy subjects. \*:  $p < 0.05$ : stress-induced vs. baseline levels.

## 4. Discussion

In response to environmental stimuli, typical behavioral, endocrine and physiological reactions are initiated which enable an organism to cope with a changing environment. These include a variety of physiological responses, such as an increased heart rate, increased plasma levels of adrenal stress hormones, differential vasoconstriction and vasodilatation, increased muscular tension and increased metabolic rates. Generally, this fine-tuned response is functional, but it can become maladaptive when it is inappropriately or chronically activated. This can result in the development of stress-related pathological conditions, such as anxiety disorders or major depressive disorder (de Kloet et al 2005). The current study reports that after exposure to the Trier Social Stress Test (TSST), the peripheral skin temperature in male and female subjects increased, whereas the intestinal core temperature decreased only in male subjects. Moreover, increases in heart rate, respiration rate and blood pressure levels were found during the TSST, accompanied by higher subjective stress levels.

Animal models examine normal and abnormal stress-related behavior by means of exposure to a threatening environment (open field, elevated plus-maze, light/dark box), social interaction tests, punishment-based conflict procedures (e.g. punished drinking), defensive burying, predator stress and stress-induced ultrasonic vocalizations (Cryan and Holmes 2005). Notwithstanding continuous progress and increased insight into the neurobiology of stress and anxiety, the search for anxiety models with potential to support the direct translation of animal studies to clinical research is challenging (Garner et al 2009). Since exposure to stress results in activation of the autonomic nervous system (ANS) in animals as well as humans with attending increases in heart rate, blood pressure and body temperature, measuring such phenomena in animals as well as in humans may constitute a translational physiological approach to study stress (Ulrich-Lai and Herman 2009). Specifically, the stress-induced hyperthermia (SIH) response is the transient rise in body temperature in response to stress that is mediated by the ANS. A SIH response is present in a variety of species, including rodents, baboons, impalas and chimpanzees (Vinkers et al 2008), and there is evidence that a SIH response exists in humans (Briese 1995; Marazziti et al 1992). Therefore, the present study aimed to study the putative body temperature changes in response to stress in healthy young subjects of either sex. In addition, we measured other ANS-mediated parameters including heart rate, blood pressure and respiration rate.

Here, we report that exposure to the Trier Social Stress Test (TSST), a standardized laboratory stress task, increased skin temperature on the upper arm in male and female subjects, whereas it resulted in a small but statistically significant decrease in intestinal core temperature in male subjects only. To our knowledge, this is the first study to establish a decrease in intestinal body temperature in response to stress. Interestingly, the drop in core body temperature in male as well as female volunteers negatively correlated with the increase in subjective stress levels, suggesting that intestinal temperature may constitute a sensitive physiological parameter to detect stress levels. Also, a robust increase in skin temperature over the deltoid muscle was established, which may be the result of both cutaneous vasodilatation as well as increased muscular metabolic rates. A number of studies have addressed the effects of stress on peripheral body temperature in

humans. Stress has been shown to increase axillary temperature (Gotsev and Ivanov 1950; Gotsev and Ivanov 1954; Marazziti et al 1992) as well as oral temperature (Briese 1995; Renbourn 1960). In contrast to axillary and oral temperature, peripheral finger temperature was found to decrease when listening to “negative” music (McFarland 1985). In support, finger temperature decreased up to 13.5 °C (!) in 45 out of 47 subjects in affective states, whereas only a slight increase of forehead and cheek temperature in some subjects was observed (Mittelman and Wolff 1939). There are non-human primate studies that report temperature changes after stress exposure with increased tympanic membrane temperature in chimpanzees (Parr and Hopkins 2000a) and decreased nasal temperature of rhesus monkeys (Nakayama et al 2005). Therefore, taken together, all previous studies suggest that both the direction and magnitude of stress-induced temperature changes may strongly depend on the sites of measurement.

Our finding that intestinal core temperature decreased in response to stress is surprising, as rodent core temperature generally increases in response to stress, at least when body temperature is measured using either rectal temperature probes or telemetric equipment located in the abdominal cavity (Bouwknicht et al 2007; Vinkers et al 2008). The fact that we measured core body temperature inside the lumen of the small intestine may at least partially explain this discrepancy. Thus, it may be speculated that vasoconstriction inside the small intestine decreases core temperature, whereas a stress-induced vasodilatation could be present in the colon and/or peritoneal cavity. These findings are compatible with the concept of decreased gastric and small intestinal activity in response to stress when a fight-or-flight response is initiated (Martinez et al 2004). Preclinical stress-induced increases in the abdominal cavity temperature may then be the result of increased metabolic activity of abdominal organs including the liver, whereas stress-induced activation of the colon (see (Tache et al 2005)) results in increased colon evacuation and increases rectal temperature. The fact that abdominal organs including the liver actively produce heat complicates the interpretation of intestinal core temperature decrease. Unfortunately, studies addressing this specific topic are lacking.

Moreover, we confirm and extend earlier findings that the exposure to the TSST leads to consistent increases in subjective stress levels as well as in heart rate and blood pressure independent of gender, whereas saliva cortisol increases were higher in male subjects (Fries et al 2006; Kirschbaum et al 1993; Kudielka et al 2004). The relative drop in heart rate levels during the middle part of the TSST can be ascribed to the transition between the first and second TSST task. Interestingly, respiration rate significantly increased prior to and during the first encounter with the stress committee but subsequently decreased throughout the TSST, while heart rate remained elevated. This may indicate that stress only induces short-lasting increases in respiration, or, alternatively, that orthostatic changes are responsible for these respiration-induced changes.

In conclusion, the present study found robust increases in skin temperature but decreased intestinal core temperature in response to stress. Thus, an unconditioned stress-induced temperature response seems to be present in humans and may possess translational potential to study stress and anxiety in rodents as well as humans. Moreover, the fact that anxiolytic drugs including benzodiazepines and 5-HT<sub>1A</sub> receptor agonists consistently reduce the SIH response in rodents suggests that the SIH response could also

be applied as a translational pharmacological tool (Bouwknicht et al 2007; Vinkers et al 2008). If human central or peripheral changes in body temperature after stress exposure could be altered using a clinically effective anxiolytic drug (e.g. benzodiazepines), this would increase the translational potential of this approach. Moreover, as a dysfunctional autonomic nervous system is often present in anxiety disorders, heightened or blunted physiological stress responsivity may be detected with body temperature measurements. Our results also indicate that a direct translation of the preclinical SIH paradigm to a human version is not without difficulty as the direction of stress-induced temperature changes does not coincide and human thermoregulation may be more complex and differentiated compared to rodents. However, our finding that stress-induced changes in body temperature are present in humans indicates that body temperature changes possess translational potential and may constitute a novel approach to study stress responsivity in humans.

### **Acknowledgements**

The authors would like to thank Thomas Scheewe and Sandor Schmikli for their help with the Suunto equipment, and Monique Mets and Martje de Groot for their help with the TSST protocol.

# **Chapter 15**

## **Part I**

### **General discussion**

# 15

## General discussion

The research described in this thesis shows that the body temperature response to stress (stress-induced hyperthermia (SIH)) can be employed to study stress in its broadest sense with a wide variety of applications. In this thesis, we examined the pharmacological, genetic and mechanistic backgrounds of stress and their possible consequences at the receptor level using the SIH paradigm. An important finding of the current research is that the SIH paradigm is suitable to assess the acute and chronic effects of  $\alpha$  subunit-selective GABA<sub>A</sub> receptor agonists. Using novel and selective ligands, the SIH paradigm is able to dissect the contributions of the different  $\alpha$  subunits (chapter 3 and 5). Importantly, we confirm putative anxiolytic effects for GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonists (chapter 3) which do not result in the development of tolerance after chronic treatment (chapter 5). Moreover, we show that the  $\alpha_1$  subunit may be involved in hypothermia, whereas the  $\alpha_5$  subunit is not directly involved in the anxiolytic or hypothermic effects of benzodiazepines (chapter 3 and 5) (for a review on the GABA<sub>A</sub> and GABA<sub>B</sub> receptor and the SIH paradigm see part II of chapter 15). Importantly, rapid tolerance occurred after chronic treatment with the non subunit-selective benzodiazepine diazepam but not after chronic activation of the  $\alpha_1$ ,  $\alpha_{2/3}$  or  $\alpha_5$  subunit. Together, these data indicate that selective GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonists constitute a promising class of novel anxiolytics. In addition to the GABA<sub>A</sub> receptor studies, we confirm and extend the findings that a variety of putative anxiolytic ligands including CRF<sub>1</sub>, nicotinic, serotonergic and glutamatergic receptor ligands reduce the SIH response (chapter 8, 10). In contrast, the SIH response is not sensitive to the effects of non-steroidal anti-inflammatory drugs (NSAIDs), indicating that the SIH is distinct from infection-induced fever (chapter 7). Genetically modified point-mutated mice have importantly contributed to the elucidation of the function of different GABA<sub>A</sub> receptor  $\alpha$  subunits. Therefore, future studies could adopt this genetic strategy and combine it with the SIH paradigm to confirm and extend the results described in this thesis. Based on the current findings, it may be interesting to study whether benzodiazepine-induced hypothermia is reduced or absent in  $\alpha_1$ -point mutated mice.

Moreover, we provide evidence that the SIH response can be used to investigate the functional interactions of different neurotransmitter systems in response to stress (chapter 4 and 6). This is important as different neurotransmitter systems intricately cooperate to generate a coordinated stress response, and multiple dysfunctional neurotransmitter systems may contribute to the development of stress-related disorders. In this thesis, we show that high levels of CRF in the central nervous system modulate the GABA<sub>A</sub> and metabotropic glutamate receptor system (chapter 10). Moreover, the SIH paradigm is sufficiently sensitive to establish adult benzodiazepine insensitivity which is the result of a transient early-life disruption of the serotonin system (chapter 6) (for a review on the role of the 5-HT system in the SIH paradigm see part III of chapter 15).

The consistent and rather selective effects of different drug classes suggest that the SIH paradigm may be of additional value in stress-research and could be employed to assess the putative anxiolytic potential of a drug. Because the SIH response is unconditioned and normally does not habituate, pharmacological interventions can be repeatedly carried

out. This constitutes a major advantage of the SIH paradigm over other stress and anxiety paradigms, and acute SIH tests may cover a long period of time (we have tested mice once per week for up to one year without interfering habituation processes). This not only reduces the number of animals but, essentially, allows for repeated acute pharmacological challenges during chronic treatments (chapter 5). Future studies may adopt this approach in a variety of approaches. Benzodiazepine sensitivity may for example be studied during chronic serotonergic interventions. Also, a study that examines the putative protective effects of low doses of a GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonist during chronic mild stress or social defeat using the SIH paradigm is feasible.

Interestingly, we found that the medial amygdala is not only directly involved in the SIH response but also alters sensorimotor gating and light-enhanced startle. However, the currently employed lesion technique also destroys fibers of passage, and SIH studies that use infusions of GABAergic drugs into the medial amygdala could be a valuable addition. Specifically, local infusions of  $\alpha_{2/3}$  subunit-selective GABA<sub>A</sub> receptor agonist into the medial amygdala may thus combine the pharmacological SIH evidence with the mechanistical SIH evidence to further elucidate the neurobiological basis of the SIH response.

From a translational perspective, the fact that we found that stress exposure differentially affects core and peripheral body temperature in healthy human volunteers, indicates that the SIH paradigm may indeed possess translational potential (chapter 14). The fact that a decrease in core temperature correlated with the subjective stress increase suggests that stress-induced core intestinal temperature changes may constitute a valid stress read out parameter. A placebo-controlled follow up study could examine the effects of a benzodiazepine on peripheral and core temperature. This way, the direction and amplitude of stress-induced temperature changes that are shown in the present study could be confirmed, but, more importantly, the translational pharmacological potential of the SIH paradigm could be assessed. If a benzodiazepine affects the SIH response in humans, this could open up possibilities to directly apply the SIH paradigm in a preclinical as well as clinical setting (for a review on the translational potential of the SIH paradigm see part IV of chapter 15). Another approach could constitute the examination of the SIH response in psychiatric patients. So far, studies on the basal SIH response in genetically modified mice with increased anxiety-like behavior have produced mixed results. However, it would be useful to study the basal body temperature and stress-induced body temperature levels in patients that suffer from stress-related disorders.

Altogether, the present thesis shows that the SIH paradigm can be employed in preclinical and possibly clinical setups and provides an additional tool to examine the effects of stress in pharmacological, genetic and mechanistic studies.



# Chapter 15

## Part II

### **Elucidating GABA<sub>A</sub> and GABA<sub>B</sub> receptor functions in anxiety using the stress-induced hyperthermia model: a review**

Christiaan H. Vinkers

John F. Cryan

Berend Olivier

# 15

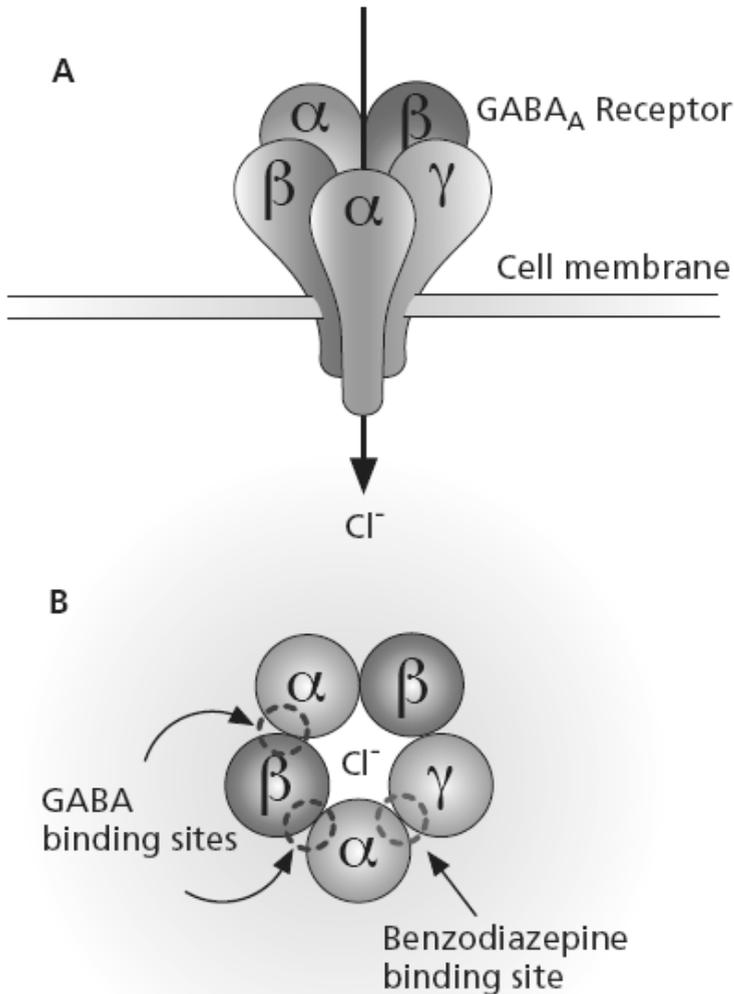
*The Open Pharmacology Journal, in press*

## 1. The SIH paradigm

Exposure to acute psychological or physical stress robustly increases core body temperature (stress-induced hyperthermia, SIH) which is part of the autonomic stress response (Vinkers et al 2008). The fact that perceived stressful occasions (e.g. during a movie or prior to a sporting contest) increase body temperature in humans has been known for a long time (Kleitman and Jackson 1950; Renbourn 1960). However, it was not until decades later that the SIH response was used as a putative rodent anxiety model when it was noticed that removing mice one by one from a group-housed cage reproducibly increased body temperature of the last mouse compared to the first (Borsini et al 1989). Later, this putative anticipatory anxiety model was improved to a singly-housed version in which the rectal temperatures are measured twice with an interval of 10 minutes (representing basal and stressed temperature values) (Zethof et al 1995). More recently, the advent of telemetric systems that can accurately measure body temperature has led to increasing application of such systems in SIH experiments (van Bogaert et al 2006b; Vinkers et al 2009f).

The predictive validity of the SIH model has proven to be very good, making it very suitable to screen putative anxiolytic drugs (Vinkers et al 2008). So far, drugs with anxiolytic properties such as GABA<sub>A</sub> and 5-HT<sub>1A</sub> receptor agonists as well as CRF antagonists have proven to attenuate the SIH response, whereas non-anxiolytic dopaminergic or noradrenergic drugs generally do not affect the SIH response (Bouwknrecht et al 2007). Moreover, acute administration of selective serotonin reuptake inhibitors and tricyclic antidepressants have no effect on the SIH response (Bouwknrecht et al 2007). So far, chronic treatment with antidepressants has not resulted in altered SIH responses either.

Ionotropic (GABA<sub>A</sub>) and metabotropic (GABA<sub>B</sub>) receptors are ubiquitously expressed in the central nervous system (Castelli et al 1999; Pirker et al 2000). The GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) has been known to be closely involved in the acute stress response and clinically relevant anxiolytic drugs such as benzodiazepines and most anesthetics act on this receptor (Rudolph and Mohler 2006), whereas evidence for a role of the GABA<sub>B</sub> receptor in anxiety has more recently accumulated (Cryan and Holmes 2005; Cryan et al 2004). Both drugs acting on the GABA<sub>A</sub> and the GABA<sub>B</sub> receptor are generally able to attenuate the SIH response, and this review therefore presents a detailed overview on the effects of both drug classes in the SIH paradigm. SIH is an unconditioned, consistent and robust response, and the SIH test is easy to perform in acute and chronic setups. Moreover, the SIH model is able to measure the effects of anxiolytic drugs on the SIH response as well as basal body temperature. As the GABA receptor family is diverse and complex, this model may contribute to the elucidation of the putative effects of GABAergic drugs in emotional disorders such as anxiety and depression.



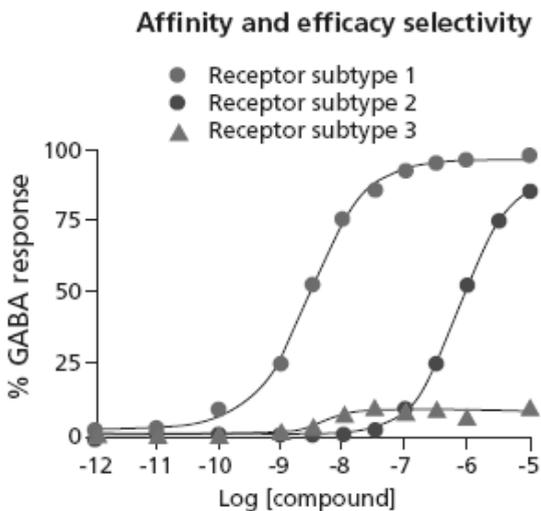
**Figure 1:** Schematic representation of the GABA<sub>A</sub> receptor.

## 2. The GABA<sub>A</sub> receptor (GABA<sub>A</sub>R)

GABA<sub>A</sub>Rs are ligand-gated ion channels that mediate fast inhibitory effects and are ubiquitously present in the central nervous system (Figure 1), even though a typical central subunit distribution seems to exist (Pirker et al 2000; Wisden et al 1992). When GABA binds, chloride ions flow into the neuron, resulting in a hyperpolarization of the cell membrane. GABA<sub>A</sub>Rs are found synaptically as well as extrasynaptically and are composed of five subunits with various possibilities per subunit ( $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$  and  $\pi$ ) that assemble to form a pentameric ligand-gated chloride channel. The GABA<sub>A</sub>R displays a large molecular heterogeneity that depends on a variable subunit composition which

ultimately determines physiological and pharmacological receptor properties and contributes to flexibility in signal transduction and modulation (Rudolph and Mohler 2006). The most common subtype is a pentamer with 2  $\alpha$ , 2  $\beta$  and 1  $\gamma$  subunit (Sibille et al 2000). The fact that two different  $\alpha$  subunits can co-exist in a single GABA<sub>A</sub> receptor adds to the variability of this receptor (Benke et al 2004). Different classes of pharmacological agents act on different sites on the GABA<sub>A</sub>R. Classic benzodiazepines bind to the GABA<sub>A</sub>R benzodiazepine modulatory site between the  $\alpha$  and  $\gamma$  subunit. Other drug classes also bind to the GABA<sub>A</sub>R, such as alcohol, barbiturates and neurosteroids (Sieghart 1995).

Classical (non-selective) benzodiazepines allosterically enhance the inhibitory actions of GABA by binding to GABA<sub>A</sub>R that contain  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  or  $\alpha_5$  subunits in combination with a  $\beta$  and a  $\gamma_2$  subunit. Recently, genetic and pharmacological evidence has indicated that  $\alpha$  subunits may differentially contribute to the various classical benzodiazepine effects such as anxiolysis, dependence, anticonvulsant activity, sedation and amnesia (Crestani et al 2001; Rudolph et al 1999). More specifically, the  $\alpha_1$  subunit (present in over 50% of all GABA<sub>A</sub>R) is thought to mediate the sedative and amnestic actions of benzodiazepines, whereas  $\alpha_2$  and  $\alpha_3$  subunits (present in 10–20% of all GABA<sub>A</sub>R) probably mediate the anxiolytic action of benzodiazepines (Dias et al 2005; Low et al 2000; McKernan et al 2000). GABA<sub>A</sub>R  $\alpha_2$  and  $\alpha_3$  subunit involvement in the anxiolytic effects of benzodiazepines is derived from studies of knock-in mice that point to a role for the  $\alpha_2$  subunit, whereas pharmacological experiments suggest a role for the  $\alpha_3$  subunit. Currently, there is no good explanation for these apparent discrepancies.

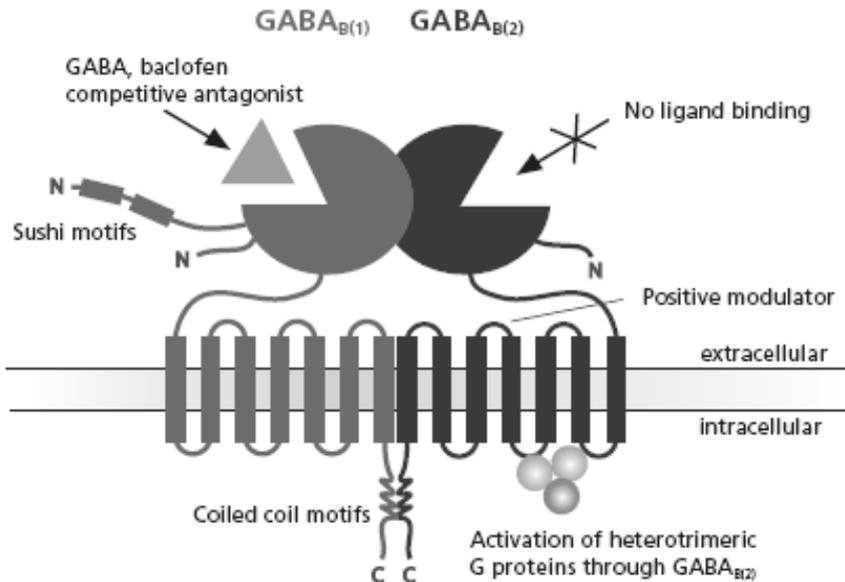


**Figure 2:** Concept of compound with a lower affinity (circles, right line) or efficacy (triangles) for a GABA<sub>A</sub> receptor  $\alpha$  subunits.

The development of compounds with contrasting binding affinities for all  $\alpha$  subunits has proven to be difficult as the benzodiazepine binding site is probably highly conserved between different  $\alpha$  subunits. Thus, compounds that are affinity-selective in vitro are generally less or even non-selective in vivo, and finding compounds with differential  $\alpha$  subunit affinity is a difficult goal to achieve. More recently, an alternative approach resulted in the development of compounds with selective efficacy for different  $\alpha$  subunits of the GABA<sub>A</sub>R. Such drugs generally bind with equal affinity to all  $\alpha$  subunits, but selectively modulate the activity of one or some of them (Figure 2). Already earlier, partial agonists with lower efficacy at the GABA<sub>A</sub>R compared to classical benzodiazepines such as bretazenil and abecarnil were developed. These drugs display overall lower efficacy at all  $\alpha$  subunits and were thought to have decreased side effects, but resulted in severe sedation in humans (van Steveninck et al 1996). Moreover, in contrast to the GABA<sub>A</sub>R subtype specificity hypothesis, recently developed compounds with more  $\alpha_1$  agonistic activity compared to the  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  subunit appeared to be anxiolytic in humans, indicating that the preclinical profile of GABA<sub>A</sub>ergic compounds does not always predict the clinical effects (Lippa et al 2005; Popik et al 2006). A possible explanation for these results could be that compounds only need moderate efficacy at  $\alpha_2$  subunits to produce anxiolysis, whereas high efficacy at  $\alpha_1$  subunits is required for sedation. However, these results show that GABA<sub>A</sub>R pharmacology is complex and not fully understood. The concept that distinct GABA<sub>A</sub>R  $\alpha$  subtypes generate the various clinical benzodiazepine effects remains valuable, and research on the GABA<sub>A</sub>R in stress and anxiety processes therefore presents opportunities for the development of novel anxiolytic compounds.

In addition to the efforts that have been made on compounds targeting the GABA<sub>A</sub>R  $\alpha$  subunits, the  $\gamma$  subunit has also proven to influence benzodiazepine efficacy as the benzodiazepines binding site is located between the  $\alpha$  and the  $\gamma$  subunit (Rudolph et al 1999). Interestingly, exchanging the abundant  $\gamma_2$  subunit for a  $\gamma_3$  subunit resulted in decreased benzodiazepine affinity, whereas the hypnotic CL218,872 (with 17-fold selectivity for the  $\alpha_1$  subunit, (Wafford et al 1993)) displayed an increased affinity (Graham et al 1990). This suggests that a compound with  $\alpha_x\gamma_3$  affinity over  $\alpha_x\gamma_2$  affinity may constitute a novel target for the development of hypnotic or anxiolytic drugs.

In summary, the search for new anxiolytic drugs has focused on subunit specific GABA<sub>A</sub>R agonists as such drugs are expected to dissociate anxiolytic from sedative effects. Prime candidates for non-sedating anxiolytic drugs appear to be compounds that lack activity at the  $\alpha_1$ -containing GABA<sub>A</sub>R while modulating the  $\alpha_2$  and/or  $\alpha_3$  GABA<sub>A</sub>R subunit. These compounds could exert anxiolytic effects, whereas side effects which currently limit benzodiazepine use (among which sedation, ataxia, amnesia, alcohol potentiation, tolerance development and abuse potential) would be absent.



**Figure 3:** Schematic representation of the GABA<sub>B</sub> receptor.

### 3. The GABA<sub>B</sub> receptor (GABA<sub>B</sub>R)

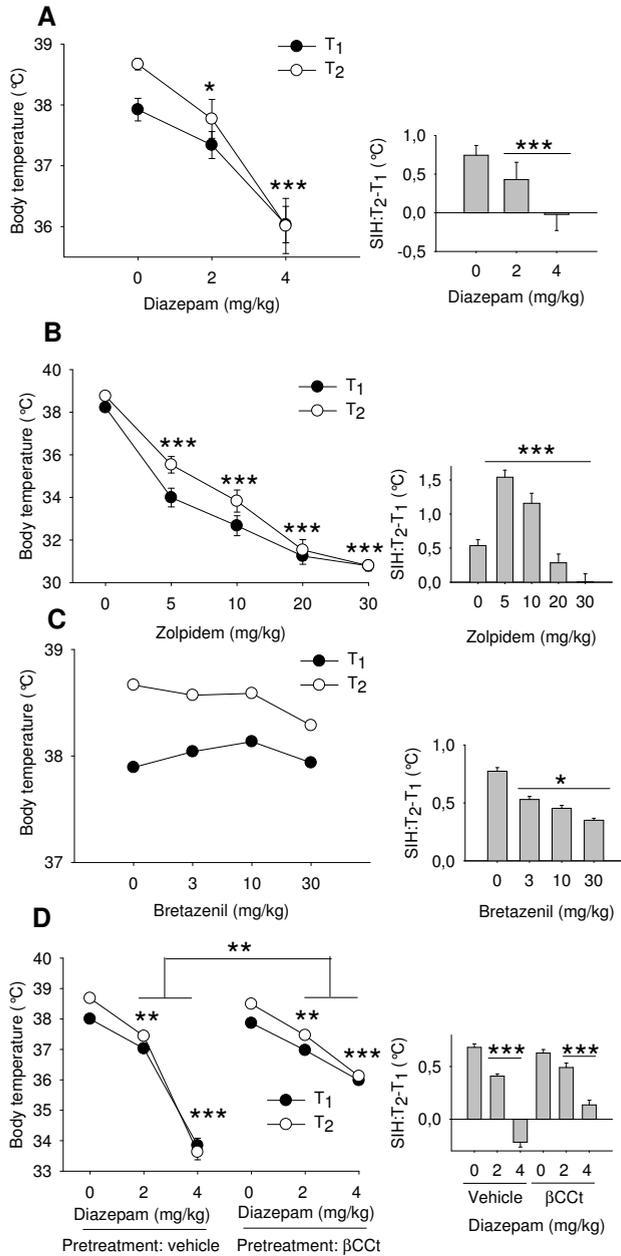
The GABA<sub>B</sub> receptor is a G-protein coupled receptor consisting of a heterodimer made up of two subunits, GABA<sub>B(1)</sub> and GABA<sub>B(2)</sub>, both of which are necessary for GABA<sub>B</sub> receptors to be functionally active (Calver et al 2002) (Figure 3). They are expressed both as presynaptic heteroreceptors and also postsynaptically, where they respectively modulate neuronal excitability. Heteroreceptors modulate the release of (excitatory) neurotransmitters, mainly via actions on presynaptic Ca<sup>2+</sup> channels, and postsynaptic GABA<sub>B</sub> receptors activate slow inhibitory postsynaptic potentials via activation of inwardly-rectifying K<sup>+</sup> channels. GABA<sub>B</sub> receptors also function as autoreceptors on interneurons. Additionally, GABA<sub>B</sub> receptors are negatively coupled to adenylyl cyclase, through which they influence downstream molecular pathways (Bettler et al 2004).

There is a growing body of evidence indicating that GABA<sub>B</sub> receptors play a critical role in anxiety (Cryan and Kaupmann 2005). The prototypical GABA<sub>B</sub> receptor agonist, baclofen, has shown anxiolytic activity in some clinical settings. Baclofen reduced anxiety in post-traumatic stress disorder (PTSD) patients (Drake et al 2003), in alcoholics following alcohol withdrawal (Addolorato et al 2002; Addolorato et al 2006), in panic disorder (Breslow et al 1989) and in patients suffering from acute spinal trauma (Hinderer 1990). Baclofen has also demonstrated anxiolytic effects in several preclinical studies including ultrasonic vocalisation in rat pups (Nastiti et al 1991), increased punished drinking (Shephard et al 1992), elevated plus maze (Andrews and File 1993) (but see (Dalvi and

Rodgers 1996)) and in the social interaction and elevated plus maze tests following withdrawal of dependent rats from either diazepam or alcohol (File et al 1992; File et al 1991a; File et al 1991b).

Perhaps the strongest preclinical evidence to date for a role of GABA<sub>B</sub> receptors in anxiety was demonstrated by the phenotype of GABA<sub>B</sub> receptor-deficient mice. Deletion of either the GABA<sub>B(1)</sub> or GABA<sub>B(2)</sub> receptor subunits results in a complete loss of typical GABA<sub>B</sub> functions and induces a highly anxious phenotype in mice in exploratory-based tests of anxiety (Mombereau et al 2004; Mombereau et al 2005). Furthermore, the GABA<sub>B(1)</sub> subunit is predominantly expressed as one of two isoforms: GABA<sub>B(1a)</sub> or GABA<sub>B(1b)</sub>, and deletion of these isoforms has differential effects on the acquisition and extinction of amygdala dependent conditioned aversive learning tasks (Shaban et al 2006).

Although studies with the GABA<sub>B</sub> receptor agonist baclofen have supported a role for GABA<sub>B</sub> receptors in anxiety its hypothermic, sedative and muscle-relaxant profile in a wide range of different species limits its applicability as a tool for behavioral research and as a therapy in psychiatry (Bettler et al 2004; Cryan and Kaupmann 2005; Jacobson and Cryan 2005). Recently, positive modulators of the GABA<sub>B</sub> receptor have been developed. CGP7930 and its aldehyde analogue CGP13501 were the first GABA<sub>B</sub> receptor positive modulators to be characterised *in vitro* (Urwyler et al 2001). A subsequent, structurally distinct chemical series which includes the more efficacious GS39783 were published shortly thereafter (Urwyler et al 2003). More recently two new classes have been identified: rac-BHFF (Malherbe et al 2008) and BHF177 (Jacobson et al 2008; Paterson et al 2008). All of these compounds enhance both the potency and the maximal efficacy of GABA at GABA<sub>B</sub> receptors *in vitro*, *via* interactions with the 7-transmembrane domain of the GABA<sub>B(2)</sub> subunit, although they have little to no intrinsic action by themselves (Cryan et al 2004; Dupuis et al 2006). CGP7930, rac-BHFF and GS39783 have also demonstrated GABA<sub>B</sub> receptor positive modulation properties *in vivo*. CGP7930 and rac-BHF177 potentiated the loss-of righting effects of the GABA<sub>B</sub> receptor agonists baclofen and gamma-hydroxybutyrate (GHB) (Carai et al 2004; Malherbe et al 2008), while in a microdialysis study, GS39783 potentiated the inhibitory effects of baclofen on forskolin-induced cAMP production in the rat striatum (Gjoni et al 2006). Of particular note, GABA<sub>B</sub> receptor positive modulators demonstrated anxiolytic profile in multiple rodent tests (Cryan et al 2004; Malherbe et al 2008) without showing the motor impairing hypothermic or cognitive impairing actions that are characteristic of full GABA<sub>B</sub> receptor agonists (Cryan et al 2004; Jacobson and Cryan 2008; Mombereau et al 2004).



**Figure 4:** Effects of non-subunit selective classical GABA<sub>A</sub> receptor agonist diazepam (A), the GABA<sub>A</sub> receptor α<sub>1</sub> subunit-selective agonist zolpidem (B), the partial GABA<sub>A</sub> receptor agonist bretazenil (C), and the combination of diazepam and GABA<sub>A</sub> receptor α<sub>1</sub> subunit antagonist βCCT (D) on the SIH response in 129Sv/Ev mice. \*: p<0.05; \*\*: p<0.01, \*\*\*: p<0.001. Unpublished data.

## 4. Effects of GABA<sub>A</sub>ergic drugs on the SIH response

### 4.1 Effects of benzodiazepine site ligands

#### **Classical benzodiazepines**

Classical benzodiazepines (among which chlordiazepoxide, diazepam, oxazepam, nitrazepam and alprazolam) dose-dependently reduce the SIH response, and, at higher doses, also reduce basal body temperature in rodents (Bouwknrecht et al 2007; Vinkers et al 2008). So far, all benzodiazepines that have been studied in the original group-housed and the singly-housed SIH model reduce the SIH response (Table 1). Therefore, studies that aim to assess the anxiolytic effects of different drug classes via the SIH paradigm often use benzodiazepines as a positive reference compound (Iijima et al 2007; Nordquist et al 2007; Stemmelin et al 2008). A typical example of the effects of the classical benzodiazepine diazepam on the SIH response in mice is shown in figure 4A. The SIH response is significantly decreased in drug-treated mice compared to vehicle-treated animals (one-way ANOVA with T<sub>1</sub> and T<sub>2</sub> as within-subject factor (SIH) and treatment as between-subject factor, diazepam x SIH interaction  $F_{2,26}=3.27$ ,  $p<0.05$ ). Moreover, diazepam significantly reduced basal body temperature at both doses (main diazepam effect  $F_{1,26}=15.67$ ,  $p<0.001$  with Dunnett's multiple comparison as post-hoc test). As classical benzodiazepines bind to  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  subunits (Pritchett et al 1989; Pritchett and Seeburg 1990), their effects on both the SIH response and basal body temperature are mediated via these subunits. Flumazenil (Ro 15-1788), a silent non-selective GABA<sub>A</sub>R  $\alpha$  subunit antagonist, dose-dependently reversed the diazepam effects on the SIH response and basal body temperature in mice (Olivier et al 2002). This illustrates the close involvement of the GABA<sub>A</sub>R  $\alpha$  subunit in the benzodiazepine action on the SIH response as repeatedly has been shown that flumazenil does not influence the SIH response or basal body temperature levels (Olivier et al 2002; Pattij et al 2002b; Van Bogaert et al 2006a).

#### **Benzodiazepine agonists**

If GABA<sub>A</sub>R  $\alpha$  subunits indeed differentially contribute to the various effects of classical benzodiazepines, the question remains how more selective drugs for these GABA<sub>A</sub>R subtypes influence the SIH response and body temperature. So far, a number of drugs with  $\alpha$  subtype selective activity has been tested in the SIH model (Table 3). Zolpidem, with 5-10 fold more selectivity for  $\alpha_1$  subunits compared to  $\alpha_2/\alpha_3$  subunits (Ebert et al 2006), generally causes hypothermia without attenuating of the SIH response, indicating that the GABA<sub>A</sub>R  $\alpha_1$  subunit is not directly involved in anxiolytic effects but plays a role in thermoregulatory processes. In rats, zolpidem reduced the SIH response, but this was most likely the result of strong hypothermic effects on basal body temperature that disturbed physiological homeostatic mechanisms (Vinkers et al 2009f). The hypothermic effects of zolpidem in mice are illustrated in figure 4B (main zolpidem effect  $F_{4,72}=83.24$ ,  $p<0.001$ ). Zolpidem also affected the SIH response (zolpidem x SIH interaction  $F_{4,72}=27.81$ ,  $p<0.001$ ). The apparent increase of the SIH response at lower doses is caused by a general body temperature reduction, allowing the SIH response to increase as the maximum body temperature is limited due to ceiling effects. At the highest dose, the reduction of the SIH response can be explained by the strong hypothermic and not necessarily anxiolytic

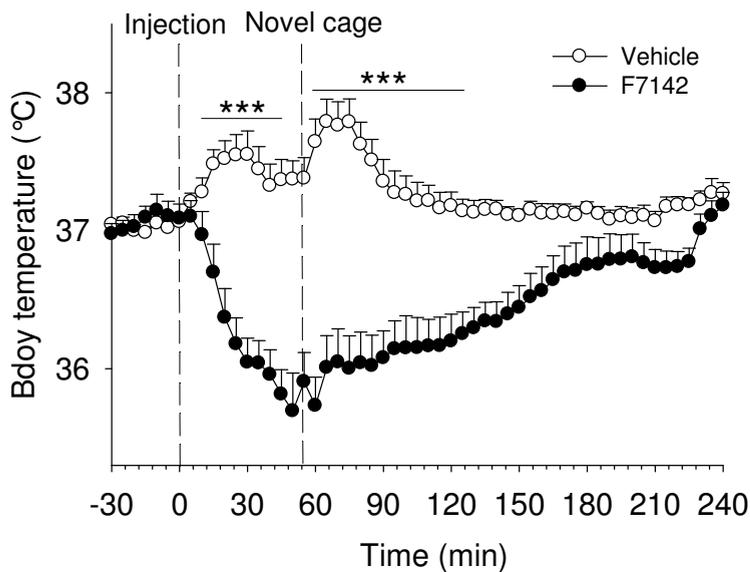
effects of zolpidem. However, zolpidem may demonstrate less  $\alpha_1$  subunit selectivity in vivo compared to in vitro studies using recombinant receptors, and it is possible that zolpidem may exert anxiolytic effects and reduce the SIH response in vivo via  $\alpha_{2/3}$  subunits (Atack et al 1999). L838,417 is a compound with comparable affinity for the  $\alpha_{1,2,3,5}$  subunits, but with no efficacy at the  $\alpha_1$  subtype and partial agonistic efficacy at  $\alpha_2$ ,  $\alpha_3$  and  $\alpha_5$  subtypes (McKernan et al 2000). In three different mouse strains, L838,417 dose-dependently reduced the SIH response without affecting basal body temperature (Van Bogaert et al 2006a), indicating that the SIH response and basal body temperature can be independently altered depending on the drug properties. A putative role for the GABA<sub>A</sub>R  $\alpha_3$  subunit in the SIH response was confirmed using the GABA<sub>A</sub>R  $\alpha_3$  subunit agonist TP003 that attenuated the SIH response without affecting basal body temperature levels in both rats and mice (Dias et al 2005; Vinkers et al 2009e). Recently, we tested an essentially  $\alpha_5$  selective compound which neither affected the SIH response nor caused hypothermia (unpublished data). This confirmed that activation of the  $\alpha_5$  subunit is not essential for anxiolytic effects of classical benzodiazepines. There is increasing evidence for a role of the  $\alpha_5$  subunit in cognitive processes however (Collinson et al 2002), and as a result, inverse  $\alpha_5$  subunit agonists are being developed as cognition enhancers (Dawson et al 2006). The low efficacy positive GABA<sub>A</sub>R modulator bretazenil was found to be ineffective in the SIH model in NMRI mice (Olivier et al 2002). The marginal effects of bretazenil on either the SIH response or basal body temperatures were confirmed in 129Sv/Ev mice (main bretazenil effect  $F_{3,61}=0.82$ ,  $p=0.49$ , NS, figure 4C). Interestingly, bretazenil significantly reduced the SIH response in this strain (bretazenil x SIH interaction  $F_{3,61}=2.70$ ,  $p=0.05$ ). These results are in line with previous research that showed an excellent non-sedating preclinical profile for this drug (Potier et al 1988), even though later clinical studies showed that bretazenil caused sedation (van Steveninck et al 1996).

### ***Benzodiazepine antagonists***

Based on these results, we hypothesize that the  $\alpha_2$  and/or the  $\alpha_3$  GABA<sub>A</sub>R subunit is involved in the attenuation of the SIH response, whereas GABA<sub>A</sub>R  $\alpha_1$  subunit activation results in hypothermia. If hypothermia and sedation are both the result of GABA<sub>A</sub>R  $\alpha_1$  subunit activation, an absence of lower body temperatures after drug administration may indicate reduced sedative side effects. To test this hypothesis, we combined the classical benzodiazepine diazepam and the hypnotic zolpidem with the  $\alpha_1$  subunit antagonist  $\beta$ CCt in rats (Vinkers et al 2009e) (Table 2).  $\beta$ CCt is a compound with high affinity for the GABA<sub>A</sub> receptor  $\alpha_1$  subunit compared to the  $\alpha_2$ ,  $\alpha_3$ , and  $\alpha_4$  subunits and with comparable low efficacy at all  $\alpha$  subunits (Huang et al 2000). We found that administration of  $\beta$ CCt alone had no effect on either basal body temperature or novel cage-induced temperatures. However, prior injection with  $\beta$ CCt antagonized hypothermic effects of both diazepam and zolpidem. We replicated this finding in mice (figure 4D, unpublished results). Again,  $\beta$ CCt was able to reduce the diazepam-induced hypothermia (diazepam effect  $F_{2,64}=34.17$ ,  $p<0.001$ ;  $\beta$ CCt x diazepam interaction  $F_{2,64}=5.38$ ,  $p<0.01$ ) without affecting the diazepam effects on the SIH response (diazepam x SIH interaction  $F_{2,64}=12.05$ ,  $p<0.001$ ; Diazepam x  $\beta$ CCt x SIH interaction  $F_{2,64}=2.41$ ,  $p=0.10$ , NS).  $\beta$ CCt alone did not affect the SIH response ( $\beta$ CCt x SIH interaction  $F_{1,64}=0.23$ ,  $p=0.64$ , NS). This supports the hypothesis that different GABA<sub>A</sub>R  $\alpha$  subunits are responsible for SIH attenuation and hypothermia after benzodiazepine administration (Vinkers et al 2009e).

### Inverse benzodiazepine agonists

Recently, we showed that the inverse benzodiazepine agonist F7142 indeed resulted in hypothermia in rats, although only we only used a high dose (15 mg/kg) (Figure 5, unpublished results). However, acute administration of the inverse benzodiazepine agonists pentylentetrazole and FG-7142 did not result in an increased SIH response (Table 3). Inverse benzodiazepine agonists allosterically decrease the binding of GABA and negatively influence constitutive GABA<sub>A</sub>R activity. These compounds display anxiogenic effects in various animal models of anxiety (Atack et al 2005; File and Baldwin 1987). In the SIH model, anxiogenic drugs would thus theoretically lead to an exaggerated SIH response. The fact that stress-induced body temperatures display a consistent maximum value above which stress does not further increase body temperature may explain why the SIH model is less appropriate for the screening of anxiogenic properties of drugs. Other temperature parameters, such as the area under the curve or latency to decrease to baseline after stress exposure could be used to screen for anxiogenicity instead, although such parameters have not been evaluated in the SIH model. Moreover, bimodal influences of inverse benzodiazepines on locomotor responses have been described (Savic et al 2006). Therefore, inverse benzodiazepine agonists may exert increased hyperthermia or hypothermia as well depending on the dose. Further research with  $\alpha$ 3IA, (Atack et al 2005) is needed to elucidate the exact effects of inverse benzodiazepine agonists in the SIH model.



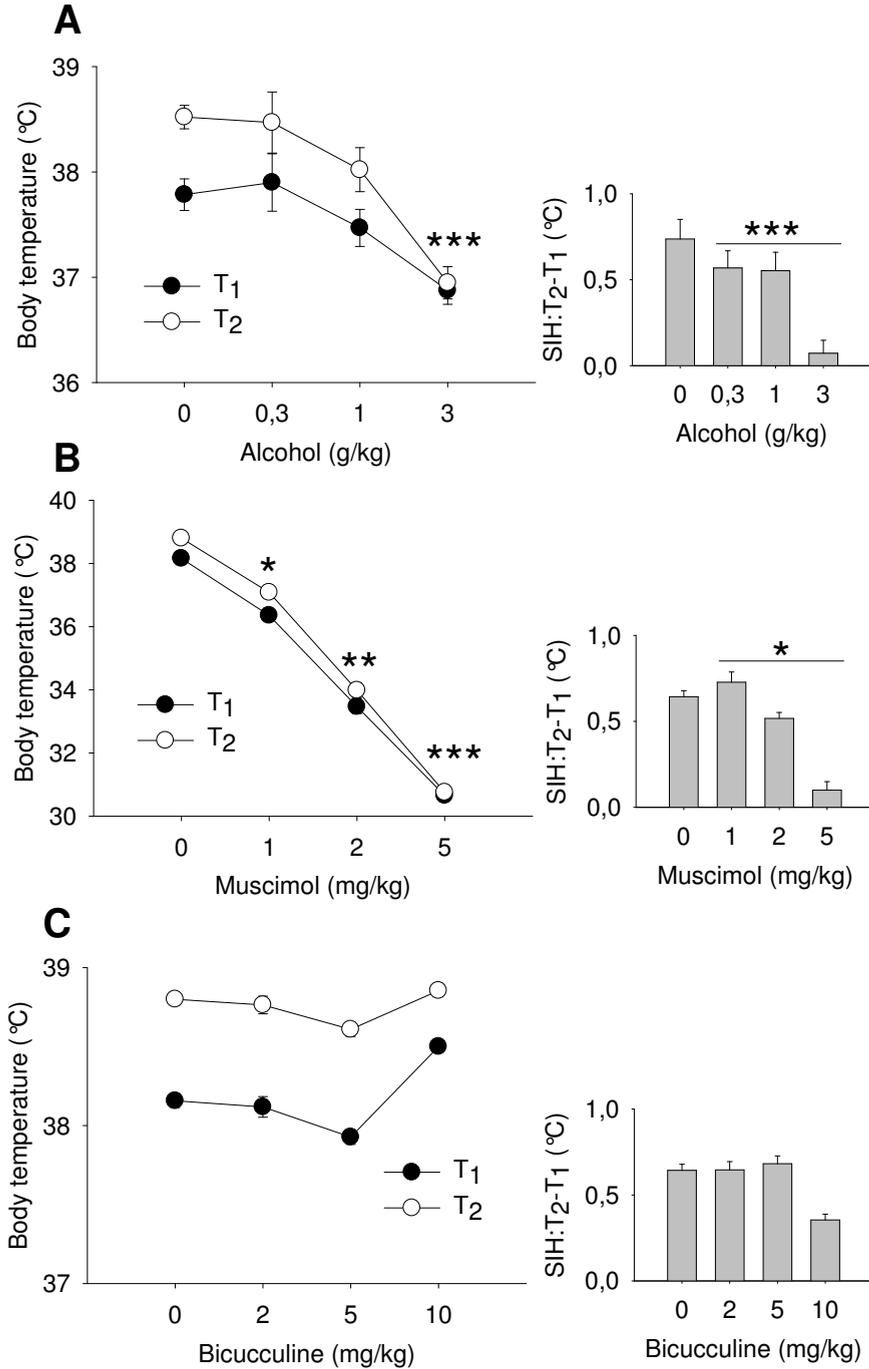
**Figure 5:** Effects of GABA<sub>A</sub> receptor inverse agonist F7142 (15 mg/kg, IP) on the SIH response in Wistar rats (n=9) \*\*\*: p<0.001. Unpublished data.

## 4.2 Effects of other drugs binding to the GABA<sub>A</sub>R

### **Alcohol**

The GABA<sub>A</sub>R has been implicated in the anxiolytic effects of alcohol. Generally, alcohol is thought to affect the tonic inhibition generated by extrasynaptic GABA<sub>A</sub>Rs that contain  $\alpha_4$ ,  $\alpha_6$  and  $\delta$  subunits (Wallner and Olsen 2008), whereas modulation of synaptic GABA<sub>A</sub>Rs is only present at higher concentrations (Jia et al 2008). However, alcohol also enhances inhibition via a GABA<sub>A</sub>ergic presynaptic mechanism in various brain areas including the amygdala (Roberto et al 2003). At higher doses, alcohol can also modulate excitatory N-methyl-D-aspartic acid (NMDA) and non- NMDA glutamate receptors, serotonin and glycine receptors, as well as potassium and calcium channels (Dopico and Lovinger 2009; Harris 1999).

In the SIH model, alcohol consistently decreases the SIH response in rats as well as mice, although the effects on basal body temperature appear strain dependent (Table 5). In figure 6A, a typical example of the effects of alcohol on the SIH response is shown. Here, alcohol reduced the SIH response (alcohol x SIH interaction,  $F_{3,72}=8.58$ ,  $p<0.001$ ), whereas it reduced the basal body temperature at the highest dose (main alcohol effect,  $F_{3,72}=10.28$ ,  $p<0.001$ , with Dunnett's multiple comparison as post-hoc test). Although acute administration of alcohol is known to possess an anxiolytic profile, these effects are not identical to those of classic benzodiazepines (Langen et al 2002). The question remains whether this putative anxiolytic alcohol effect is mediated by synaptic or extrasynaptic GABA<sub>A</sub>R activation. The fact that  $\delta$ -subunit deficient mice demonstrate a normal anxiolytic and hypothermic response to alcohol suggests that the discussion on the (extra)synaptic mechanism by which alcohol activates the GABA<sub>A</sub>R is ongoing (Mihalek et al 1999).



**Figure 6:** effects of GABA<sub>A</sub> receptor modulator alcohol (A), GABA<sub>A</sub> receptor GABA site agonist muscimol (B) and GABA<sub>A</sub> receptor GABA site antagonist bicuculline (C) on the SIH response in 129Sv/Ev mice (n=10-16). \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001. Unpublished data.

**Table 1:** Effects of classical benzodiazepines on the basal body temperature ( $T_1$ , hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Compound	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref.
<b>Alprazolam</b>	Mouse (NMRI)	0.3-3	PO	Y	Y	G-SIH	(Zethof et al 1995)
	Mouse (Swiss)	0.15-0.6	IP	N	Y	G-SIH, 45min	(Lecci et al 1990b)
	Mouse (NMRI)	0.3-3	PO	Y	Y		(Olivier et al 2002)
	Mouse (NMRI)	0.3-3	PO	Y	Y		(Olivier et al 2003)
	Mouse (129/Sv)	0.3-3	PO	Y	Y	vs. 5-HT <sub>1A</sub> R KO mice	(Pattij et al 2002b)
	Mouse (DBA/J)	0.3-3	PO	N	Y		(Rorick-Kehn et al 2005)
	Mouse (DBA/J)	2.5-10	PO	N	Y		(Rorick-Kehn et al 2005)
	Mouse (OF1/IC)	0.3-10	PO	N	Y		(Spooren et al 2002)
<b>Chlor-diazepoxide</b>	Mouse	15	IP	N	Y	vs $\alpha_2$ KO mice	(Dias et al 2005)
	Mouse (Swiss)	7.5-25	PO	N	Y	G-SIH	(Lecci et al 1990b)
	Mouse (NMRI)	3-30	PO	Y	Y		(Olivier et al 2003)
	Mouse (NMRI)	3-30	PO	Y	Y		(Olivier et al 2002)
	Mouse (OF1, NMRI, FVB/NJ)	10	PO	N	Y	As a reference	(Jacobson and Cryan 2008; Nordquist et al 2007; Stemmelin et al 2008)
	Mouse (DBA/J)	2.5-10	PO	N	Y		(Rorick-Kehn et al 2005)
	Mouse (OF1/IC)	0.3-10	PO	N	Y		(Spooren et al 2002)

Table 1 (continued)

Compound	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref
Diazepam	Mouse (Swiss)	2.5-5	PO	?	Y	G-SIH, 30 min	(Borsini et al 1989)
	Mouse (Swiss)	1.25-5	PO	N	Y	G-SIH, 50 min	(Chen et al 2003)
	Mouse (NMRI)	3-12	PO	Y	Y	G-SIH	(Zethof et al 1995)
	Mouse (NMRI)	3-12	PO	Y	Y		(Olivier et al 2003)
	Mouse (NMRI)	3-12	PO	N	Y		(Groenink et al 1996b)
	Mouse (129Sv, B6, SW)	1-4	IP	Y	Y		(Van Bogaert et al 2006a; Vinkers et al 2008)
	Rat (Wistar)	1-4	IP	N	Y		(Vinkers et al 2009e)
	Mouse (NMRI, Balb/c)	1-12	PO	Y	Y		(Olivier et al 2002)
	Mouse (129Sv)	1-4	SC	N	Y		(Pattij et al 2001)
	Mouse (ICR)	1	IP	N	Y	as a reference	(Iijima et al 2007; Satow et al 2008)
	Mouse (OF1/IC)	0.1-3	PO	N	Y		(Spooren et al 2002)
	Mouse (Swiss)	5	PO	N	Y		(Chen et al 2004a)
	Mouse (NMRI)	0.3-3	PO	Y	Y		(Van der Heyden et al 1997)
Estazolam	Mouse (Swiss)	0.5-1	PO	N	Y	G-SIH, 45min	(Lecci et al 1990b)
Nitrazepam	Mouse (Swiss)	2-4	PO	?	Y	G-SIH, 30 min	(Borsini et al 1989)
Oxazepam	Mouse (NMRI)	0.3-3	PO	N	Y		(Olivier et al 2002)
	Mouse (OF1/IC)	5-10	PO	N	Y		(Spooren et al 2002)
	Mouse (NMRI)	0.3-3	PO	N	Y		(Olivier et al 2003)

**Table 2:** Effects of benzodiazepines inverse agonists and antagonists on the basal body temperature ( $T_b$ , hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Compound	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref
<b>βCCt</b>	Rat (Wistar)	3-20	IP	N	N		(Vinkers et al 2009e)
<b>Flumazenil</b>	Mouse (129Sv, B6, SW)	3-30	PO	N	N		(Van Bogaert et al 2006a)
	Mouse (NMRI)	1-30	PO	N	N		(Olivier et al 2002)
	Mouse (129Sv)	3-30	SC	N	N	vs. 5-HT <sub>1A</sub> R KO	(Pattij et al 2002b)
<b>FG-7142</b>	Mouse (NMRI)	1-10	PO	N	N		(Olivier et al 2002)
	Rat (Wistar)	15	IP	Y	Y		Present study
<b>Pentylentetrazole</b>	Mouse (NMRI)	7.5-30	SC	Y	Y	G-SIH, only at 30 mg/kg	(Zethof et al 1995)
	Mouse (NMRI)	7.5-30	PO	Y	N		(Olivier et al 2002)
	Mouse (129Sv)	7.5-30	SC	Y	N	vs. 5-HT <sub>1A</sub> R KO mice	(Pattij et al 2002b)

**Table 3:** Effects of benzodiazepines agonists (including combinations with antagonists) on basal body temperature (T<sub>1</sub>, hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Compound	Species	Dose (mg/kg)	Route	Hyp o ther mia	SIH ↓	Remarks	Ref
<b>Alpidem</b>	Mouse (NMRI)	1-30	PO	Y	Y		(Olivier et al 2002)
<b>βCCt + diazepam</b>	Rat (Wistar)	10 + 4	IP	N	Y		(Vinkers et al 2009e)
<b>βCCt + zolpidem</b>	Rat (Wistar)	10 + 10	IP	N	Y		(Vinkers et al 2009e)
<b>Bretazenil</b>	Mouse (NMRI)	1-30	PO	N	N		(Olivier et al 2002)
	Mouse (129Sv/Ev)	3-30	IP	N	Y		Current study(Olivier et al 2002)
<b>Flumazenil +diazepam</b>	Mouse (NMRI)	10-30 + 3-6	PO	N	Y/N	Flumazenil reverses DZP effects on SIH and T <sub>1</sub>	(Olivier et al 2002)
<b>L838,417</b>	Mouse (129Sv, B6, SW)	3-30	PO	N	Y		(Van Bogaert et al 2006a)
<b>TP003</b>	Mouse (α2 and WT)	1	IP	N	Y		(Zethof et al 1995)
	Rat (Wistar)	0.3-	IP	N	Y		(Vinkers et al 2009e)
<b>Zolpidem</b>	Rat (Wistar)	3-30	IP	Y	Y		(Vinkers et al 2009e)
	Mouse (129Sv, B6, SW)	3-30	PO	Y	Y/N	No SIH reduction in 129Sv mice	(Vinkers et al 2009e)
	Mouse (NMRI)	0.3-30	PO	Y	N	Only highest dose effect on T <sub>1</sub>	(Olivier et al 2002)
	Mouse (NMRI)	0.3-30	PO	Y	N	Only highest dose effect on T <sub>1</sub>	(Olivier et al 2003)

**Table 4:** Effects of GABA site binding GABA<sub>A</sub>R agonists on the basal body temperature (T<sub>1</sub>, hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Compound	Species	Dose (mg/kg)	Route	Hypot hermia	SIH ↓	Remarks	Ref
THIP	Rat (Wistar)	0.3-10	IP	Y	Y		(Vinkers et al 2009e)
Muscimol	Mouse (129Sv/Ev)	1-5	IP	Y	Y		Current article
Bicuculline	Mouse (129Sv/Ev)	2-10	IP	N	N	Only at highest dose due to hyperthermia	Current article

### **Compounds acting on the GABA binding site**

Endogenous GABA can bind at two different GABA binding sites located between the  $\alpha$  and  $\beta$  GABA<sub>A</sub>R subunits (Amin and Weiss 1993; Boileau et al 1999). Exogenous compounds that can bind to the same binding sites include agonists muscimol and gaboxadol as well as the antagonist bicuculline (Ebert et al 1994). Endogenous and exogenous ligands have different affinity for the two GABA binding sites as one GABA binding site is flanked by a  $\beta$  and a  $\gamma$  subunit and the other by an  $\alpha$  and a  $\gamma$  subunit (Baumann et al 2003) (Figure 1).

Drugs that directly act on the GABA binding site have received limited attention in the SIH model. The putative anxiolytic drug gaboxadol (THIP) has a high efficacy at extrasynaptic receptors compared to GABA (Saarelainen et al 2008; Wafford and Ebert 2006). The  $\delta$  subunit-containing GABA<sub>A</sub>Rs are often located extrasynaptically and perisynaptically and are thought to be involved in a continuous active inhibitory tone instead of the phasic inhibitory tone caused by intrasynaptic agonists (Farrant and Nusser 2005; Nusser et al 1998). In rats, gaboxadol reduced basal body temperature and the SIH response only at the highest dose tested (10 mg/kg), whereas lower doses were ineffective (Vinkers et al 2009e). The GABA binding site agonist muscimol dose-dependently reduced basal body temperature (Figure 6B, main muscimol effect,  $F_{3,42}=43.34$ ,  $p<0.001$ , with Dunnett's multiple comparison as post-hoc test) and affected the SIH response due to basal temperature lowering effects (muscimol x SIH interaction,  $F_{3,42}=2.90$ ,  $p<0.05$ ). Thus, GABA binding site agonists possess limited or no anxiolytic effect in the SIH model. The GABA binding site antagonist bicuculline did not alter the SIH response or basal body temperature (Bicuculline effect  $F_{3,43}=1.40$ ,  $p=0.26$ , NS; bicuculline x SIH interaction  $F_{3,43}=1.13$ ,  $p=0.35$ , NS), although an increased body temperature at the highest dose is apparent (Figure 6C, unpublished results). Overall, for drugs acting at the GABA site, it seems likely that, at higher doses, agonists cause hypothermia whereas antagonists increase basal body temperature. The SIH response is generally unaffected except at high doses when interference with physiological thermoregulation occurs. So far, no clear anxiolytic effects of drugs acting at the GABA binding site have been found in the SIH model.

**Table 5:** Effects of GABA<sub>A</sub>R agonists acting on other sites than the benzodiazepine and GABA binding site on the basal body temperature (T<sub>1</sub>, hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Compound	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref
<b>Alcohol</b>	Rat (Wistar)	0.3-3 g/kg	PO	Y	Y	Only at 3 g/kg	(Vinkers et al 2009e)
	Mouse (NMRI)	2-4 g/kg	PO	Y	Y	G-SIH	(Zethof et al 1995)
	Mouse (Swiss)	2-4 g/kg	PO	Y	Y	G-SIH, 45 min	(Lecci et al 1990b)
	Mouse (NMRI)	2-4 g/kg	PO	Y	Y	Only at 4 g/kg	(Olivier et al 2002)
	Mouse (129Sv, C57Bl/6, SW)	2-4 g/kg	PO	Y	Y		(Vinkers et al 2008)
	Mouse (129Sv)	1-4 g/kg	PO	N	Y	Vs 5-HT <sub>1A</sub> KO	(Pattij et al 2002b)
	Mouse (OF1/IC)	15-30%, 10ml/kg	PO	N	Y		(Spooren et al 2002)
<b>Phenobarbital</b>	Mouse (NMRI)	1	IP	N	Y	G-SIH	(Dias et al 2005)
	Mouse (Swiss)	10-20	IP	N	Y	G-SIH, 75 min	(Lecci et al 1990b)
	Mouse (NMRI)	30-100	PO	N	N		(Olivier et al 2002)

### Neurosteroids, barbiturates and general anesthetics

Neurosteroids are strong and rapid potentiators of GABA<sub>A</sub>Rs, interacting with more than one steroid-binding site (Akk et al 2008; Hosie et al 2007). Recently, the GABA<sub>A</sub>R  $\alpha_1$  subunit was found to be essential for the response to neurosteroids (Akk et al 2009). This generic pharmacological profile of neurosteroids is ascribed to a highly conserved amino acid (glutamine, Q241) in the GABA<sub>A</sub>R  $\alpha$  subunits (Hosie et al 2009). However, neurosteroids have an increased potency at the  $\alpha_5$  subunit (Rahman et al 2006) and extrasynaptic  $\delta$  subunit-containing GABA<sub>A</sub>R receptors (Belelli and Lambert 2005). No neurosteroids have been tested in the SIH model yet. The putative anxiolytic effects of neurosteroids in other studies suggest that these drugs might be effective in the SIH model (Bitran et al 1991; Crawley et al 1986).

Barbiturates bind to the GABA<sub>A</sub>R at the  $\alpha$  subunit with distinct binding sites from the GABA and the benzodiazepine binding site (Sieghart 1995). Two studies found that phenobarbital was able to reduce the SIH response in mice, whereas another study that used higher doses did not find any effects (Table 5). At lower doses, barbiturates enhance GABA binding, although it potentiates GABA<sub>A</sub>Rs at moderate doses in the absence of GABA, and even block GABA<sub>A</sub>Rs at high doses (Steinbach and Akk 2001). Therefore, the anxiolytic effects of phenobarbital on the SIH response may depend on the applied dose. GABA<sub>A</sub>R-sensitive general anesthetics such as etomidate and propofol cause unconsciousness and immobility by acting on extrasynaptic tonic inhibitory  $\alpha_4/\beta_3\delta$  and  $\alpha_4\beta_3$  GABA<sub>A</sub>Rs (Meera et al 2009). No anxiolytic properties have been ascribed to general

anesthetics, and, to our knowledge, these compounds have not been applied in the SIH model.

### 4.3 Conclusion

Altogether, a wide variety of GABA<sub>A</sub>ergic compounds has been applied using the SIH paradigm. There is overwhelming evidence that classical benzodiazepines dose-dependently reduce the SIH response. Subsequent studies that have applied GABA<sub>A</sub> subunit selective compounds as well as combination of agonists and antagonists have confirmed a role for the GABA<sub>A</sub>R  $\alpha_{2/3}$  subunit in the reduction of the SIH response (anxiolytic effect), whereas the GABA<sub>A</sub>R  $\alpha_1$  subunit primarily causes hypothermia. Thus, the effects of GABA<sub>A</sub>R compounds on basal body temperature and the SIH response can be dissociated. The effects of benzodiazepine inverse agonists on the SIH response and basal body temperature are complex and remain to be elucidated. However, a clear anxiogenic effect resulting in an increased SIH response does not seem likely. Drugs that act on the GABA site of the GABA<sub>A</sub>R did not result in a reduction of the SIH response, although all of them caused hypothermia. Whereas alcohol consistently decreases the SIH response, the effects of the barbiturate phenobarbital are not easily interpreted and might depend on the applied dose.

## 5. Effects of GABA<sub>B</sub>ergic drugs on the SIH response

Baclofen's effects on SIH have been assessed (Olivier et al 2003). These studies show little anxiolytic effects at doses that do not alter baseline temperature. Given the ability of full GABA<sub>B</sub> receptor agonists to produce dose-dependent mechanistically-predicted temperature decreases (Jacobson and Cryan 2005) it is unlikely that the SIH paradigm will be sensitive enough to dissociate baseline changes in homeostatic physiology with the potential ability of full agonists to reverse stress-induced autonomic responses. Similar problems also lie with assessing the effects of other classes of ligands such as nicotine (see (Bouwknicht et al 2007) for discussion) or certain GABA<sub>A</sub>R ligands (see above; and (Vinkers et al 2009f). The development of GABA<sub>B</sub> receptor positive modulators, which on the whole have no intrinsic effects on temperature, has allowed for the contribution of GABA<sub>B</sub> receptors to the SIH response to be better elaborated (Table 6).

**Table 6:** Effects of various GABA<sub>B</sub>ergic compounds on the SIH response and basal body temperature.

Compound	Species	SIH ↓	Hypo thermia	Dose (mg/kg)	Ref
Baclofen	Mouse	N	Y	0-10	(Olivier et al 2003)
Baclofen	Mouse	-	Y	0-12	(Jacobson and Cryan 2005)
GS39783	Mouse	Y	Y	0-100 PO	(Cryan et al 2004)
rac-BHFF	Mouse	Y	N	0-100	(Malherbe et al 2008)
CGP7930	Mouse	Y	N	0-100	(Jacobson and Cryan 2008)
BHF177	Mouse	Y	Y	0-100 PO	(Jacobson et al 2008)

Initial studies characterized the effects of GS39783 on SIH where it was demonstrated that at oral doses from 0.1 – 30 mg/kg. GS39783 was able to counteract the SIH response (Cryan et al 2004) although the effect size was less than that garnered with benzodiazepines. The effects of CGP7930 on SIH were also demonstrated but these were less potent than chlordiazepoxide and than previously shown for GS39783, with only the 100 mg/kg dose effective (Jacobson and Cryan 2008). Interestingly, the magnitude of the effect was relatively similar between both GABA<sub>B</sub> receptor modulators. Recent studies with the novel modulator rac-BHFF (doses 3, 10, 30 and 100mg/kg, p.o.) demonstrated anxiolytic effects at all doses but with significance reached for 100 mg/kg dose only (Malherbe et al 2008). Preliminary data demonstrates that BHF177 at oral doses of 20 and 30 mg/kg displayed an anxiolytic-like SIH test in mice (Jacobson et al 2008). However, given that BHF177, at doses over 40 mg/kg caused hypothermia - distinct from the lack of effect of other GABA<sub>B</sub> receptor positive modulators on temperature - the observed anxiolytic property in the SIH test must be viewed with caution and such effects requires further confirmation in paradigms not reliant on body temperature.

## 6. Conclusion

Although baseline temperature effects of GABA<sub>B</sub> receptor agonists preclude the drawing of any decisive conclusions on the role of this receptor in SIH, the development of novel GABA<sub>B</sub> receptor positive modulators does indeed suggest that this receptor is a novel mechanism for counteracting SIH. To date, all of the four classes of modulators have been able to significantly counteract SIH and this paradigm is ideal for assessing the anxiolytic potential of future GABA<sub>B</sub> receptor modulators.



# Chapter 15

## Part III

### **Stress-induced hyperthermia, the serotonin system and anxiety**

Christiaan H. Vinkers

Berend Olivier

J. Adriaan Bouwknecht

Lucianne Groenink

Jocelien D.A. Olivier

# 15

*The Open Pharmacology Journal, in press*

## 1. Introduction

The serotonin (5-HT) system plays a key role in the pathophysiology of psychiatric disorders including mood and anxiety disorders (Millan 2003). Specifically, the serotonergic system has been implicated in changes that are present in stress-related disorders including alterations in appetite, sleep, mood, and cognition. In support, depressed patients have decreased plasma levels of tryptophan (Coppen et al 1973; Cowen et al 1989) and decreased CSF levels of the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) (Asberg et al 1976; Owens and Nemeroff 1998).

A role for serotonin in stress-related disorders is further supported by the fact that clinically effective treatments for these disorders alter serotonergic neurotransmission. Selective serotonin reuptake inhibitors (SSRIs) increase serotonergic signaling by blocking the serotonin transporter (SERT) and are widely used in anxiety disorders and major depressive disorder (Vaswani et al 2003). Although evidence points at a dysfunctional serotonin system in depression and anxiety disorders, the underlying causes of these disorders are complex and may also involve other neurotransmitter systems including the noradrenaline and dopamine systems (D'Aquila et al 2000; Ressler and Nemeroff 2000). Moreover, serotonin can modulate the dopaminergic and noradrenalin systems, and vice versa (Benloucif et al 1993; Esposito 2006; Iyer and Bradberry 1996; Salomon et al 2006). Therefore, recent efforts have been made to develop serotonin-noradrenalin-dopamine reuptake inhibitors (which block the transporters for all three key biogenic amines, so-called "triple reuptake inhibitors") for the treatment of stress-related disorders (Skolnick et al 2003)

The therapeutic potential of serotonergic interventions has resulted in a variety of preclinical approaches have been employed to study the serotonin system. Of these, the stress-induced hyperthermia (SIH) paradigm has been extensively used to study the serotonin system at a preclinical level (Borsini et al 1989; Vinkers et al 2008). The SIH response uses the transient rise in body temperature in response to a stressor which can be reduced using anxiolytic drugs including benzodiazepines and various serotonergic ligands (Bouwknicht et al 2007).

Of the different neurotransmitter systems, the serotonin system has received ample attention in the SIH paradigm. Therefore, the present review aims to discuss the acute and chronic effects of serotonergic ligands on the SIH response as well as to present an overview of the SIH response and drug responsiveness of genetically modified mice that lack or overexpress specific serotonergic receptor subtypes. First, the SIH paradigm will be shortly introduced, followed by an introduction on the 5-HT system and its role in affective disorders. Then, an overview of the different receptor subtypes of the 5-HT system that have been studied using the SIH paradigm is presented, including acute and chronic drug treatment as well as data from genetically modified animals. Finally, the possible role for the SIH model in the search for serotonergic interactions with other neurotransmitter systems is touched upon.

## 2. The SIH paradigm

The first use of SIH in anxiety research occurred after it was noted that removing mice one by one from a group-housed cage increased body temperature of the last mouse compared to the first (the group-housed version of the SIH model) (Borsini et al 1989). Later on, this model was refined to a singly-housed version in which the rectal temperature was measured twice with an interval of 10 min (Van der Heyden et al 1997). Here, the first rectal temperature measurement ( $T_1$ ) is the basal unstressed core temperature that also functions as a stressor, whereas the second rectal temperature measurement ( $T_2$ ) is the stress-induced body temperature which is increased due to the stress experienced from the first temperature measurement. The difference in temperature ( $\Delta T = T_2 - T_1$ ) is defined as the SIH response. A typical SIH response differs from species to species, but may range from 0.5 to 2°C. More recently, telemetric setups have enabled the continuous registration of body temperature responses to stress which opens up new possibilities for more advanced SIH studies (Vinkers et al 2009d; Vinkers et al 2009f). In anxiety research, it is difficult to find models with sufficient clinical predictive validity to support the translation of animal studies on anxiolytic drugs to clinical research. The highly reproducible and robust SIH response, combined with ease of testing, make the SIH paradigm very suitable for drug screening. It is a commonly used paradigm and different studies have investigated the effects of acute and chronic serotonergic drugs on the SIH response. Before we will discuss these studies into detail, we will take a closer look at the 5-HT system and discuss why the serotonin system may be important in stress and anxiety.

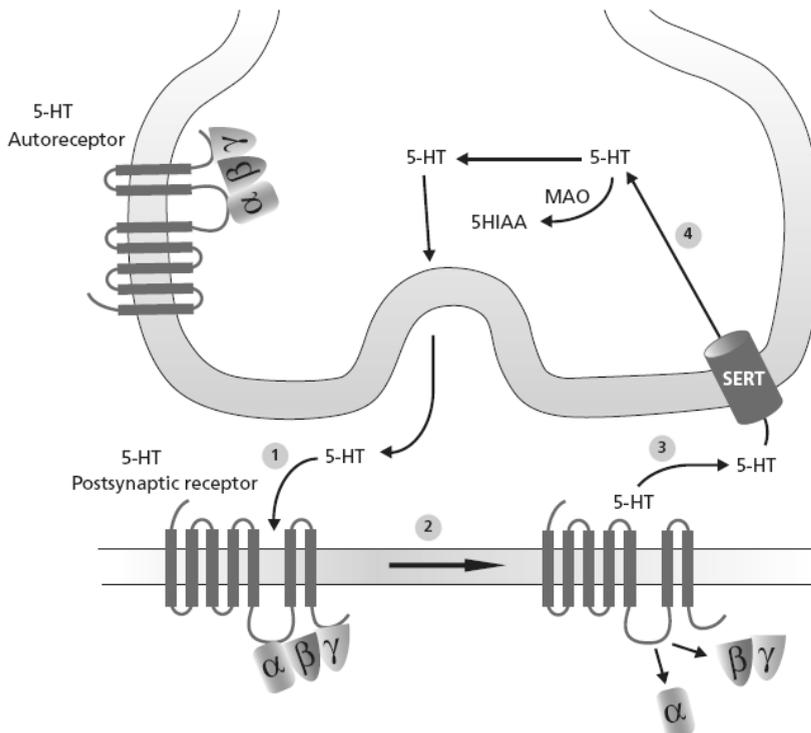
## 3. Serotonin receptors and transporter

### 3.1 The serotonin system

Serotonergic effects are mediated by multiple receptor subtypes with distinct distribution, localization, receptor structure, and second messenger systems that exert many functions in the central nervous system (Nichols and Nichols 2008). The 5-HT receptor family consists of at least 14 structurally and pharmacologically distinct receptors, of which 13 are G-protein-coupled receptors and one (the 5-HT<sub>3</sub> receptor) is a ligand-gated ion channel. Serotonergic neurons in the dorsal and median raphe nuclei of the brain stem are the main source of serotonin in the central nervous system. Serotonin, which is synthesized via tryptophan hydroxylase and L-amino acid decarboxylase, binds to all these different pre- and postsynaptic 5-HT receptors (Figure 1). Serotonergic receptors are distributed throughout the CNS, with a preferential presynaptic location in the raphe nuclei and a postsynaptic location in various limbic structures including the amygdala and the hippocampus (Burnet et al 1995; Chalmers and Watson 1991; Pompeiano et al 1992). In addition to a general pre- and postsynaptic distribution, 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub> and 5-HT<sub>1D</sub> receptors are also found as somatodendritic autoreceptors in the raphe nuclei which are pivotal in the control of serotonergic output to the frontal cortex as well as the hippocampus and is thought to be involved in the effects of SSRIs (Hjorth et al 1997). Moreover, the fact that specific 5-HT receptor subtypes may exist in different

isoforms (after gene splicing or post-transcriptional processes) indicates the true complexity of the 5-HT system (Barnes and Sharp 1999).

Another important modulator of the 5-HT system is the serotonin transporter (SERT), an integrated membrane protein which is both localized at the terminal portion of the axon as well as at the cell body of 5-HT neurons (Chen et al 1992; D'Amato et al 1987; Hrdina et al 1990). The SERT is pivotal in the reuptake of serotonin from the extracellular space into the presynaptic neuron, regulating the extracellular serotonin concentration and thus affecting the 5-HT system. After reuptake, 5-HT is stored in vesicles or is degraded via the monoamine oxidase enzyme to 5-hydroxy-indole acetaldehyde (5-HIAA) (Murphy et al 1998).



**Figure 1:** schematic representation of serotonin (5-HT) in the terminal and synapse. G-protein coupled receptors are located presynaptically (5HT autoreceptor (5-HT<sub>1A/1B</sub>) or postsynaptically (5-HT<sub>1/2/4/5/6/7</sub> receptors). 1) 5-HT is released from presynaptic neuron and binds to heterotrimeric G-protein postsynaptic receptor. Heterotrimeric G protein complexes contain an alpha, beta and gamma subunit, which in the inactive state are bound to GDP. 2) 5-HT acts on postsynaptic receptor and a change in the conformation of the postsynaptic receptor is induced. GDP is phosphorylated to GTP and binds to the  $\alpha$  subunit, which then becomes active. The  $\beta$  and  $\gamma$  subunits are freed. 3) Extracellular 5-HT is taken up by the SERT into the presynaptic neuron. 4) Back in the presynaptic neuron 5-HT is broken down by MOA to 5-HIAA or is being stored for future release. MAO: Mono-amine oxidase. SERT: serotonin transporter; 5HIAA: 5-hydroxyindole acetic acid.

### 3.2 The role of the 5-HT receptor family in stress and anxiety

Serotonergic receptors play a pivotal role in the modulation of behavioral, autonomic and endocrine stress responses. Of the serotonin receptor subtypes, the 5-HT<sub>1A</sub> receptor has been suggested to play a pivotal role in the pathophysiology of anxiety and depression (Gingrich and Hen 2001; Pucadyil et al 2005). Specifically, depression is associated with presynaptic 5-HT<sub>1A</sub> receptor upregulation and postsynaptic 5-HT<sub>1A</sub> receptor downregulation (van Praag 2004). Generally, 5-HT<sub>1A</sub> receptor agonists exert anxiolytic actions in rodents and humans (Millan 2003), and genetically modified 5-HT<sub>1A</sub> receptor knockout mice display increased anxiety behavior (Heisler et al 1998; Parks et al 1998; Ramboz et al 1998). Analysis of postmortem 5-HT<sub>1A</sub> receptor levels in humans after suicide have yielded inconclusive results ranging from an increase in 5-HT<sub>1A</sub> receptor binding (Arango et al 1995; Matsubara et al 1991; Meltzer 1990), to no differences (Arranz et al 1994; Dillon et al 1991; Lowther et al 1997a; Stockmeier et al 1997). A recent postmortem study found decreased binding of the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 to the orbitofrontal cortex in major depressive disorder patients (Stockmeier et al 2009). Imaging studies confirm that the WAY-100635 binding potential is significantly decreased in depressed patients (Drevets et al 2007), although the evidence is inconsistent (Parsey et al 2006). The delay in the onset of SSRI efficacy may be ascribed to developing 5-HT<sub>1A</sub> receptor desensitization which may be pivotal to establish a clinical response to SSRIs. This idea is supported by the fact that co-administration of the beta-adrenergic/5-HT<sub>1A</sub> receptor antagonist pindolol may decrease SSRI latency of onset (Kinney et al 2000).

In addition to 5-HT<sub>1A</sub> receptors, animal studies have implicated 5-HT<sub>1B</sub> receptors in the development of stress-related disorders. However, the exact involvement of the 5-HT<sub>1B</sub> receptor subtype in stress-related disorders is unclear due to the lack of a receptor-specific ligand. 5-HT<sub>1B</sub> receptors are found as autoreceptors regulating the release of 5-HT and also act as heteroreceptors on non-serotonergic neurons (Moret and Briley 1997; Morikawa et al 2000).

Studies on the involvement of 5-HT<sub>1B</sub> receptors in anxiety-related behavior are contradictory. 5-HT<sub>1B</sub> receptor agonists show anxiogenic-like effects (Lin and Parsons 2002; Moret and Briley 2000) and overexpression of 5-HT<sub>1B</sub> autoreceptors results in increased basal but reduced stress-induced anxiety-like behavior in rats (Clark et al 2002; Clark et al 2004). In contrast, higher 5-HT<sub>1B</sub> autoreceptor mRNA levels are correlated with decreased anxiety-like behavior (Clark et al 2004; Kaiyala et al 2003; Neumaier et al 2002). Moreover, 5-HT<sub>1B</sub> receptor knockout mice do not display clear alterations in anxiety-like behavior, even though altered autonomic stress responses have been reported (Groenink et al 2003b; Zhuang et al 1999). Altogether, the role of 5-HT<sub>1B</sub> receptors in anxiety and depression is at best circumstantial and more research is necessary to draw conclusions on the involvement of these receptors in stress-related disorders. A far more convincing role for the 5-HT<sub>1B</sub> receptor is the involvement in impulse control and aggressivity that has been convincingly demonstrated (de Boer and Koolhaas 2005; Olivier et al 1995; Olivier and van Oorschot 2005; Saudou et al 1994).

The precise function of 5-HT<sub>2</sub> receptors in anxiety states is complex. There are studies that report 5-HT<sub>2A</sub> receptor upregulation on platelets in depression (Biegonek et al 1990; Pandey

et al 1995; Pandey et al 1990), whereas others have reported a downregulation (Audenaert et al 2001). Therefore, it may be hypothesized that the effects of a dysfunctional 5-HT<sub>2A</sub> system may differentially affect receptor levels depending on the brain structure. In support, expression of the 5-HT<sub>2A</sub> receptor in the cortex of 5-HT<sub>2A</sub> knockout mice (with an increased anxiety-like behavior) normalized anxiety levels, suggesting that 5-HT<sub>2A</sub> receptors in the cortex are involved in anxiety-related processes (Weisstaub et al 2006). So far, brain imaging studies have shown mixed results (van Praag 2004).. Activation of 5-HT<sub>2C</sub> receptors, including administration of agonists like 1-(m-Chlorophenyl)piperazine (mCPP) and 6-Chloro-2-(1-piperazinyl)pyrazine (MK-212) results in anxiogenic and panic-like responses in humans (Charney et al 1987; Klein et al 1991; Lowy and Meltzer 1988) as well as animals (Bilkei-Gorzo et al 1998; Campbell and Merchant 2003; Kennett et al 1989), even though this seems to be dose-dependent (Kahn et al 1990). In line with this observation, 5-HT<sub>2C</sub> receptor antagonists have been reported to exert anxiolytic effects (Hackler et al 2007; Kennett et al 1995; Kennett et al 1997b; Wood et al 2001) and 5-HT<sub>2C</sub> knockout mice show decreased anxiety-like behavior (Heisler et al 2007). Interestingly, anxiogenic behavior induced by administration of SSRIs (e.g. fluoxetine) may be blocked by 5-HT<sub>2C</sub> receptor antagonists, indicating that acute anxiogenic effects that acutely occur after SSRIs may be attributed to stimulation of the 5-HT<sub>2C</sub> receptor (Bagdy et al 2001).

Overall, both 5-HT<sub>2A</sub> as well as 5-HT<sub>2C</sub> receptors are implicated in anxiety-like behavior and the contribution to an overall dysfunctional 5-HT system in various psychiatric disorders. As 5-HT<sub>2</sub> receptors are known shown to modulate other neurotransmitter systems including GABAergic (Millan 2003), glutamatergic (Beique et al 2007; Celada et al 2004) and dopaminergic neurons (Pehek et al 2006), 5-HT<sub>2</sub> receptor-mediated effects may be at least partially attributable to the downstream modulation of other neurotransmitter systems.

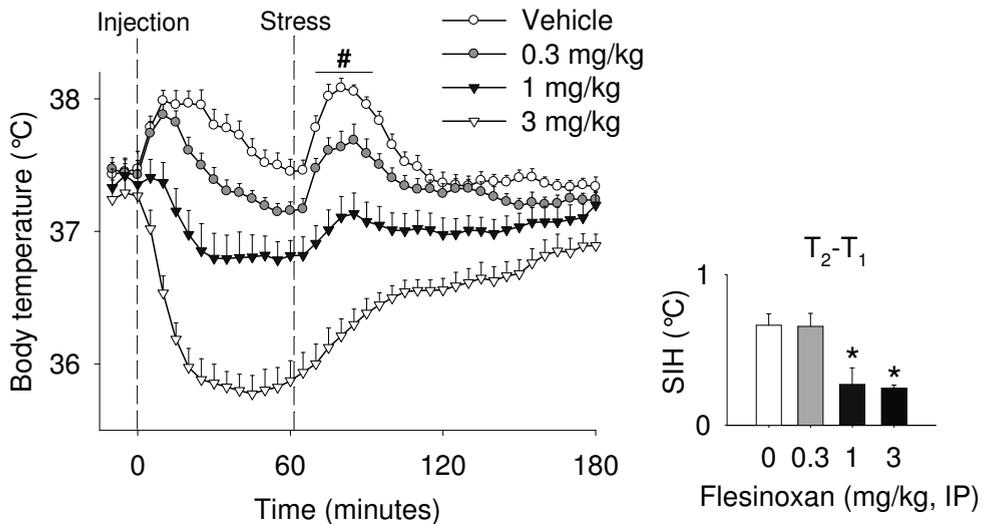
The 5-HT<sub>3</sub> receptor is a ligand-gated ion channel in contrast to the other G-protein-coupled 5-HT receptors (Derkach et al 1989). Putative anxiolytic effects have been attributed to 5-HT<sub>3</sub> receptor antagonists which may be mediated through 5-HT<sub>3A</sub> receptors in limbic structures (Artaiz et al 1995; Costall et al 1990; Jones et al 1988). However, knock out studies have not yielded a clear role for the 5-HT<sub>3A</sub> receptor in the regulation of anxiety and depression (Bhatnagar et al 2004). The contributions of 5-HT<sub>3A</sub> receptors may alternatively affect the genetic vulnerability to develop a dysfunctional 5-HT system, and humans carrying an allelic variation (single nucleotide polymorphism) in the 5-HT<sub>3A</sub> receptor gene (C178T) exhibit lower scores for anxiety-related traits (Melke et al 2003). Overall, the exact role of the 5-HT<sub>3</sub> receptors in stress-related disorders has not been fully elucidated yet but does not seem to present a breakthrough in the treatment of anxiety disorders.

5-HT<sub>4</sub>, 5-HT<sub>5</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub> receptors have received less attention compared to the other serotonergic receptors. Nonetheless, there is some evidence that some of these receptors may be involved in anxiety processes. Antagonizing or knocking out 5-HT<sub>4</sub> receptors reduces anxiety like behavior (Compan et al 2004; Kennett et al 1997a), although conflicting results exist (Artaiz et al 1998). No clear effects were found after knocking out 5-HT<sub>5A</sub> receptors in mice (Grailhe et al 1999). Pharmacological antagonism of

5-HT<sub>6</sub> receptors is known to produce anxiolytic-like effects (Wesolowska 2008; Wesolowska and Nikiforuk 2007; Woolley et al 2004), although no anxious phenotype was found in 5-HT<sub>6</sub> receptor knockout mice (Bonasera et al 2006). 5-HT<sub>7</sub> receptor antagonists exert anxiolytic effects (Wesolowska et al 2006), and 5-HT<sub>7</sub> receptor knockout mice do not display altered anxiety-like behavior (Guscott et al 2005; Roberts et al 2004a). In the various studies that address the role 5-HT<sub>4,5,6,7</sub> receptors, there appears to be a discrepancy between pharmacological (anxiolytic effect) and genetic knockout studies (no anxiolytic effect), indicating that these receptors may not be constitutionally necessary to develop a normal anxiety-related phenotype.

Beside 5-HT receptors, the SERT may also impact anxiety-like behavior. The SERT modulates the magnitude and duration of action of serotonin on both pre- and postsynaptic 5-HT receptors. Knocking out the SERT gene or pharmacologically blocking the SERT increases anxiety-like behavior in mice (Ansorge et al 2004; Holmes et al 2003a; Holmes et al 2003b) and rats (Olivier et al 2008b). Moreover, the link between the polymorphism in the promoter region of the human SERT gene (5-HTTLPR) and stress-related disorders has been established (Brown and Harris 2008), although a recent meta-analysis yielded no evidence for an interaction between 5-HTTLPR genotype and adult stress-related behaviors (Risch et al 2009). Thus, if genetic vulnerability to depression is at least partially attributed to allelic variations in genes that influence the 5-HT system, such a predisposition to develop a dysfunctional 5-HT system does not seem to be the sole explaining factor in the development of stress-related disorders.

The majority of the 5-HT receptor subtypes may be differentially involved in normal and stress-related behaviors. Of these different 5-HT receptors, the 5-HT<sub>1A</sub> and 5-HT<sub>2</sub> receptor have been consistently shown to be closely involved in stress-related behavior. A complex interaction between the various 5-HT receptors, the SERT and other neurotransmitter systems is apparent, and it is beyond doubt that these interactions yield an extremely fine-tuned system. Therefore, it may not be surprising that disturbances in this system may lead to the development of psychiatric disorders.



**Figure 2:** Acute administration of the 5-HT<sub>1A</sub> receptor agonist flesinoxan (0-3 mg/kg, IP) reduced the SIH response as well lowers basal body temperature in *Wistar rats* (n=12). Error bars represent the S.E.M.. \*: significant reduction of the SIH response (p<0.05); #: overall drug effect on basal body temperature (p<0.05). Unpublished data.

## 4. Effects of serotonergic compounds on the SIH response

### 4.1 Effects of 5-HT<sub>1A</sub> receptor ligands on the SIH response (Table 1)

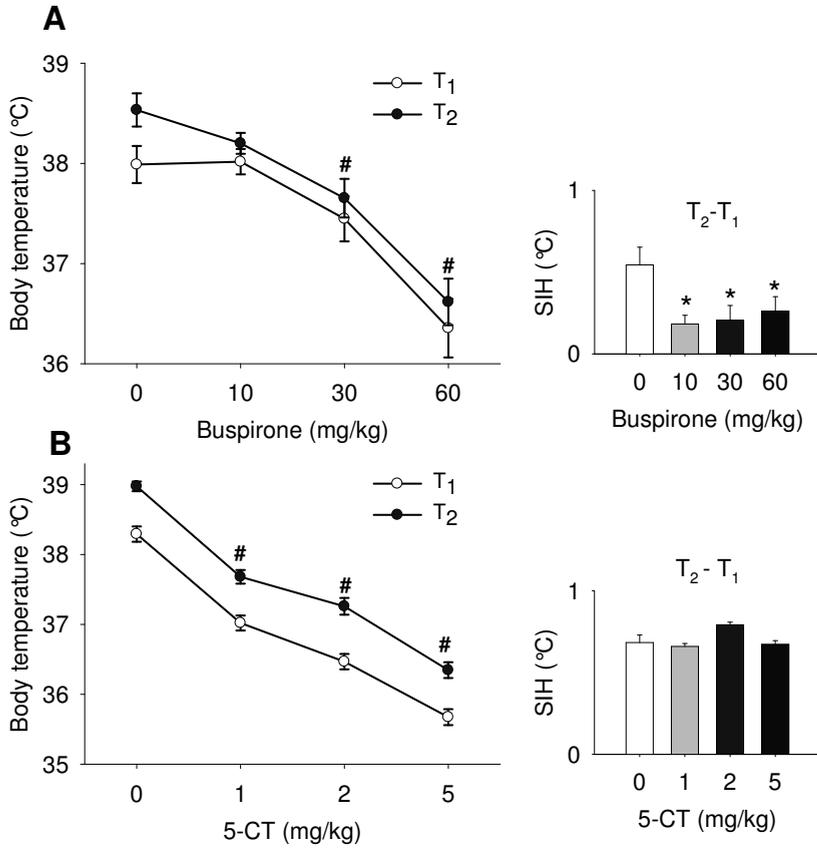
In line with a prominent role for the 5-HT<sub>1A</sub> receptor in anxiety processes, the studies on serotonergic drugs in the SIH paradigm has focused on 5-HT<sub>1A</sub> receptor agonists. The 5-HT<sub>1A</sub> receptor agonist flesinoxan has received ample attention as an anxiolytic attenuating the SIH response (Bouwknicht et al 2000; Bouwknicht et al 2004a; Groenink et al 1996b; Olivier et al 2003; Zethof et al 1995). In rodents, flesinoxan generally reduces the SIH response at lower doses, although hypothermia at higher doses makes the anxiolytic effects less readily interpretable. In figure 2, a typical example is shown of the SIH-reducing and hypothermic effects of flesinoxan in rats. Also, other 5-HT<sub>1A</sub> receptor agonists like 8-OH-DPAT (Lecci et al 1990a) and flibanserine (Borsini et al 1999), have also been shown to attenuate the SIH response. Using the classical group-housed SIH test, ipsapirone did not affect the SIH response (Zethof et al 1995), although it was effective in the singly-housed paradigm though only at high doses (40 and 60 mg/kg) that were not tested in the group-housed mice (Olivier et al 2003). Also, buspirone, registered as an anxiolytic in humans, dose-dependently decreases the SIH response in mice (Figure 3A), confirming earlier SIH studies (Lecci et al 1990a; Lecci et al 1990b; Spooen et al 2002; Zethof et al 1995). This effect is absent in 5-HT<sub>1A</sub> receptor KO mice (Van Bogaert et al 2006a).

**Table 1:** Effects of 5-HT<sub>1A</sub> receptor ligands on basal body temperature (T<sub>1</sub>, hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Drug	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref
Ipsapirone	Mouse (NMRI)	10-20	PO	N	N	G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	10-60	PO	N	Y		(Olivier et al 2003)
Flesinoxan	Mouse (NMRI)	0.3-3	PO	N	Y	G-SIH, 60 min	(Zethof et al 1995)
	Mouse (129Sv/Ola)	0.3-3	SC	Y	Y		(Bouwknicht et al 2000)
	Mouse (129Sv/EvTac and C57Bl/6J)	0.3-3	SC	Y	Y		(Bouwknicht et al 2004b)
	Mouse (NMRI)	0.1-10	PO	Y	Y		(Olivier et al 2003)
	Mouse (129Sv)	0.3-3	SC	Y	Y		(Pattij et al 2002a)
	Mouse (129Sv)	0.3-3	SC	Y	Y		(Pattij et al 2001)
	Mouse (129Sv, C57Bl/6J, Swiss)	0.3-3	IP	Y	Y	Three mouse strains compared	(Vinkers et al 2008)
Buspirone	Mouse (Swiss)	10	IP	N	Y	G-SIH, 45 min	(Lecci et al 1990a)
	Mouse (NMRI)	10-20	PO	N	Y	G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	10-60	PO	N	Y		(Olivier et al 2003)
	Mouse (129Sv, C57Bl/6J, Swiss)	1-4	IP	N	Y	Three strains compared	(Van Bogaert et al 2006a)
	Mouse (CD-1)	5-10	IP	Y	Y	G-SIH, 30 min	(Borsini et al 1999)
8-OH-DPAT	Mouse (Swiss)	2.5-10	SC	Y	Y	G-SIH, 30 min	(Lecci et al 1990a)
	Mouse (NMRI)	1-10	SC	Y	Y		(Olivier et al 2003)
Flibanserin	Mouse (CD-1)	8-16	IP	N	Y	5-HT <sub>1A</sub> R agonist, G-SIH, 30 min	(Borsini et al 1999)
WAY100635	Mouse (NMRI)	0.001-10	SC	N	N	Hyperthermia at higher doses	(Olivier et al 2003)
WAY100635 + flesinoxan	Mouse (129Sv)	1.0 + 0.3-3	SC	N	N	WAY blocks flesinoxan effects on SIH	(Pattij et al 2001)
S-UH301	Mouse (NMRI)	1-30	SC	N	N	5-HT <sub>1A</sub> R antagonist	(Olivier et al 2003)
DU125530	Mouse (NMRI)	3-30	PO	N	N	5-HT <sub>1A</sub> R antagonist	(Olivier et al 2003)

The hypothermic effects of 5-HT<sub>1A</sub> receptor agonists are in line with several preclinical and clinical studies (Bouwknicht et al 2000; Cryan et al 1999; Pitchot et al 2002; Pitchot et al 2004). This effect is probably induced through presynaptic 5-HT<sub>1A</sub> autoreceptors (Cowen 2000). The hypothermic effects of 5-HT<sub>1A</sub> receptor agonists are probably mediated via the medullary rostral raphe pallidus, leading to cutaneous vasodilatation and decreased brown adipose tissue thermogenesis (Dimicco and Zaretsky 2007; Ootsuka and Blessing 2006a), and common descending thermoregulatory pathways via the rostral raphe pallidus may explain the fact that 5-HT<sub>1A</sub> receptor agonists reduce lipopolysaccharide-induced fever (Blessing 2004; Nalivaiko et al 2005). Chronic treatment with SSRIs attenuates 5-HT<sub>1A</sub> agonist-induced hypothermia in healthy subjects (Lerer et al 1999; Sargent et al 1997) as well as in patients diagnosed with anxiety disorders and depression (Broocks et al 2003; Lesch et al 1991; Navines et al 2007), suggesting that desensitization of the somatodendritic 5-HT<sub>1A</sub> receptor is involved in SSRI effects. A more selective 5-HT<sub>1A</sub> receptor agonist that preferentially acts on postsynaptic receptors would be of value as it may aid in distinguishing putative postsynaptic anxiolytic effects from presynaptic hypothermic processes (Maurel et al 2007). 5-HT<sub>1A</sub> receptor knockout mice (1AKO) display an increased SIH response compared to wildtype mice after novel cage stress using telemetry but not after the manual rectal temperature measurement methods (Pattij et al 2002a; Pattij et al 2001). This difference could be attributable to a differential stress responsivity in which a rectal temperature measurement - in contrast to novel cage stress - would not yield a different SIH response.

Altogether, 5-HT<sub>1A</sub> receptor ligands reduce the SIH response and the SIH paradigm is thus sensitive to detect the anxiolytic effects of 5-HT<sub>1A</sub> receptor agonists. In support, the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 is able to block the SIH-attenuating effects of 5-HT<sub>1A</sub> receptor agonists, while WAY-100635 (or other 5-HT<sub>1A</sub> receptor antagonists such as S-5-fluoro-8-hydroxy-2-(dipropylamino)tetralin (S-UH-301) and 2-[4-[4-(7-Chloro-2,3-dihydro-1,4-benzodioxyn-5-yl)-1-piperazinyl]butyl]-1,2-benzisothiazol-3-(2H)-one-1,1-dioxide (DU125530)) have no intrinsic effects on the SIH response or basal body temperature (Olivier et al 2003).



**Figure 3:** Effects of 5-HT<sub>1A</sub> receptor agonist buspirone (0-60 mg/kg, IP) and 5-HT<sub>7</sub> receptor agonist 5-CT (0-5 mg/kg) on basal body temperature and the SIH response in *129Sv* mice (n=10-12). \*: significant reduction of the SIH response (p<0.05); #: significant effect on basal body temperature (p<0.05). Error bars represent the S.E.M.. Unpublished data.

**Table 2:** Effects of other 5-HT receptor ligands on basal body temperature ( $T_b$ , hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

Drug	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref
TFMPP	Mouse (Swiss)	5-20	IP	N	N	5-HT <sub>1A/B</sub> agonist, G-SIH, 45 min	(Lecci et al 1990a)
	Mouse (NMRI)	3-30	PO	Y	Y	5-HT <sub>1A/B</sub> agonist	(Olivier et al 2003)
RU24969	Mouse (NMRI)	3-30	PO	Y	Y	5-HT <sub>1A/B</sub> agonist	(Olivier et al 2003)
Aniprotilin	Mouse (NMRI)	1-10	PO	Y	N	5-HT <sub>1B</sub> agonist	(Olivier et al 2003)
DOI	Mouse (NMRI)	0.3-3	PO	Hyperthermia	Y, hyperthermia	5-HT <sub>2A/C</sub> agonist, G-SIH	(Zethof et al 1995)
	Mouse (NMRI)	0.3-10	PO	N	N	5-HT <sub>2A/C</sub> agonist	(Olivier et al 2003)
mCPP	Mouse (Swiss)	2.5-5	IP	N	N	5-HT <sub>2c</sub> agonist, G-SIH, 45 min	(Lecci et al 1990a)
	Mouse (NMRI)	1-10	PO	N	N	5-HT <sub>2c</sub> agonist, G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	3-30	PO	N	N	5-HT <sub>2c</sub> agonist	(Olivier et al 2003)
	Mouse (129Sv, C57Bl/6J, Swiss)	1-10	IP	Y/N	N	5-HT <sub>2c</sub> agonist	This review
Ketanserin	Mouse (Swiss)	0.1-0.2	IP	N	N	5-HT <sub>2A/2C</sub> antagonist, G-SIH, 60 min	(Lecci et al 1990a)
	Mouse (NMRI)	1-10	PO	N	N	5-HT <sub>2A/2C</sub> antagonist, G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	1-10	PO	Y	N	5-HT <sub>2A/2C</sub> antagonist	(Olivier et al 2003)
Ritanserin	Mouse (Swiss)	0.1-0.2	IP	N	N	5-HT <sub>2A/2C</sub> antagonist, G-SIH, 60 min	(Lecci et al 1990a)
	Mouse (NMRI)	1-30	PO	Y	Y	5-HT <sub>2A/2C</sub> antagonist	(Olivier et al 2003)

Table 2 (continued).

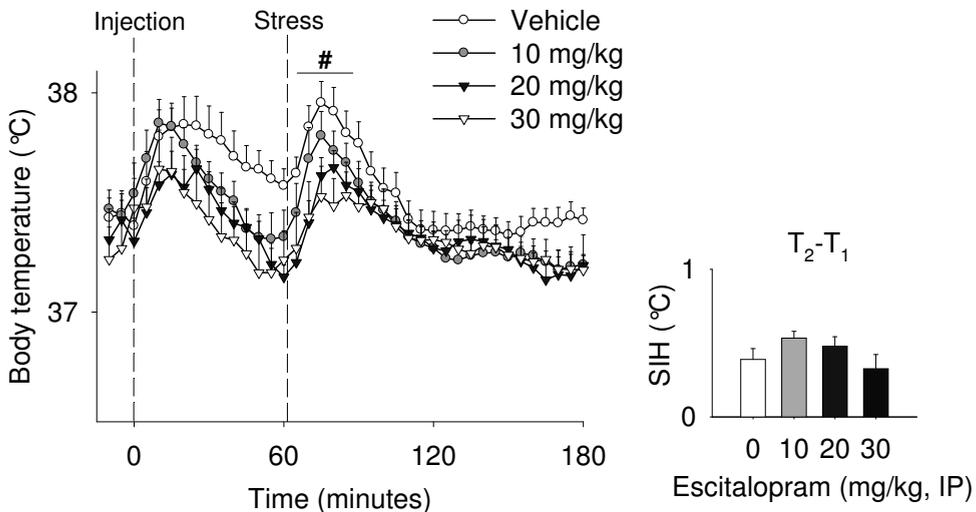
Drug	Species	Dose (mg/kg)	Route	Hypot hermia	SIH ↓	Remarks	Ref
DAU6215	Mouse (CD-1)	1-100 µg/kg	IP	N	N	5-HT <sub>3</sub> antagonist, G-SIH, 45 min	(Borsini et al 1993)
Ondansetron	Mouse (Swiss)	0.1-100 µg/kg	IP	N	N	5-HT <sub>3</sub> R antagonist, G-SIH	(Lecci et al 1990a)
	Mouse (NMRI)	0.1-100 µg/kg	IP	N	N	5-HT <sub>3</sub> R antagonist, G-SIH	(Zethof et al 1995)
	Mouse (CD-1)	1-100 µg/kg	IP	N	N	5-HT <sub>3</sub> R antagonist, G-SIH 45 min	(Borsini et al 1993)
	Mouse (NMRI)	0.001-1	IP	N	N	5-HT <sub>3</sub> R antagonist	(Olivier et al 2003)
MCPB	Mouse (NMRI)	1-10	PO	N	N	5-HT <sub>3</sub> R agonist	(Olivier et al 2003)
Etoprozine	Mouse (NMRI)	1-10 mg/kg	IP	Y/N (U shaped effect)	N	5-HT <sub>1A/B</sub> R agonist, G-SIH	(Zethof et al 1995)
LY53857	Mouse (Swiss)	1.5-3	IP	N	N	5-HT <sub>2</sub> R antagonist, G-SIH	(Lecci et al 1990a)
5-CT	Mouse (129Sv, C57Bl/6J, Swiss)	0.5-2	PO	Y	N	5-HT <sub>7</sub> R agonist	This review
	Mouse (129Sv, C57Bl/6J, Swiss)	0.5-2	IP	Y	N		(Van Bogaert et al 2006a)
	Mouse (C57Bl/6J)	1-5	IP	Y	N		(Vinkers et al 2008)

#### 4.2 Effects of other 5-HT receptor drugs on the SIH response (Table 2)

In contrast to the convincing SIH-reducing effects of 5-HT<sub>1A</sub> receptor agonists, other serotonergic drugs generally do not influence the SIH response (Bouwknicht et al 2007). Both the 5-HT<sub>1B</sub> receptor agonist eltoprozine (Zethof et al 1995) as well as TFMPP (Lecci et al 1990a) have no effect on the SIH response, and 5HT<sub>1B</sub> KO mice do not display altered SIH responses although an increased SIH response to novel cage stress as well as an increased basal body temperature have been reported (Groenink et al 2003b). Moreover, 5-HT<sub>2A/C</sub> receptor agonists and antagonists do not alter the SIH response. For example, no effects were found after administration of the 5-HT<sub>2A</sub> receptor agonist DOI (Zethof et al 1995), the 5-HT<sub>2C</sub> receptor agonist mCPP or the 5-HT<sub>2A/C</sub> receptor antagonists ketanserin or ritanserin (Lecci et al 1990a; Zethof et al 1995). Although they do not affect the SIH response, 5-HT<sub>2</sub> receptor agonists increase and 5-HT<sub>2</sub> receptor antagonists decrease basal body temperature levels (Nisijima et al 2001; Yamada et al 2001; Zethof et al 1995).

Interestingly, blockade of the 5-HT<sub>2</sub> receptor has been implicated in hypothermia during antipsychotic use (van Marum et al 2007). Similar to 5-HT<sub>2A/C</sub> receptor ligands, 5-HT<sub>3</sub> receptor antagonists are ineffective in reducing the SIH response. Both DAU6215 (Borsini et al 1993) and ondansetron (Olivier et al 2003; Zethof et al 1995) did not alter the SIH response.

The 5-HT<sub>7</sub> receptor agonist 5-carboxytryptamine (5-CT) induces hypothermia without affecting the SIH response (Figure 3B), an effect that has also been reported in guinea-pigs (Hagan et al 2000) and mice (Guscott et al 2003). 5-HT<sub>7</sub> receptor antagonists have been suggested to exert anxiolytic effects (Wesolowska et al 2006), although 5-HT<sub>7</sub> receptor agonist and antagonists do not alter the SIH response (Van Bogaert et al 2006a; Vinkers et al 2008).



**Figure 4:** Acute administration of the SSRI escitalopram (0-30 mg/kg, IP) does not affect the SIH response in Wistar rats (n=11), but it lowers basal body temperature. #: overall drug effect on basal body temperature ( $p < 0.05$ ). Error bars represent the S.E.M. Unpublished data.

### 4.3 Effects of acute and chronic serotonergic treatment on the SIH response (Table 3 and 4)

Chronic treatment with tricyclic antidepressants or SSRIs remains the mainstay in the management of major depressive disorder and anxiety disorders. SSRIs selectively bind to the SERT and inhibit 5-HT reuptake which results in an increase of extracellular 5-HT. The majority of TCAs act primarily as serotonin-norepinephrine reuptake inhibitors (SNRIs) by blocking the SERT and the norepinephrine transporter (NET), resulting in an increase of the extracellular concentrations of these neurotransmitters, and thereby enhances their neurotransmission (Gillman 2007; Tatsumi et al 1997). An acute single bolus of a SSRI or TCA does not alter the SIH response as has been reported for fluvoxamine, escitalopram, desipramine, imipramine, fluoxetine, and amitriptyline (Lecci et al 1990b; Olivier et al 2003; Zethof et al 1995). In general, some serotonergic antidepressants induce hypothermia without affecting the SIH response. Figure 4 shows the typical hypothermic (with no effect on the SIH response) of acute escitalopram administration in Wistar rats (unpublished data). Besides escitalopram, fluoxetine, desipramine and amitriptyline have been shown to cause hypothermia without altering the SIH response (Table 3). In contrast, hypothermic effects of acute SSRI, TCA or serotonin modulating drugs may also be absent, for example with fluvoxamine, clorgyline, clomipramine imipramine and tianeptine. The apparent differences are unclear and do not seem attributable to the pharmacological profile of the compounds. Alternatively, the dose and/or strain may influence the hypothermic effects of antidepressants.

In contrast to acute data, there is a paucity of data on the effects of chronic antidepressant treatment on the SIH response. Because SSRIs are effective in the treatment of anxiety disorders, chronic SSRI treatment would be expected to alter the SIH response. However, the evidence so far has been inconclusive, and the majority of the SIH studies using chronic administration do find no effect on the SIH response. In one study, chronic but not acute fluoxetine treatment reduced the SIH response in rats and mice (Conley and Hutson 2007). However, conflicting data exist whether chronic antidepressant treatment is able to reduce the SIH response, since Roche and colleagues did not find any attenuation in the SIH after chronic fluoxetine treatment in rats (Roche et al 2007). Also, no effects of chronic antidepressant treatment (fluoxetine, imipramine and amitriptyline) on the SIH response were found in mice (Table 4). Thus, the SIH model may be insensitive to detect the anxiolytic effects of chronic serotonergic antidepressants. The discrepancy between clinical drug efficacy versus inefficacy in the SIH model may at least be partially explained by the fact that the SIH response generally constitutes a normal and healthy stress response. The drugs that have been found to reduce the SIH response (e.g. benzodiazepines), acutely do so irrespective of the healthy or pathological status of an individual. This way, one may argue that chronic exposure to SSRIs would only alter the SIH response under pathological conditions. However, except the SERT knockout rat (Olivier et al 2008a), an altered SIH response is not a common finding in genetically modified animals with increased anxiety levels, such as 1AKO mice.

**Table 3:** Effects of acute administration of SSRIs, TCAs and MAOIs on basal body temperature ( $T_b$ , hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

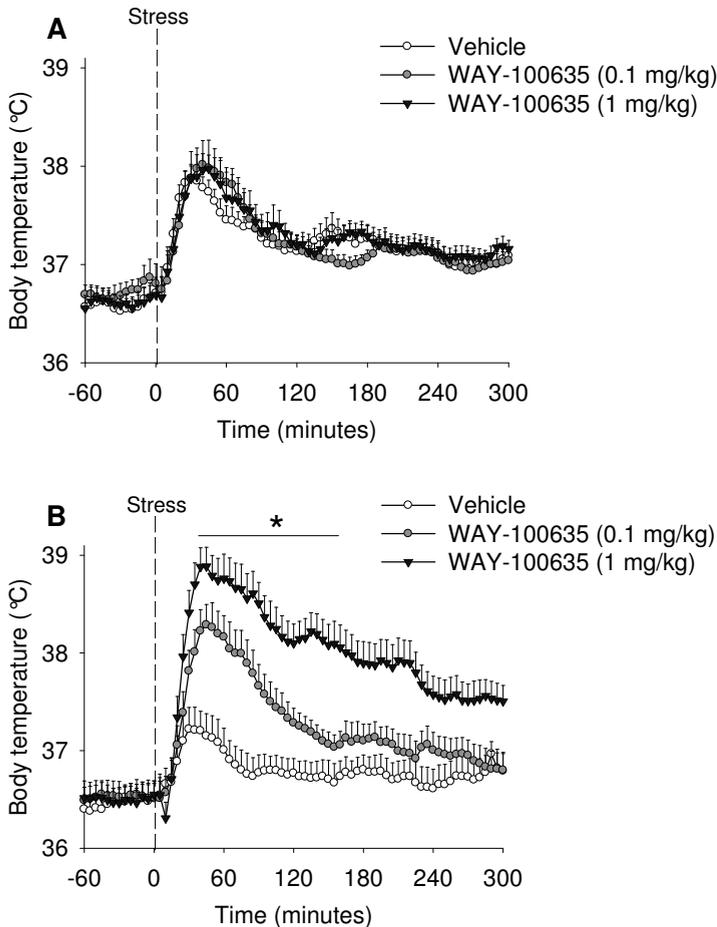
Drug	Species	Dose (mg/kg)	Route	Hypothermia	SIH ↓	Remarks	Ref
Fluvoxamine	Mouse (NMRI)	3-30	PO	N	Y, due to hyperthermia	G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	3-30	PO	N	N		(Olivier et al 2003)
Escitalopram	Mouse (C57Bl/6J)	2-10	IP	Y	N		(Vinkers et al 2008)
Fluoxetine	Mouse (Swiss)	10-20	IP	Y	N	G-SIH	(Lecci et al 1990b)
	Mouse (NMRI)	3-30	PO	N	N		(Olivier et al 2003)
Tranlycypromine	Mouse (Swiss)	5-10	IP	Y	N	MAO-I, G-SIH	(Lecci et al 1990b)
Clorgyline	Mouse (NMRI)	10-30	PO	N	N	MAO <sub>A</sub> -I	(Olivier et al 2003)
Desipramine	Mouse (Swiss)	15-30	SC	Y	N	G-SIH, 45 min	(Lecci et al 1990b)
	Mouse (NMRI)	3-30	PO	Y	N	G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	3-30	PO	N	N		(Olivier et al 2003)
Clomipramine	Mouse (NMRI)	3-30	PO	N	N	G-SIH, 60 min	(Zethof et al 1995)
	Mouse (NMRI)	3-30	PO	N	N		(Olivier et al 2003)
Amitriptyline	Mouse (Swiss)	10	IP	Y	N	G-SIH	(Lecci et al 1990b)
	Mouse (NMRI)	3-30	PO	Y	N	G-SIH	(Zethof et al 1995)
	Mouse (NMRI)	3-30	PO	Y	N		(Olivier et al 2003)
Imipramine	Mouse (Swiss)	15-30	IP	N	N	G-SIH	(Borsini et al 1989)
	Mouse (NMRI)	3-30	PO	N	N	G-SIH	(Zethof et al 1995)
	Mouse (NMRI)	10-30	PO	N	N		(Olivier et al 2003)
Tianeptine	Mouse (NMRI)	3-30	PO	N	Y, due to hyperthermia	G-SIH, serotonin enhancer	(Zethof et al 1995)
	Mouse (NMRI)	3-30	PO	N	Y, due to hyperthermia	serotonin enhancer	(Olivier et al 2003)

**Table 3 (continued)**

Drug	Species	Dose (mg/kg)	Route	Hypo thermia	SIH ↓	Remarks	Ref
PCPA	Mouse (Swiss)	75-150	IP	N	N	5-HT depleter Injected 72,48,24 before test, G-SIH	(Lecci et al 1990a)
5,7 DHT	Mouse (Swiss)	200 ug	ICV	N	N	9 days before test, G-SIH, reverses buspirone induced SIH reduction	(Lecci et al 1990a)
D,-fenfluramine	Mouse (NMRI)	3-30	PO	N	N	5-HT releaser, G-SIH	(Zethof et al 1995)
	Mouse (NMRI)	3-30	IP	N	Y	5-HT releaser	(Olivier et al 2003)

**Table 4:** Effects of chronic SSRI treatment on basal body temperature ( $T_1$ , hypothermia) and the stress-induced hyperthermia (SIH) response. G-SIH: group-house SIH model, including the injection-stressor interval (minutes). PO: oral, IP: intraperitoneal.

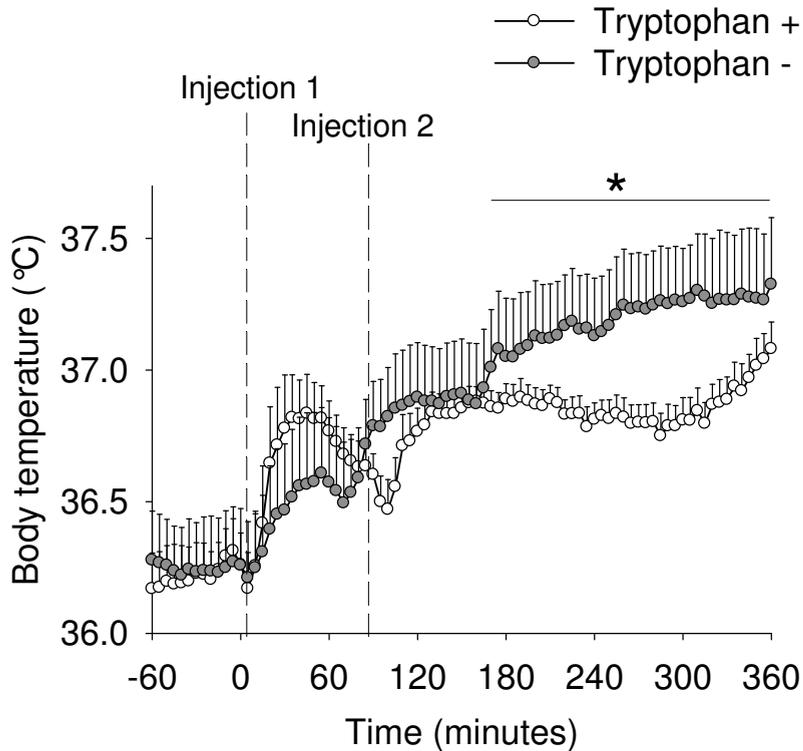
Drug	Species	Dose (mg/kg)	Route	Hypo thermia	SIH ↓	Remarks	Ref
Fluoxetine	Mouse (Swiss)	10	IP	N	N	7, 14 and 21 day period, G-SIH	(Lecci et al 1990b)
Imipramine	Mouse (Swiss)	10	IP	N	N	7, 14 and 21 day period, G-SIH	(Lecci et al 1990b)
Amitriptyline	Mouse (Swiss)	10	IP	N	N	7, 14 and 21 day period, G-SIH	(Lecci et al 1990b)
Fluoxetine	Rat (Sprague-dawley)	10	SC	N	N	35 days, SIH restoration in OBX rats	(Roche et al 2007)
Fluoxetine	Mouse (SW) and rat (CD-1)	15 and 10	PO	N	YW	21 days	(Conley and Hutson 2007)



**Figure 5:** Stress-induced hyperthermia response after administration of the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 (0.1 mg/kg, IP) in SERT<sup>+/+</sup> rats (A, n=8) and in SERT<sup>-/-</sup> rats (B, n=7-8). Values are mean  $\pm$  SEM. No drug effect was found. In SERT<sup>+/+</sup> rats, no differences in WAY-100635 response were found. In contrast, significant differences were found between WAY-100635 doses in SERT<sup>-/-</sup> rats compared to vehicle treatment ( $F_{(144,1728)} = 10.764$ ;  $p < 0.001$ ). Unpublished data.

SIH responsiveness has also been assessed in SERT knockout (SERT<sup>-/-</sup>) mice and rats (Li et al 1999; Olivier et al 2008a). The SERT<sup>-/-</sup> rat or mice has been developed as an animal model of depression, and, comparable to humans treated with SSRIs, these rats (Homberg et al 2008; Olivier et al 2008a) and mice (Bouali et al 2003; Gobbi et al 2001; Mannoury la Cour et al 2001) possess reduced 5-HT<sub>1A</sub> receptor reactivity. In SERT<sup>-/-</sup> mice, no differences in SIH responsiveness were found (Li et al 1999). In contrast, SERT<sup>-/-</sup> rats displayed a decreased SIH response after a saline injection, although novel cage stress elicited a similar SIH response in SERT<sup>-/-</sup> rats compared to wildtype animals (Olivier et al 2008a). Thus, differences in the SIH response in SERT<sup>-/-</sup> rats appear with decreased intense stressor intensity. In support, a differential SIH reactivity that depends on stressor intensity was

also found in olfactory bulbectomized animals which possess altered 5-HT functionality (Vinkers et al 2009b). Interestingly, the 5-HT<sub>1A</sub> receptor agonist flesinoxan did not result in the regular hypothermic effects in SERT<sup>-/-</sup> rats, whereas the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 increased the body temperature in SERT<sup>-/-</sup> rats compared to SERT<sup>+/+</sup> rats (Figure 5). So far, it is unclear whether this WAY-100635-induced hyperthermia in SERT<sup>-/-</sup> rats is due to an altered thermoregulation or altered anxiety-related circuitry.



**Figure 6:** The effect of acute tryptophan depletion on core body temperature in Wistar rats. Rats were treated with a protein-carbohydrate mixture containing TRP (TRP+ group, 0.30% TRP of the total protein) or lacking TRP (TRP- group). The rats received two TRP doses (PO, 10 ml/kg) with a 90-minute interval. Significant differences were found between TRP+ and TRP- treatment ( $F_{(84,1260)}=3.418$ ;  $p<0.001$ ). Unpublished data.

#### 4.4 Effects of serotonin releasers or depleters on the SIH response

Fenfluramine is a 5-HT releaser that disrupts vesicular 5-HT storage as well as binds to SERT, thereby increasing extracellular 5-HT levels. Fenfluramine was found to be ineffective in lowering the SIH response (Zethof et al 1995). Moreover, the selective serotonin reuptake enhancer tianeptine did not affect the SIH response, even though it lowered extracellular 5-HT levels (Zethof et al 1995). As tryptophan is the precursor of serotonin, diet depletion of l-tryptophan results in lower 5-HT levels in the CNS which has resulted in mood depression in at-risk individuals (Ellenbogen et al 1996; Young 1996). So far, acute tryptophan depletion has not been studied in the SIH paradigm, even though depletion resulted in core body temperature increases in rats (Figure 6).

#### 4.5 Effects of serotonergic-mediated alterations of other neurotransmitter systems

In addition to direct serotonergic effects on the SIH response, a number of studies addressed the hypothesis that serotonin may affect other neurotransmitter systems in altering the SIH response. 1AKO mice on a Swiss-Webster (SW) background possess reduced benzodiazepine sensitivity, and this serotonin-induced benzodiazepine insensitivity in mice on the SW but not the C57Bl6/J or 129Sv background was confirmed using the SIH paradigm (Groenink et al 2003a; Groenink et al 2003b; Pattij et al 2002b; Pattij et al 2001; Van Bogaert et al 2006a). 1AKO mice on a SW background were insensitive to the GABA<sub>A</sub> receptor agonists diazepam and L838,417, while SIH reduction was apparent in wildtype mice (Van Bogaert et al 2006a). Moreover, the inability of benzodiazepines to reduce the SIH response in 5-HT<sub>1A</sub> receptor KO mice was replicated in wildtype mice after the pharmacological 5-HT<sub>1A</sub> receptor blockade with WAY-100635 during the early postnatal period (Vinkers et al 2009a). This way, long-lasting benzodiazepine insensitivity was found in adolescent as well as adult mice using the SIH paradigm. WAY-100635-treated mice also showed increased cortical GABA<sub>A</sub>R  $\alpha_1$  and  $\alpha_3$  subunit levels and increased hippocampal GABA<sub>A</sub>R  $\alpha_2$  subunit levels. Thus, early-life disruption of the 5-HT system may affect benzodiazepine sensitivity in later life. Recently, the SIH-reducing effects of group II metabotropic glutamate (mGlu) 2/3 receptor agonists MGS0039 and LY341495 could be reversed after co-administration of the 5-HT<sub>1A</sub> receptor antagonist WAY-100635, suggesting that glutamatergic drugs act via the serotonin system to exert its anxiolytic effects (Iijima et al 2007). Moreover, the 5-HT<sub>1A</sub> receptor appeared to modulate the SIH-reducing effects of benzodiazepines via the GABA<sub>A</sub> receptor  $\alpha_3$  subunit (Vinkers et al., unpublished observations). Altogether, these data indicate that the SIH paradigm may be employed to study the acute and chronic interactions of different neurotransmitter systems on the autonomic stress response.

## 5. Conclusion

Preclinical studies indicate that disruption of the serotonergic system from the early-life to adult period may influence stress responsivity, and a normal functionality of the serotonergic system is essential to prevent affective disorders. The structurally, anatomically and functionally complex serotonin system is involved in stress-related behavior and vulnerability to develop psychiatric disorders. So far, some serotonergic ligands have been shown to differentially affect the autonomic SIH response. Activation of 5-HT<sub>1A</sub> receptors convincingly reduces the SIH response, confirming the anxiolytic potential of this receptor class. In contrast, modulation of other 5-HT receptor types including 5-HT<sub>1B</sub>, 5-HT<sub>2</sub> and 5-HT<sub>3</sub> receptors does generally not influence the SIH response. The effects of serotonergic drugs on basal body temperature can hamper the interpretation of SIH data as severe hypothermia can interfere with thermoregulatory homeostatic processes.

The 5-HT system is regulated via SERT modulation, and SERT dysfunction has been implicated in anxiety-related behavior. Acute and chronic administration of drugs affecting the SERT (which include TCAs and SSRIs) are generally ineffective in reducing the SIH response. The SIH paradigm is sensitive to acute anxiolytic effects of different drug classes that reduce the stress response irrespective of an individual's mental health. Hypothetically, chronic SSRIs treatment could affect the SIH response under pathological conditions. If so, it would be necessary to establish the SIH response in patients suffering from a stress-related disorder. If an increased, attenuated, or inappropriately activated SIH response is present in stress-related disorders, it would be interesting to follow the effects of chronic SSRI treatment on the autonomic SIH response.

In addition to direct serotonergic effects on the SIH response, there are indications that SIH-reducing effects of other neurotransmitter systems including the GABA<sub>A</sub> and glutamate receptor system may be at least partially mediated through the 5-HT system. Thus, the SIH paradigm may be employed to study the acute and chronic interactions of different neurotransmitter systems on the acute stress response, including putative serotonergic interactions.



# Chapter 15

## Part IV

### **Stress-induced hyperthermia in translational stress research**

Christiaan H. Vinkers

Renske Penning

Marieke M. Ebbens

Juliane Hellhammer

Joris C. Verster

Cor J. Kalkman

Berend Olivier

# 15

*The Open Pharmacology Journal, in press*

## 1. Introduction

The stress-induced hyperthermia (SIH) response is a relatively short-lasting rise in body temperature in response to stress. This temperature increase is part of the autonomic stress response which also results in tachycardia and increased blood pressure levels. So far, a SIH response has been found in a variety of species, including rodents, baboons, impalas and chimpanzees (for review see (Bouwknicht et al 2007). Non-human primate studies reported temperature changes after stress exposure with increased tympanic membrane temperature in chimpanzees (Parr and Hopkins 2000a) and decreased nasal temperature of rhesus monkeys (Nakayama et al 2005). Moreover, there are indications that stress exposure can lead to body temperature changes in human subjects (Briese 1995; Marazziti et al 1992). Consistent stress-induced temperature changes across species may possess translational value and can be applied to study stress and anxiety at a genetic and pharmacological level.

Various animal models have aided in establishing the biological basis of stress-related psychiatric disorders including anxiety disorders and depression (Cryan and Holmes 2005). However, in stress and anxiety research, there is a need for approaches with good translational potential. Any additional approach that may aid in finding an early proof-of-concept of efficacy before expensive clinical trials are initiated is valuable. Although the SIH response does not model any specific psychiatric condition, this response is useful as a read-out parameter of stress. It can be studied at the preclinical and clinical level applying different interventions and might therefore serve as an animal-to-human translational paradigm. Throughout this review, the SIH response including all pharmacological, genetic and local CNS applications in healthy and dysfunctional organisms will be referred to as “the SIH model”.

This review aims to assess the translational potential and the different aspects of the SIH response. The validity of an animal model is generally assessed by three sets of criteria: its predictive validity (does the animal respond to the treatment which is known to be effective in humans), its face validity (is the animal behavior similar to the human condition), and its construct validity (does the mechanism corresponds with the known disorder mechanism) (McKinney and Bunney 1969). In the current review, the SIH paradigm will be discussed into more detail using these criteria. As the human thermoregulatory system is complex, it will be discussed into more detail.

## 2. Human thermoregulation

Human body temperature is kept at a constant 37 °C, ranging from 35.8 and 38.2 °C by regulating the balance between heat production and heat loss. It can be challenging to determine the ‘normal’ body temperature as this depends on the location of the temperature sensor, time and measurement equipment (Mackowiak 1997). A meta-analysis of Sund and co-workers reviewed 27 temperature studies and found different ranges for oral (33.2-38.2 °C), rectal (34.4-37.8 °C), tympanic (35.4-37.8 °C) and axillary

(35.5-37.0 °C) temperatures (Sund-Levander et al 2002). There has been an ongoing debate which site of measurement most accurately reflects the true body temperature (Craig et al 2002). In rest, metabolically active organs such as the liver produce most heat, whereas during exercise, skeletal muscles account for heat production. Besides metabolic mechanisms, body temperature is regulated by environmental heat exchange. Different parts of the body have different temperatures, with the highest temperature in the brain and the organs in the thoracic and abdominal cavities (the central or 'core' temperature). In contrast, the body's shell, essentially the skin, usually has the lowest temperature (the peripheral temperature). The central temperature is precisely regulated by altering the peripheral temperature through heat exchange with blood. A high central temperature (e.g. after exercise) leads to increased capillary blood flow in the skin by opening arterial resistance vessels where it exchanges heat with the environment, leading to heat loss and a net increase of skin temperature.

Body temperature regulation is a complex and vital process which is governed by the central nervous system. The hypothalamic preoptic area (POA) is the main thermoregulatory integrating center that contains a heat-loss center with warm-sensitive neurons and a heat-promoting center with cold-sensitive neurons. The hypothalamus receives input from peripheral thermoreceptors located in the skin and central thermoreceptors sensitive to blood temperature (Boulant 2000; Marieb and Hoehn 2007). Warm-sensitive neurons inhibit cold-responsive neurons of the dorsomedial nucleus of the hypothalamus (DMH). After exposure to cold, activity of warm-sensitive neurons decreases and subsequently leads to heat conservation and/or heat production. DMH activation results in both vasoconstriction and shivering via neurons that project directly to the rostral raphe pallidus (for reviews: (DiMicco et al 2006; Dimicco and Zaretsky 2007)). The rostral raphe pallidus directly controls sympathetic preganglionic neurons in the intermediolateral cell column of the thoracic spinal cord (Nakamura et al 2004; Nakamura et al 2005a). In addition to autonomic processes, behavioral strategies (putting on warmer clothing, consumption of hot fluids) aids in keeping an optimal body temperature (Benarroch 2005; Gale 1973; Marieb and Hoehn 2007).

Stress-induced changes in body temperature are probably mediated through hypothalamic pathways including the DMH and the RPa. However, as the SIH response is an acute stress response, it is probably initiated by limbic brain areas, including various amygdala nuclei (Carrasco and Van de Kar 2003). In a recent study, we showed that the classical benzodiazepine diazepam but not the prostaglandin-blocking aspirin attenuated the SIH response in rodents, whereas aspirin but not diazepam greatly reduced LPS- and IL-1 $\beta$  induced fever states, suggesting that stress-induced hyperthermia and infection-induced fever are two distinct processes (Vinkers et al 2009d).

### **3. The SIH response in human subjects**

A variety of studies investigated the effects of stress on body temperature. Generally, these studies differ in their setup, stress-induction method as well as site of temperature measurements. In two early studies, the axillary temperature of 1068 and 1374 students

was measured before their exams (Gotsev and Ivanov 1950; Gotsev and Ivanov 1954). Compared to a 'normal' body temperature of 37 degrees, the stress-induced body temperature significantly increased. Marazzitti and co-workers conducted an investigation in which the axillary temperature was increased in 22 residents before an important exam as compared to an unstressed situation some weeks later (Marazziti et al 1992). In support, the oral temperature of 108 students was increased before a difficult exam compared to a non-stressful situation (Briese 1995). Interestingly, grade point averages increased with larger temperature increases. Male participants who watched or participated in a boxing contest displayed elevated oral temperatures compared to oral temperature levels on a regular school day (Renbourn 1960). In contrast to these studies, peripheral finger temperatures generally decrease in response to stress. In one study, finger temperature decreased up to 13.5 °C (!) in 45 out of 47 subjects in affective states, whereas only a slight increase of forehead and cheek temperature was observed in a minority of the subjects (Mittelman and Wolff 1939). In support, listening to 'positive' music elevated skin temperature of the middle finger whereas 'negative' music decreased it (McFarland 1985). Surprisingly, only when listening to music with the left ear induced these skin finger temperature responses (McFarland and Kennison 1989). We recently found that in healthy human subjects, upper arm skin temperature increased whereas intestinal core temperature decreased in male subjects during exposure to the Trier Social Stress Test (chapter 14). Interestingly, decreased core temperature correlated significantly with the subjective stress increase. However, the fact that core body temperature decreased indicates that human thermoregulation may be more complex, and that the site of temperature measurement may affect the direction and amplitude of stress-induced body temperature changes.

Taken together, the existing human studies report that body temperature either rises or drops in response to stress depending on the stress-induction method, the body location and the temperature measuring apparatus. A tentative conclusion may be that axillary and oral temperature rise in response to stress, whereas distal skin temperature decreases in stressful situations as a result of vasoconstriction. Thus, different thermoregulatory reactions in response to stress may exist depending on the site of measurement. It can be hypothesized that the skin temperature in the distal parts of the limbs (e.g. fingers) reacts differently to stress compared to the more proximal skin temperature (e.g. axilla) or even the core body temperature.

## 4. Face validity of the SIH paradigm

### ***Stress as a translational approach in psychiatric research***

Stress is an intuitively translational concept as any emotional or physical trigger will result in a physiological stress response in almost any organism (Herman and Cullinan 1997). In support, both preclinical and clinical studies suggest a pivotal role for the amygdala in stress (Davis 1997; Dayas et al 1999; Myint et al 2007; Ulrich-Lai and Herman 2009). Adaptive stress strategies exist in humans and animals that are beneficial for survival (Korte et al 2005). Moreover, the concept that the adaptive nature of stress (allostatic load) is not infinite and can lead to psychopathology when excessive or chronic stress is

experienced possesses translational potential (Joels and Baram 2009). So far, a dysfunctional stress system has been suggested in different psychiatric disorders, among which anxiety disorders, depression, burn-out, schizophrenia and post-traumatic stress disorder (PTSD) (de Kloet et al 2005; Roozendaal et al 2009).

### ***The autonomic stress response in translational research***

Emotional and psychological stress have been shown to consistently activate the autonomic nervous system. This autonomic stress response is mediated by an increased activity of the sympatho-adrenomedullary system, resulting in increased heart rate and blood pressure, cutaneous vasoconstriction in the periphery of the limbs or selective dilatation elsewhere, redistribution of organ blood flow and increased cardiac output and an increase in (non-)shivering thermogenesis (Carrasco and Van de Kar 2003; de Kloet et al 2005; Ulrich-Lai and Herman 2009). These responses are present across species (including rodents and humans), making a direct comparison between humans and animals possible (DiMicco et al 2006; Franzini et al 1981; Horiuchi et al 2006; Kuwaki et al 2008; Ulrich-Lai and Herman 2009). Altogether, the stress-induced autonomic activation seems to possess translational value as the read-out parameters (heart rate, blood pressure and body temperature) largely overlap in a stressful situation.

## **5. Predictive validity of the SIH paradigm**

A SIH response is present in all mammals that have been tested to date, including humans, chimpanzees, baboons, silver foxes, pigs, impalas, turtles, ducks, ground squirrels, rabbits, rats and mice (Bouwknicht et al 2007; Cabanac and Bernieri 2000; Gray et al 2008; Meyer et al 2008; Ritter et al 2009). In rodents, body temperature is usually determined by manual rectal temperature measurements or with telemetric equipment in the abdominal cavity. So far, the SIH has been particularly useful as a screening approach to evaluate the effects of novel anxiolytic drug candidates (Vinkers et al 2009f). Drug classes with clinically effective anxiolytic properties such as GABA<sub>A</sub> and 5-HT<sub>1A</sub> receptor agonists as well as CRF<sub>1</sub> receptor antagonists were all proven to attenuate the SIH response (Bouwknicht et al 2007; Griebel et al 2002; Olivier et al 2002; Olivier et al 2003; Spooren et al 2002). In contrast, non-anxiolytic dopaminergic or noradrenergic compounds generally do not alter the SIH response (Bouwknicht et al 2007). Therefore, the SIH model possesses good predictive validity in a preclinical setting. So far, no study has been carried out to assess whether a standard anxiolytic drug (e.g. a classical benzodiazepine) may alter a human SIH response. If clinically effective anxiolytic drugs reduce the SIH response in healthy subjects, this would yield an additional indicator of a drug's acute anxiolytic effects.

## **6. Construct validity of the SIH paradigm**

The SIH response in itself does not model any disease but rather functions as a quantitative read-out of stress in any situation. Therefore, the SIH paradigm does not possess any direct construct validity. However, the SIH response may possess construct validity in the

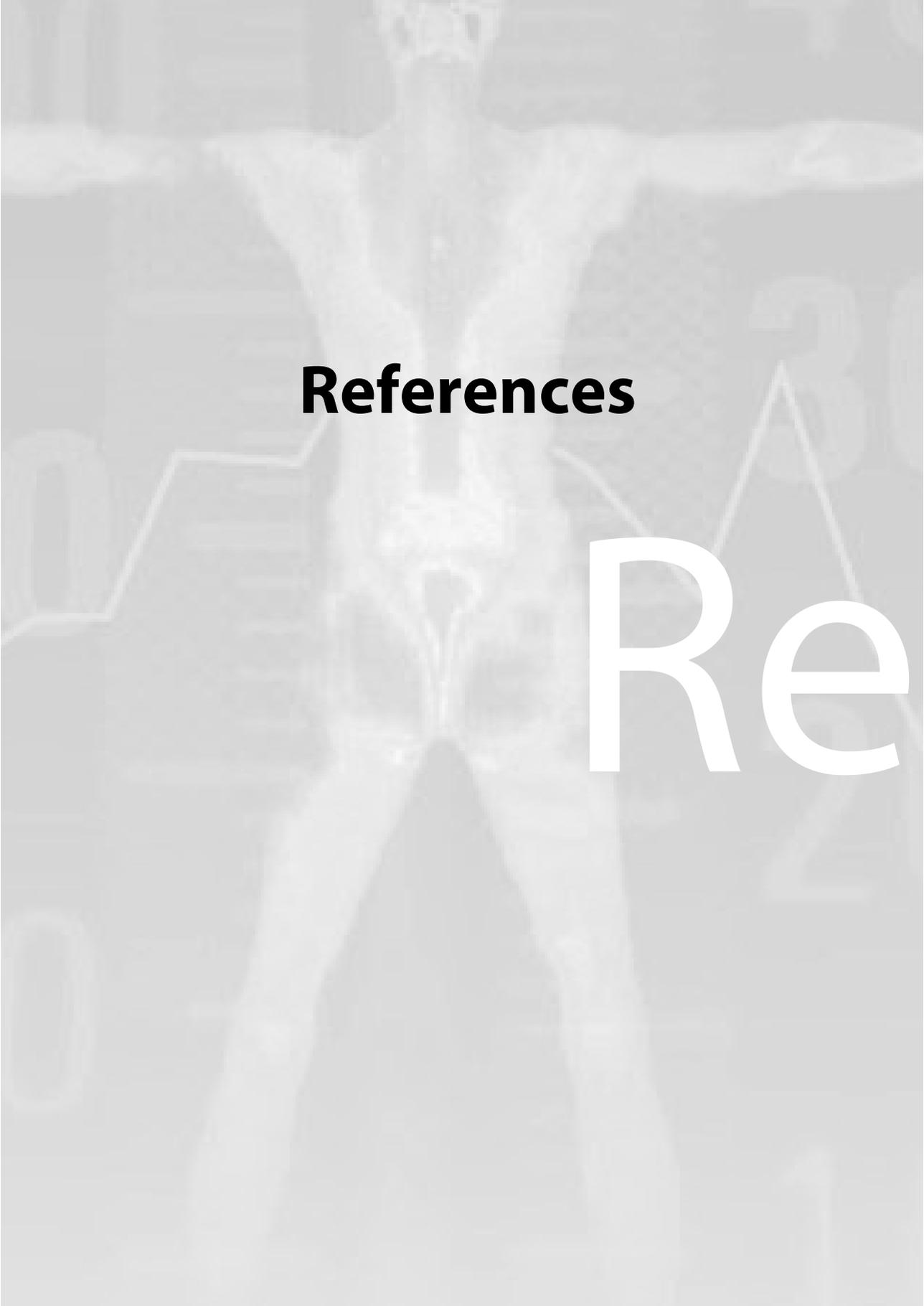
sense that it can become dysfunctional in certain circumstances (including any stress-related psychiatric disorder), and such dysfunctional temperature responses can be present in humans and animals. So far, autonomic symptoms of stress-related psychiatric disorders have been found in animals and humans (Brown and McNiff 2009). For example, a reduced autonomic flexibility (decreased heart rate variability and vagal tone) has been found in panic disorder and general anxiety disorder (Hoehn-Saric et al 2004; Stockmeier et al 2009).

## **7. The SIH response and temperature in pathophysiology**

The role of body temperature in pathophysiology has received less attention. , there is evidence for an altered thermoregulation or SIH response in various stress-related disorders. A series of articles by Shiloh et al. have suggested a dysfunctional thermoregulation in schizophrenia patients that corresponds to symptom severity (Shiloh et al 2008; Shiloh et al 2005; Shiloh et al 2007; Shiloh et al 2009a; Shiloh et al 2009b). Moreover, circadian body temperature abnormalities have been observed in depressive disorders (Daimon et al 1992), and there is evidence for a relationship between insomnia and body temperatures (Lack et al 2008). Interestingly, removal of the olfactory bulbs, an animal model of depression, results in rapid, stable and persistent changes in basal and stress-induced body temperature levels (Roche et al 2007; Vinkers et al 2009b). Also, perimenopausal women may experience hot flushes that are characterized by an acute rise in skin temperature which can be regarded as a form of SIH. In support, hot flushes are associated with anxiety and psychological factors (Blumel et al 2004; Freeman et al 2005). There is evidence that a hot flush is caused by a narrowed band around the setpoint mediated by the hypothalamus (Rapkin 2007; Sturdee et al 1978), or, alternatively, by increasing levels of norepinephrine and decreasing levels of serotonin due to declining estrogen levels after menopause (Carpenter et al 2004; Freedman 2002; Freedman 2005).

## **8. Conclusion**

A growing body of literature provides support to the existence of a human SIH response. The fact that a SIH response is consistently present across species in response to stress and that dysfunctional stress responses and/or thermoregulation are present in stress-related psychiatric disorders indicates that the SIH paradigm may prove to have translational value. However, studies investigating the human SIH response are scarce, and more research is needed to characterize the basal and stress-induced body temperature levels in humans. Nevertheless, current evidence suggests that the SIH paradigm may function well as a translational approach to study different psychiatric disorders. In animals, anxiolytic drugs acutely reduce the SIH response. So far, no human studies have been carried out to examine anxiolytic drug effects on the SIH response in human subjects. If future studies prove that a SIH response in humans can be reduced by anxiolytic drugs, this would strengthen the role of the SIH paradigm in translational stress research.



# References

Re

## A

- Addolorato G, Caputo F, Capristo E, Domenicali M, Bernardi M, Janiri L, et al (2002): Baclofen efficacy in reducing alcohol craving and intake: a preliminary double-blind randomized controlled study. *Alcohol Alcohol* 37:504-508.
- Addolorato G, Leggio L, Abenavoli L, Agabio R, Caputo F, Capristo E, et al (2006): Baclofen in the treatment of alcohol withdrawal syndrome: a comparative study vs diazepam. *Am J Med* 119:276 e213-278.
- Ader R (2003): Conditioned immunomodulation: research needs and directions. *Brain Behav Immun* 17 Suppl 1:S51-57.
- Adriaan Bouwknecht J, Olivier B, Paylor RE (2007): The stress-induced hyperthermia paradigm as a physiological animal model for anxiety: a review of pharmacological and genetic studies in the mouse. *Neurosci Biobehav Rev* 31:41-59.
- Akimova E, Lanzenberger R, Kasper S (2009): The Serotonin-1A Receptor in Anxiety Disorders. *Biol Psychiatry*.
- Akk G, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH (2008): Mutations of the GABA-A receptor alpha1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. *Mol Pharmacol* 74:614-627.
- Akk G, Li P, Bracamontes J, Steinbach JH (2009): Activation and modulation of concatemeric GABA-A receptors expressed in human embryonic kidney cells. *Mol Pharmacol*.
- Alexandre C, Popa D, Fabre V, Bouali S, Venault P, Lesch KP, et al (2006): Early life blockade of 5-hydroxytryptamine 1A receptors normalizes sleep and depression-like behavior in adult knock-out mice lacking the serotonin transporter. *J Neurosci* 26:5554-5564.
- Ali NJ, Olsen RW (2001): Chronic benzodiazepine treatment of cells expressing recombinant GABA(A) receptors uncouples allosteric binding: studies on possible mechanisms. *J Neurochem* 79:1100-1108.
- Allison C, Pratt JA (2006): Differential effects of two chronic diazepam treatment regimes on withdrawal anxiety and AMPA receptor characteristics. *Neuropsychopharmacology* 31:602-619.
- Amin J, Weiss DS (1993): GABAA receptor needs two homologous domains of the beta-subunit for activation by GABA but not by pentobarbital. *Nature* 366:565-569.
- Anderson AK, Phelps EA (2001): Lesions of the human amygdala impair enhanced perception of emotionally salient events. *Nature* 411:305-309.
- Andrews N, File SE (1993): Handling history of rats modifies behavioral effects of drugs in the elevated plus-maze test of anxiety. *Eur J Pharmacol* 235:109-112.
- Ansoorge MS, Zhou M, Lira A, Hen R, Gingrich JA (2004): Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* 306:879-881.
- Arango V, Underwood MD, Gubbi AV, Mann JJ (1995): Localized alterations in pre- and postsynaptic serotonin binding sites in the ventrolateral prefrontal cortex of suicide victims. *Brain Res* 688:121-133.
- Arborelius L, Hawks BW, Owens MJ, Plotsky PM, Nemeroff CB (2004): Increased responsiveness of presumed 5-HT cells to citalopram in adult rats subjected to prolonged maternal separation relative to brief separation. *Psychopharmacology (Berl)* 176:248-255.
- Arnot MI, Davies M, Martin IL, Bateson AN (2001): GABA(A) receptor gene expression in rat cortex: differential effects of two chronic diazepam treatment regimes. *J Neurosci Res* 64:617-625.
- Arranz B, Eriksson A, Mellerup E, Plenge P, Marcusson J (1994): Brain 5-HT1A, 5-HT1D, and 5-HT2 receptors in suicide victims. *Biol Psychiatry* 35:457-463.
- Artaiz I, Romero G, Zazpe A, Monge A, Caldero JM, Roca J, et al (1995): The pharmacology of VA21B7: an atypical 5-HT<sub>3</sub> receptor antagonist with anxiolytic-like properties in animal models. *Psychopharmacology (Berl)* 117:137-148.

- Artaiz I, Zazpe A, Del Rio J (1998): Characterization of serotonergic mechanisms involved in the behavioral inhibition induced by 5-hydroxytryptophan in a modified light-dark test in mice. *Behav Pharmacol* 9:103-112.
- Asberg M, Traskman L, Thoren P (1976): 5-HIAA in the cerebrospinal fluid. A biochemical suicide predictor? *Arch Gen Psychiatry* 33:1193-1197.
- Atack JR (2005): The benzodiazepine binding site of GABA<sub>(A)</sub> receptors as a target for the development of novel anxiolytics. *Expert Opin Investig Drugs* 14:601-618.
- Atack JR, Hutson PH, Collinson N, Marshall G, Bentley G, Moyes C, et al (2005): Anxiogenic properties of an inverse agonist selective for alpha3 subunit-containing GABA A receptors. *Br J Pharmacol* 144:357-366.
- Atack JR, Smith AJ, Emms F, McKernan RM (1999): Regional differences in the inhibition of mouse in vivo [3H]Ro 15-1788 binding reflect selectivity for alpha 1 versus alpha 2 and alpha 3 subunit-containing GABAA receptors. *Neuropsychopharmacology* 20:255-262.
- Atack JR, Wafford KA, Tye SJ, Cook SM, Sohal B, Pike A, et al (2006): TPA023 [7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for alpha2- and alpha3-containing GABAA receptors, is a nonsedating anxiolytic in rodents and primates. *J Pharmacol Exp Ther* 316:410-422.
- Audenaert K, Van Laere K, Dumont F, Slegers G, Mertens J, van Heeringen C, Dierckx RA (2001): Decreased frontal serotonin 5-HT 2a receptor binding index in deliberate self-harm patients. *Eur J Nucl Med* 28:175-182.
- Autà J, Impagnatiello F, Kadriu B, Guidotti A, Costa E (2008): Imidazenil: a low efficacy agonist at alpha1- but high efficacy at alpha5-GABAA receptors fail to show anticonvulsant cross tolerance to diazepam or zolpidem. *Neuropharmacology* 55:148-153.
- Avery DH, Wildschiodt G, Rafaelsen OJ (1982): Nocturnal temperature in affective disorder. *J Affect Disord* 4:61-71.
- Avitsur R, Donchin O, Barak O, Cohen E, Yirmiya R (1995): Behavioral effects of interleukin-1 beta: modulation by gender, estrus cycle, and progesterone. *Brain Behav Immun* 9:234-241.
- B**
- Bachtell RK, Tsivkovskaia NO, Ryabinin AE (2003): Identification of temperature-sensitive neural circuits in mice using c-Fos expression mapping. *Brain Res* 960:157-164.
- Bagdy E, Kiraly I, Harsing LG, Jr. (2000): Reciprocal innervation between serotonergic and GABAergic neurons in raphe nuclei of the rat. *Neurochem Res* 25:1465-1473.
- Bagdy G, Graf M, Anheuer ZE, Modos EA, Kantor S (2001): Anxiety-like effects induced by acute fluoxetine, sertraline or m-CPP treatment are reversed by pretreatment with the 5-HT2C receptor antagonist SB-242084 but not the 5-HT1A receptor antagonist WAY-100635. *Int J Neuropsychopharmacol* 4:399-408.
- Bagosi Z, Jaszberenyi M, Szabo G, Telegdy G (2008): The effects of CRF and the urocortins on [3H]GABA release from the rat amygdala—an in vitro superfusion study. *Brain Res Bull* 75:15-17.
- Bailey SJ, Toth M (2004): Variability in the benzodiazepine response of serotonin 5-HT1A receptor null mice displaying anxiety-like phenotype: evidence for genetic modifiers in the 5-HT-mediated regulation of GABA(A) receptors. *J Neurosci* 24:6343-6351.
- Baker DG, West SA, Nicholson WE, Ekhaton NN, Kasckow JW, Hill KK, et al (1999): Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. *Am J Psychiatry* 156:585-588.
- Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, et al (1998): International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50:291-313.
- Barnes NM, Sharp T (1999): A review of central 5-HT receptors and their function. *Neuropharmacology* 38:1083-1152.

- Barnum CJ, Blandino P, Jr., Deak T (2007): Adaptation in the corticosterone and hyperthermic responses to stress following repeated stressor exposure. *J Neuroendocrinol* 19:632-642.
- Baumann SW, Baur R, Sigel E (2003): Individual properties of the two functional agonist sites in GABA(A) receptors. *J Neurosci* 23:11158-11166.
- Beique JC, Imad M, Mladenovic L, Gingrich JA, Andrade R (2007): Mechanism of the 5-hydroxytryptamine 2A receptor-mediated facilitation of synaptic activity in prefrontal cortex. *Proc Natl Acad Sci U S A* 104:9870-9875.
- Bellelli D, Lambert JJ (2005): Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nat Rev Neurosci* 6:565-575.
- Belknap JK (2003): Chromosome substitution strains: some quantitative considerations for genome scans and fine mapping. *Mamm Genome* 14:723-732.
- Belzung C, Berton F (1997): Further pharmacological validation of the BALB/c neophobia in the free exploratory paradigm as an animal model of trait anxiety. *Behav Pharmacol* 8:541-548.
- Benarroch EE (2005): Paraventricular nucleus, stress response, and cardiovascular disease. *Clin Auton Res* 15:254-263.
- Benke D, Fakitsas P, Roggenmoser C, Michel C, Rudolph U, Mohler H (2004): Analysis of the presence and abundance of GABAA receptors containing two different types of alpha subunits in murine brain using point-mutated alpha subunits. *J Biol Chem* 279:43654-43660.
- Benloucif S, Keegan MJ, Galloway MP (1993): Serotonin-facilitated dopamine release in vivo: pharmacological characterization. *J Pharmacol Exp Ther* 265:373-377.
- Bertaina-Anglade V, La Rochelle CD, Scheller DK (2006): Antidepressant properties of rotigotine in experimental models of depression. *Eur J Pharmacol* 548:106-114.
- Bethea CL, Lu NZ, Gundlach C, Streicher JM (2002): Diverse actions of ovarian steroids in the serotonin neural system. *Front Neuroendocrinol* 23:41-100.
- Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004): Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev* 84:835-867.
- Bhatnagar S, Sun LM, Raber J, Maren S, Julius D, Dallman MF (2004): Changes in anxiety-related behaviors and hypothalamic-pituitary-adrenal activity in mice lacking the 5-HT-3A receptor. *Physiol Behav* 81:545-555.
- Bhatnagar S, Vining C, Iyer V, Kinni V (2006): Changes in hypothalamic-pituitary-adrenal function, body temperature, body weight and food intake with repeated social stress exposure in rats. *J Neuroendocrinol* 18:13-24.
- Biegon A, Grinspoon A, Blumenfeld B, Bleich A, Apter A, Mester R (1990): Increased serotonin 5-HT2 receptor binding on blood platelets of suicidal men. *Psychopharmacology (Berl)* 100:165-167.
- Bilkei-Gorzo A, Gyertyan I, Levay G (1998): mCPP-induced anxiety in the light-dark box in rats--a new method for screening anxiolytic activity. *Psychopharmacology (Berl)* 136:291-298.
- Bitran D, Hilvers RJ, Kellogg CK (1991): Anxiolytic effects of 3 alpha-hydroxy-5 alpha[beta]-pregnan-20-one: endogenous metabolites of progesterone that are active at the GABAA receptor. *Brain Res* 561:157-161.
- Blanchard DC, Blanchard RJ (1972): Innate and conditioned reactions to threat in rats with amygdaloid lesions. *J Comp Physiol Psychol* 81:281-290.
- Blank T, Nijholt I, Grammatopoulos DK, Randeva HS, Hillhouse EW, Spiess J (2003): Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. *J Neurosci* 23:700-707.
- Blasi C (2000): Influence of benzodiazepines on body weight and food intake in obese and lean Zucker rats. *Prog Neuropsychopharmacol Biol Psychiatry* 24:561-577.
- Blatteis CM, Sehic E (1998): Cytokines and fever. *Ann N Y Acad Sci* 840:608-618.
- Blatteis CM, Sehic E, Li S (2000): Pyrogen sensing and signaling: old views and new concepts. *Clin Infect Dis* 31 Suppl 5:S168-177.

- Blessing WW (2004): 5-hydroxytryptamine 1A receptor activation reduces cutaneous vasoconstriction and fever associated with the acute inflammatory response in rabbits. *Neuroscience* 123:1-4.
- Blumel JE, Castelo-Branco C, Cancelo MJ, Cordova AT, Binfa LE, Bonilla HG, et al (2004): Relationship between psychological complaints and vasomotor symptoms during climacteric. *Maturitas* 49:205-210.
- Boileau AJ, Evers AR, Davis AF, Czajkowski C (1999): Mapping the agonist binding site of the GABA<sub>A</sub> receptor: evidence for a beta-strand. *J Neurosci* 19:4847-4854.
- Boiten FA, Frijda NH, Wientjes CJ (1994): Emotions and respiratory patterns: review and critical analysis. *Int J Psychophysiol* 17:103-128.
- Bonasera SJ, Chu HM, Brennan TJ, Tecott LH (2006): A null mutation of the serotonin 6 receptor alters acute responses to ethanol. *Neuropsychopharmacology* 31:1801-1813.
- Borella A, Bindra M, Whitaker-Azmitia PM (1997): Role of the 5-HT<sub>1A</sub> receptor in development of the neonatal rat brain: preliminary behavioral studies. *Neuropharmacology* 36:445-450.
- Borghese CM, Storustovu S, Ebert B, Herd MB, Belelli D, Lambert JJ, et al (2006): The delta subunit of gamma-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. *J Pharmacol Exp Ther* 316:1360-1368.
- Borsini F, Brambilla A, Cesana R, Donetti A (1993): The effect of DAU 6215, a novel 5HT-3 antagonist, in animal models of anxiety. *Pharmacol Res* 27:151-164.
- Borsini F, Brambilla A, Grippa N, Pitsikas N (1999): Behavioral effects of flibanserin (BIMT 17). *Pharmacol Biochem Behav* 64:137-146.
- Borsini F, Lecci A, Volterra G, Meli A (1989): A model to measure anticipatory anxiety in mice? *Psychopharmacology (Berl)* 98:207-211.
- Borsini F, Podhorna J, Marazziti D (2002): Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *Psychopharmacology (Berl)* 163:121-141.
- Bouali S, Evrard A, Chastanet M, Lesch KP, Hamon M, Adrien J (2003): Sex hormone-dependent desensitization of 5-HT<sub>1A</sub> autoreceptors in knockout mice deficient in the 5-HT transporter. *Eur J Neurosci* 18:2203-2212.
- Boulant JA (2000): Role of the preoptic-anterior hypothalamus in thermoregulation and fever. *Clin Infect Dis* 31 Suppl 5:S157-161.
- Bouwknacht JA, Hijzen TH, van der Gugten J, Maes RA, Hen R, Olivier B (2001): Absence of 5-HT(1B) receptors is associated with impaired impulse control in male 5-HT(1B) knockout mice. *Biol Psychiatry* 49:557-568.
- Bouwknacht JA, Hijzen TH, van der Gugten J, Maes RA, Olivier B (2000): Stress-induced hyperthermia in mice: effects of flesinoxan on heart rate and body temperature. *Eur J Pharmacol* 400:59-66.
- Bouwknacht JA, Olivier B, Paylor RE (2007): The stress-induced hyperthermia paradigm as a physiological animal model for anxiety: a review of pharmacological and genetic studies in the mouse. *Neurosci Biobehav Rev* 31:41-59.
- Bouwknacht JA, Paylor R (2002): Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains. *Behav Brain Res* 136:489-501.
- Bouwknacht JA, van der Gugten J, Groenink L, Olivier B, Paylor RE (2004a): Behavioral and physiological mouse models for anxiety: effects of flesinoxan in 129S6/SvEvTac and C57BL/6J mice. *Eur J Pharmacol* 494:45-53.
- Bouwknacht JA, van der Gugten J, Groenink L, Olivier B, Paylor RE (2004b): Effects of repeated testing in two inbred strains on flesinoxan dose-response curves in three mouse models for anxiety. *Eur J Pharmacol* 494:35-44.
- Bouwknacht AJ, Olivier B, Paylor RE (2007): The stress-induced hyperthermia paradigm as a physiological animal model for anxiety: a review of pharmacological and genetic studies in the mouse. *Neurosci Biobehav Rev* 31:41-59.

- Boyle AE, Gill KJ (2008): Confirmation of provisional quantitative trait loci for voluntary alcohol consumption: genetic analysis in chromosome substitution strains and F2 crosses derived from A/J and C57BL/6J progenitors. *Pharmacogenet Genomics* 18:1071-1082.
- Boyle AE, Gill KJ (2009): Genetic analysis of the psychostimulant effects of nicotine in chromosome substitution strains and F2 crosses derived from A/J and C57BL/6J progenitors. *Mamm Genome* 20:34-42.
- Brambilla P, Perez J, Barale F, Schettini G, Soares JC (2003): GABAergic dysfunction in mood disorders. *Mol Psychiatry* 8:721-737, 715.
- Bremner JD, Innis RB, Southwick SM, Staib L, Zoghbi S, Charney DS (2000a): Decreased benzodiazepine receptor binding in prefrontal cortex in combat-related posttraumatic stress disorder. *Am J Psychiatry* 157:1120-1126.
- Bremner JD, Innis RB, White T, Fujita M, Silbersweig D, Goddard AW, et al (2000b): SPECT [<sup>123</sup>I]-123iomazenil measurement of the benzodiazepine receptor in panic disorder. *Biol Psychiatry* 47:96-106.
- Breslow MF, Fankhauser MP, Potter RL, Meredith KE, Misiaszek J, Hope DG, Jr. (1989): Role of gamma-aminobutyric acid in antipanic drug efficacy. *Am J Psychiatry* 146:353-356.
- Briese E (1995): Emotional hyperthermia and performance in humans. *Physiol Behav* 58:615-618.
- Briese E, Cabanac M (1991): Stress hyperthermia: physiological arguments that it is a fever. *Physiol Behav* 49:1153-1157.
- Broocks A, Meyer T, Opitz M, Bartmann U, Hillmer-Vogel U, George A, et al (2003): 5-HT<sub>1A</sub> responsivity in patients with panic disorder before and after treatment with aerobic exercise, clomipramine or placebo. *Eur Neuropsychopharmacol* 13:153-164.
- Brown GW, Harris TO (2008): Depression and the serotonin transporter 5-HTTLPR polymorphism: a review and a hypothesis concerning gene-environment interaction. *J Affect Disord* 111:1-12.
- Brown TA, McNiff J (2009): Specificity of autonomic arousal to DSM-IV panic disorder and posttraumatic stress disorder. *Behav Res Ther* 47:487-493.
- Buchanan JB, Peloso E, Satinoff E (2006): Influence of ambient temperature on peripherally induced interleukin-1 beta fever in young and old rats. *Physiol Behav* 88:453-458.
- Bull DF, Husband AJ, Munro KI, Exton MS, Pfister HP, King MG (1994): Inhibition of endotoxin-induced temperature change by behavioral conditioning using alpha-melanocyte-stimulating hormone as an unconditioned stimulus. *Peptides* 15:139-142.
- Bull DF, King MG, Pfister HP, Singer G (1990): Alpha-melanocyte-stimulating hormone conditioned suppression of a lipopolysaccharide-induced fever. *Peptides* 11:1027-1031.
- Buller KM, Day TA (2002): Systemic administration of interleukin-1beta activates select populations of central amygdala afferents. *J Comp Neurol* 452:288-296.
- Burnet PW, Eastwood SL, Lacey K, Harrison PJ (1995): The distribution of 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptor mRNA in human brain. *Brain Res* 676:157-168.
- Buwalda B, de Boer SF, Van Kalkeren AA, Koolhaas JM (1997): Physiological and behavioral effects of chronic intracerebroventricular infusion of corticotropin-releasing factor in the rat. *Psychoneuroendocrinology* 22:297-309.
- Byrnes EM, Lee JO, Bridges RS (2007): Alterations in GABA<sub>(A)</sub> receptor alpha2 subunit mRNA expression following reproductive experience in rats. *Neuroendocrinology* 85:148-156.

**C**

- Cabanac M, Bernieri C (2000): Behavioral rise in body temperature and tachycardia by handling of a turtle (*Clemmys insculpta*). *Behav Processes* 49:61-68.
- Cabanac M, Dardashti M (1999): Emotional fever in rats persists after vagotomy. *Physiol Behav* 67:347-350.
- Cabrera R, Korte SM, Lentjes EG, Romijn F, Schonbaum E, De Nicola A, De Kloet ER (2000): The amount of free corticosterone is increased during lipopolysaccharide-induced fever. *Life Sci* 66:553-562.

- Cairncross KD, Wren A, Cox B, Schnieden H (1977): Effects of olfactory bulbectomy and domicile on stress-induced corticosterone release in the rat. *Physiol Behav* 19:485-487.
- Caldji C, Diorio J, Anisman H, Meaney MJ (2004): Maternal behavior regulates benzodiazepine/GABAA receptor subunit expression in brain regions associated with fear in BALB/c and C57BL/6 mice. *Neuropsychopharmacology* 29:1344-1352.
- Calver AR, Davies CH, Pangalos M (2002): GABA(B) receptors: from monogamy to promiscuity. *Neurosignals* 11:299-314.
- Cameron OG, Huang GC, Nichols T, Koeppe RA, Minoshima S, Rose D, Frey KA (2007): Reduced gamma-aminobutyric acid(A)-benzodiazepine binding sites in insular cortex of individuals with panic disorder. *Arch Gen Psychiatry* 64:793-800.
- Campbell BM, Merchant KM (2003): Serotonin 2C receptors within the basolateral amygdala induce acute fear-like responses in an open-field environment. *Brain Res* 993:1-9.
- Canteras NS, Simerly RB, Swanson LW (1995): Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. *J Comp Neurol* 360:213-245.
- Cao BJ, Rodgers RJ (1997): Influence of 5-HT<sub>1A</sub> receptor antagonism on plus-maze behavior in mice. II. WAY 100635, SDZ 216-525 and NAN-190. *Pharmacol Biochem Behav* 58:593-603.
- Carai MA, Colombo G, Froestl W, Gessa GL (2004): In vivo effectiveness of CGP7930, a positive allosteric modulator of the GABA<sub>B</sub> receptor. *Eur J Pharmacol* 504:213-216.
- Caramaschi D, de Boer SF, Koolhaas JM (2007): Differential role of the 5-HT<sub>1A</sub> receptor in aggressive and non-aggressive mice: an across-strain comparison. *Physiol Behav* 90:590-601.
- Cardinal RN, Parkinson JA, Hall J, Everitt BJ (2002): Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev* 26:321-352.
- Carli M, Bonalumi P, Samanin R (1997): WAY 100635, a 5-HT<sub>1A</sub> receptor antagonist, prevents the impairment of spatial learning caused by intrahippocampal administration of scopolamine or 7-chloro-kynurenic acid. *Brain Res* 774:167-174.
- Carling RW, Madin A, Guiblin A, Russell MG, Moore KW, Mitchinson A, et al (2005): 7-(1,1-Dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluoro phenyl)-1,2,4-triazolo[4,3-b]pyridazine: a functionally selective gamma-aminobutyric acid(A) (GABA(A)) alpha2/alpha3-subtype selective agonist that exhibits potent anxiolytic activity but is not sedating in animal models. *J Med Chem* 48:7089-7092.
- Carney RM, Freedland KE, Veith RC (2005): Depression, the autonomic nervous system, and coronary heart disease. *Psychosom Med* 67 Suppl 1:S29-33.
- Carney RM, Freedland KE, Veith RC, Cryer PE, Skala JA, Lynch T, Jaffe AS (1999): Major depression, heart rate, and plasma norepinephrine in patients with coronary heart disease. *Biol Psychiatry* 45:458-463.
- Carobrez SG, Gasparotto OC, Buwalda B, Bohus B (2002): Long-term consequences of social stress on corticosterone and IL-1beta levels in endotoxin-challenged rats. *Physiol Behav* 76:99-105.
- Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P (2002): Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behavior in inbred mice. *Behav Brain Res* 134:49-57.
- Carpenter JS, Gilchrist JM, Chen K, Gautam S, Freedman RR (2004): Hot flashes, core body temperature, and metabolic parameters in breast cancer survivors. *Menopause* 11:375-381.
- Carrasco GA, Van de Kar LD (2003): Neuroendocrine pharmacology of stress. *Eur J Pharmacol* 463:235-272.
- Cash DJ, Serfozo P, Allan AM (1997): Desensitization of a gamma-aminobutyric acid type A receptor in rat is increased by chronic treatment with chlordiazepoxide: a molecular mechanism of dependence. *J Pharmacol Exp Ther* 283:704-711.
- Castelli MP, Ingianni A, Stefanini E, Gessa GL (1999): Distribution of GABA(B) receptor mRNAs in the rat brain and peripheral organs. *Life Sci* 64:1321-1328.
- Celada P, Puig M, Amargos-Bosch M, Adell A, Artigas F (2004): The therapeutic role of 5-HT<sub>1A</sub> and 5-HT<sub>2A</sub> receptors in depression. *J Psychiatry Neurosci* 29:252-265.

- Cerri M, Morrison SF (2006): Corticotropin releasing factor increases in brown adipose tissue thermogenesis and heart rate through dorsomedial hypothalamus and medullary raphe pallidus. *Neuroscience* 140:711-721.
- Cervo L, Mocaer E, Bertaglia A, Samanin R (2000): Roles of 5-HT<sub>1A</sub> receptors in the dorsal raphe and dorsal hippocampus in anxiety assessed by the behavioral effects of 8-OH-DPAT and S 15535 in a modified Geller-Seifter conflict model. *Neuropharmacology* 39:1037-1043.
- Chalmers DT, Watson SJ (1991): Comparative anatomical distribution of 5-HT<sub>1A</sub> receptor mRNA and 5-HT<sub>1A</sub> binding in rat brain--a combined in situ hybridisation/in vitro receptor autoradiographic study. *Brain Res* 561:51-60.
- Chan AW, Leong FW, Schanley DL, Langan MC, Penetrante ML (1989): A liquid diet model of chlordiazepoxide dependence in mice. *Pharmacol Biochem Behav* 34:839-845.
- Charney DS, Woods SW, Goodman WK, Heninger GR (1987): Serotonin function in anxiety. II. Effects of the serotonin agonist MCPP in panic disorder patients and healthy subjects. *Psychopharmacology (Berl)* 92:14-24.
- Chase TN, Katz RI, Kopin IJ (1970): Effect of diazepam on fate of intracisternally injected serotonin-C14. *Neuropharmacology* 9:103-108.
- Chen HT, Clark M, Goldman D (1992): Quantitative autoradiography of 3H-paroxetine binding sites in rat brain. *J Pharmacol Toxicol Methods* 27:209-216.
- Chen NH, Reith ME (1995): Monoamine interactions measured by microdialysis in the ventral tegmental area of rats treated systemically with (+/-)-8-hydroxy-2-(di-n-propylamino)tetralin. *J Neurochem* 64:1585-1597.
- Chen SW, Min L, Li WJ, Kong WX, Li JF, Zhang YJ (2004a): The effects of angelica essential oil in three murine tests of anxiety. *Pharmacol Biochem Behav* 79:377-382.
- Chen SW, Shemyakin A, Wiedenmayer CP (2006): The role of the amygdala and olfaction in unconditioned fear in developing rats. *J Neurosci* 26:233-240.
- Chen SW, Xin Q, Kong WX, Min L, Li JF (2003): Anxiolytic-like effect of succinic acid in mice. *Life Sci* 73:3257-3264.
- Chen X, Herbert J (1995): The effect of long-term castration on the neuronal and physiological responses to acute or repeated restraint stress: interactions with opioids and prostaglandins. *J Neuroendocrinol* 7:137-144.
- Chen Y, Brunson KL, Adelman G, Bender RA, Frotscher M, Baram TZ (2004b): Hippocampal corticotropin releasing hormone: pre- and postsynaptic location and release by stress. *Neuroscience* 126:533-540.
- Clark MS, Sexton TJ, McClain M, Root D, Kohen R, Neumaier JF (2002): Overexpression of 5-HT<sub>1B</sub> receptor in dorsal raphe nucleus using Herpes Simplex Virus gene transfer increases anxiety behavior after inescapable stress. *J Neurosci* 22:4550-4562.
- Clark MS, Vincow ES, Sexton TJ, Neumaier JF (2004): Increased expression of 5-HT<sub>1B</sub> receptor in dorsal raphe nucleus decreases fear-potentiated startle in a stress dependent manner. *Brain Res* 1007:86-97.
- Cloos JM, Ferreira V (2009): Current use of benzodiazepines in anxiety disorders. *Curr Opin Psychiatry* 22:90-95.
- Colino A, Halliwell JV (1987): Differential modulation of three separate K-conductances in hippocampal CA1 neurons by serotonin. *Nature* 328:73-77.
- Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, et al (2002): Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha<sub>5</sub> subunit of the GABAA receptor. *J Neurosci* 22:5572-5580.
- Compaan JC, Groenink L, van der Gugten J, Maes RA, Olivier B (1996): 5-HT<sub>1A</sub> receptor agonist flesinoxan enhances Fos immunoreactivity in rat central amygdala, bed nucleus of the stria terminalis and hypothalamus. *Eur J Neurosci* 8:2340-2347.

- Compaan JC, Groenink L, Van der Gugten J, Maes RA, Olivier B (1997): Pretreatment with 5-HT<sub>1A</sub> receptor agonist flesinoxan attenuates Fos protein in rat hypothalamus. *Eur J Pharmacol* 324:161-168.
- Compan V, Zhou M, Grailhe R, Gazzara RA, Martin R, Gingrich J, et al (2004): Attenuated response to stress and novelty and hypersensitivity to seizures in 5-HT<sub>4</sub> receptor knock-out mice. *J Neurosci* 24:412-419.
- Conley RK, Hutson PH (2007): Effects of acute and chronic treatment with fluoxetine on stress-induced hyperthermia in telemetered rats and mice. *Eur J Pharmacol* 564:138-145.
- Connor TJ, Leonard BE (1998): Depression, stress and immunological activation: the role of cytokines in depressive disorders. *Life Sci* 62:583-606.
- Connor TJ, Song C, Leonard BE, Anisman H, Merali Z (1999): Stressor-induced alterations in serotonergic activity in an animal model of depression. *Neuroreport* 10:523-528.
- Cook MN, Bolivar VJ, McFadyen MP, Flaherty L (2002): Behavioral differences among 129 substrains: implications for knockout and transgenic mice. *Behav Neurosci* 116:600-611.
- Cooper SJ, Estall LB (1985): Behavioral pharmacology of food, water and salt intake in relation to drug actions at benzodiazepine receptors. *Neurosci Biobehav Rev* 9:5-19.
- Coppen A, Eccleston E, Craft I, Bye P (1973): Letter: Total and free plasma-tryptophan concentration and oral contraception. *Lancet* 2:1498.
- Corradetti R, Le Poul E, Laaris N, Hamon M, Lanfumey L (1996): Electrophysiological effects of N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridinyl) cyclohexane carboxamide (WAY 100635) on dorsal raphe serotonergic neurons and CA1 hippocampal pyramidal cells in vitro. *J Pharmacol Exp Ther* 278:679-688.
- Costall B, Naylor RJ, Tyers MB (1990): The psychopharmacology of 5-HT<sub>3</sub> receptors. *Pharmacol Ther* 47:181-202.
- Coull MA, Lowther S, Katona CL, Horton RW (2000a): Altered brain protein kinase C in depression: a post-mortem study. *Eur Neuropsychopharmacol* 10:283-288.
- Coull MA, Lowther S, Katona CL, Horton RW (2000b): Post-mortem studies of brain phosphatidylinositol hydrolysis in depression and the effect of antidepressant treatment. *Int J Neuropsychopharmacol* 3:109-115.
- Coupland NJ, Ogilvie CJ, Hegadoren KM, Seres P, Hanstock CC, Allen PS (2005): Decreased prefrontal Myo-inositol in major depressive disorder. *Biol Psychiatry* 57:1526-1534.
- Cowen PJ (2000): Psychopharmacology of 5-HT<sub>(1A)</sub> receptors. *Nucl Med Biol* 27:437-439.
- Cowen PJ, Parry-Billings M, Newsholme EA (1989): Decreased plasma tryptophan levels in major depression. *J Affect Disord* 16:27-31.
- Coyle SM, Calvano SE, Lowry SF (2006): Gender influences in vivo human responses to endotoxin. *Shock* 26:538-543.
- Crabbe JC, Wahlsten D, Dudek BC (1999): Genetics of mouse behavior: interactions with laboratory environment. *Science* 284:1670-1672.
- Craig JV, Lancaster GA, Taylor S, Williamson PR, Smyth RL (2002): Infrared ear thermometry compared with rectal thermometry in children: a systematic review. *Lancet* 360:603-609.
- Crane JW, Buller KM (2007): Systemic blockade of complement C5a receptors reduces lipopolysaccharide-induced responses in the paraventricular nucleus and the central amygdala. *Neurosci Lett* 424:10-15.
- Craske MG, Waters AM (2005): Panic disorder, phobias, and generalized anxiety disorder. *Annu Rev Clin Psychol* 1:197-225.
- Crawforth J, Atack JR, Cook SM, Gibson KR, Nadin A, Owens AP, et al (2004): Tricyclic pyridones as functionally selective human GABA $\alpha$  2/3 receptor-ion channel ligands. *Bioorg Med Chem Lett* 14:1679-1682.
- Crawley JN, Glowa JR, Majewska MD, Paul SM (1986): Anxiolytic activity of an endogenous adrenal steroid. *Brain Res* 398:382-385.

- Crestani F, Low K, Keist R, Mandelli M, Mohler H, Rudolph U (2001): Molecular targets for the myorelaxant action of diazepam. *Mol Pharmacol* 59:442-445.
- Crestani F, Martin JR, Mohler H, Rudolph U (2000): Mechanism of action of the hypnotic zolpidem in vivo. *Br J Pharmacol* 131:1251-1254.
- Crews FT, Morrow AL, Criswell H, Breese G (1996): Effects of ethanol on ion channels. *Int Rev Neurobiol* 39:283-367.
- Croiset G, Heijnen CJ, Veldhuis HD, de Wied D, Ballieux RE (1987): Modulation of the immune response by emotional stress. *Life Sci* 40:775-782.
- Cryan JF, Holmes A (2005): The ascent of mouse: advances in modelling human depression and anxiety. *Nat Rev Drug Discov* 4:775-790.
- Cryan JF, Kaupmann K (2005): Don't worry 'B' happy!: a role for GABA(B) receptors in anxiety and depression. *Trends Pharmacol Sci* 26:36-43.
- Cryan JF, Kelliher P, Kelly JP, Leonard BE (1999): Comparative effects of serotonergic agonists with varying efficacy at the 5-HT(1A) receptor on core body temperature: modification by the selective 5-HT(1A) receptor antagonist WAY 100635. *J Psychopharmacol* 13:278-283.
- Cryan JF, Kelly PH, Chaperon F, Gentsch C, Mombereau C, Lingenhoehl K, et al (2004): Behavioral characterization of the novel GABAB receptor-positive modulator GS39783 (N,N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine): anxiolytic-like activity without side effects associated with baclofen or benzodiazepines. *J Pharmacol Exp Ther* 310:952-963.
- Cullinan WE (2000): GABA(A) receptor subunit expression within hypophysiotropic CRH neurons: a dual hybridization histochemical study. *J Comp Neurol* 419:344-351.
- Cullinan WE, Helmreich DL, Watson SJ (1996): Fos expression in forebrain afferents to the hypothalamic paraventricular nucleus following swim stress. *J Comp Neurol* 368:88-99.
- Cullinan WE, Herman JP, Battaglia DF, Akil H, Watson SJ (1995): Pattern and time course of immediate early gene expression in rat brain following acute stress. *Neuroscience* 64:477-505.
- Cullinan WE, Ziegler DR, Herman JP (2008): Functional role of local GABAergic influences on the HPA axis. *Brain Struct Funct* 213:63-72.

**D**

- D'Amato RJ, Largent BL, Snowman AM, Snyder SH (1987): Selective labeling of serotonin uptake sites in rat brain by [3H]citalopram contrasted to labeling of multiple sites by [3H]mipramine. *J Pharmacol Exp Ther* 242:364-371.
- D'Amico EJ, Neilands TB, Zambarano R (2001): Power analysis for multivariate and repeated measures designs: a flexible approach using the SPSS MANOVA procedure. *Behav Res Methods Instrum Comput* 33:479-484.
- D'Aquila PS, Collu M, Gessa GL, Serra G (2000): The role of dopamine in the mechanism of action of antidepressant drugs. *Eur J Pharmacol* 405:365-373.
- Daenen EW, Wolterink G, Van Der Heyden JA, Kruse CG, Van Ree JM (2003): Neonatal lesions in the amygdala or ventral hippocampus disrupt prepulse inhibition of the acoustic startle response; implications for an animal model of neurodevelopmental disorders like schizophrenia. *Eur Neuropsychopharmacol* 13:187-197.
- Daimon K, Yamada N, Tsujimoto T, Takahashi S (1992): Circadian rhythm abnormalities of deep body temperature in depressive disorders. *J Affect Disord* 26:191-198.
- Dalvi A, Rodgers RJ (1996): GABAergic influences on plus-maze behavior in mice. *Psychopharmacology (Berl)* 128:380-397.
- Daniels WM, Richter L, Stein DJ (2004): The effects of repeated intra-amygdala CRF injections on rat behavior and HPA axis function after stress. *Metab Brain Dis* 19:15-23.
- Davies M (2003): The role of GABA<sub>A</sub> receptors in mediating the effects of alcohol in the central nervous system. *J Psychiatry Neurosci* 28:263-274.

- Davies MF, Onaivi ES, Chen SW, Maguire PA, Tsai NF, Loew GH (1994): Evidence for central benzodiazepine receptor heterogeneity from behavior tests. *Pharmacol Biochem Behav* 49:47-56.
- Davis M (1997): Neurobiology of fear responses: the role of the amygdala. *J Neuropsychiatry Clin Neurosci* 9:382-402.
- Davis M (2006): Neural systems involved in fear and anxiety measured with fear-potentiated startle. *Am Psychol* 61:741-756.
- Davis M, Whalen PJ (2001): The amygdala: vigilance and emotion. *Mol Psychiatry* 6:13-34.
- Davis S, Heal DJ, Stanford SC (1995): Long-lasting effects of an acute stress on the neurochemistry and function of 5-hydroxytryptaminergic neurones in the mouse brain. *Psychopharmacology (Berl)* 118:267-272.
- Davydov DM, Shapiro D, Cook IA, Goldstein I (2007): Baroreflex mechanisms in major depression. *Prog Neuropsychopharmacol Biol Psychiatry* 31:164-177.
- Dawson GR, Crawford SP, Collinson N, Iversen SD, Tricklebank MD (1995): Evidence that the anxiolytic-like effects of chlordiazepoxide on the elevated plus maze are confounded by increases in locomotor activity. *Psychopharmacology (Berl)* 118:316-323.
- Dawson GR, Maubach KA, Collinson N, Cobain M, Everitt BJ, MacLeod AM, et al (2006): An inverse agonist selective for alpha5 subunit-containing GABA<sub>A</sub> receptors enhances cognition. *J Pharmacol Exp Ther* 316:1335-1345.
- Dawson LA, Nguyen HQ, Smith DL, Schechter LE (2002): Effect of chronic fluoxetine and WAY-100635 treatment on serotonergic neurotransmission in the frontal cortex. *J Psychopharmacol* 16:145-152.
- Dayas CV, Buller KM, Crane JW, Xu Y, Day TA (2001): Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups. *Eur J Neurosci* 14:1143-1152.
- Dayas CV, Buller KM, Day TA (1999): Neuroendocrine responses to an emotional stressor: evidence for involvement of the medial but not the central amygdala. *Eur J Neurosci* 11:2312-2322.
- Dayas CV, Day TA (2002): Opposing roles for medial and central amygdala in the initiation of noradrenergic cell responses to a psychological stressor. *Eur J Neurosci* 15:1712-1718.
- de Boer SF, Koolhaas JM (2005): 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptor agonists and aggression: a pharmacological challenge of the serotonin deficiency hypothesis. *Eur J Pharmacol* 526:125-139.
- de Haas SL, de Visser SJ, van der Post JP, de Smet M, Schoemaker RC, Rijnbeek B, et al (2007): Pharmacodynamic and pharmacokinetic effects of TPA023, a GABA<sub>A</sub> alpha(2,3) subtype-selective agonist, compared to lorazepam and placebo in healthy volunteers. *J Psychopharmacol* 21:374-383.
- de Jongh R, Groenink L, van Der Gugten J, Olivier B (2002): The light-enhanced startle paradigm as a putative animal model for anxiety: effects of chlordiazepoxide, flesinoxan and fluvoxamine. *Psychopharmacology (Berl)* 159:176-180.
- de Kloet ER, Joels M, Holsboer F (2005): Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6:463-475.
- De Souza EB (1995): Corticotropin-releasing factor receptors: physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. *Psychoneuroendocrinology* 20:789-819.
- de Visser L, van den Bos R, Kuurman WW, Kas MJ, Spruijt BM (2006): Novel approach to the behavioral characterization of inbred mice: automated home cage observations. *Genes Brain Behav* 5:458-466.
- Decker MW, Curzon P, Brioni JD (1995): Influence of separate and combined septal and amygdala lesions on memory, acoustic startle, anxiety, and locomotor activity in rats. *Neurobiol Learn Mem* 64:156-168.

- Del Rio G, Velardo A, Zizzo G, Avogaro A, Cipolli C, Della Casa L, et al (1994): Effect of estradiol on the sympathoadrenal response to mental stress in normal men. *J Clin Endocrinol Metab* 79:836-840.
- Delgado MR, Olsson A, Phelps EA (2006): Extending animal models of fear conditioning to humans. *Biol Psychol* 73:39-48.
- Den Boer JA, Bosker FJ, Slaap BR (2000): Serotonergic drugs in the treatment of depressive and anxiety disorders. *Hum Psychopharmacol* 15:315-336.
- Derkach V, Surprenant A, North RA (1989): 5-HT<sub>3</sub> receptors are membrane ion channels. *Nature* 339:706-709.
- Dias R, Sheppard WF, Fradley RL, Garrett EM, Stanley JL, Tye SJ, et al (2005): Evidence for a significant role of alpha 3-containing GABA<sub>A</sub> receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci* 25:10682-10688.
- Dickerson SS, Kemeny ME (2004): Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. *Psychol Bull* 130:355-391.
- Dielenberg RA, Hunt GE, McGregor IS (2001): "When a rat smells a cat": the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor. *Neuroscience* 104:1085-1097.
- Dillon KA, Gross-Isseroff R, Israeli M, Biegon A (1991): Autoradiographic analysis of serotonin 5-HT<sub>1A</sub> receptor binding in the human brain postmortem: effects of age and alcohol. *Brain Res* 554:56-64.
- DiMicco JA, Sarkar S, Zaretskaia MV, Zaretsky DV (2006): Stress-induced cardiac stimulation and fever: common hypothalamic origins and brainstem mechanisms. *Auton Neurosci* 126-127:106-119.
- Dimicco JA, Zaretsky DV (2007): The dorsomedial hypothalamus: a new player in thermoregulation. *Am J Physiol Regul Integr Comp Physiol* 292:R47-63.
- Dirks A, Groenink L, Bouwknegt JA, Hijzen TH, Van Der Gugten J, Ronken E, et al (2002): Overexpression of corticotropin-releasing hormone in transgenic mice and chronic stress-like autonomic and physiological alterations. *Eur J Neurosci* 16:1751-1760.
- Dong HW, Petrovich GD, Swanson LW (2001): Topography of projections from amygdala to bed nuclei of the stria terminalis. *Brain Res Rev* 38:192-246.
- Dopico AM, Lovinger DM (2009): Acute alcohol action and desensitization of ligand-gated ion channels. *Pharmacol Rev* 61:98-114.
- Drake RG, Davis LL, Cates ME, Jewell ME, Ambrose SM, Lowe JS (2003): Baclofen treatment for chronic posttraumatic stress disorder. *Ann Pharmacother* 37:1177-1181.
- Drevets WC, Thase ME, Moses-Kolko EL, Price J, Frank E, Kupfer DJ, Mathis C (2007): Serotonin-1A receptor imaging in recurrent depression: replication and literature review. *Nucl Med Biol* 34:865-877.
- Duffy ME, Stewart-Knox BJ, McConville C, Bradbury I, O'Connor J, Helander A, Strain JJ (2006): The relationship between whole-blood serotonin and subjective mood in apparently healthy postmenopausal women. *Biol Psychol* 73:165-168.
- Dunn AJ, Swiergiel AH, de Beaurepaire R (2005): Cytokines as mediators of depression: what can we learn from animal studies? *Neurosci Biobehav Rev* 29:891-909.
- Dunn JD, Whitener J (1986): Plasma corticosterone responses to electrical stimulation of the amygdaloid complex: cytoarchitectural specificity. *Neuroendocrinology* 42:211-217.
- Dupuis DS, Relkovic D, Lhuillier L, Mosbacher J, Kaupmann K (2006): Point mutations in the transmembrane region of GABAB2 facilitate activation by the positive modulator N,N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) in the absence of the GABAB1 subunit. *Mol Pharmacol* 70:2027-2036.
- Dymond KE, Fewell JE (1999): Gender influences the core temperature response to a simulated open field in adult guinea pigs. *Physiol Behav* 65:889-892.

**E**

- Ebert B, Wafford KA, Deacon S (2006): Treating insomnia: Current and investigational pharmacological approaches. *Pharmacol Ther* 112:612-629.
- Ebert B, Wafford KA, Whiting PJ, Krogsgaard-Larsen P, Kemp JA (1994): Molecular pharmacology of gamma-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different alpha, beta, and gamma receptor subunit combinations. *Mol Pharmacol* 46:957-963.
- Elfline GS, Branda EM, Babich M, Quock RM (2004): Antagonism by NOS inhibition of the behavioral effects of benzodiazepine and GABA<sub>A</sub> receptor agonists in the mouse elevated plus-maze. *Neuropsychopharmacology* 29:1419-1425.
- Ellenbogen MA, Young SN, Dean P, Palmour RM, Benkelfat C (1996): Mood response to acute tryptophan depletion in healthy volunteers: sex differences and temporal stability. *Neuropsychopharmacology* 15:465-474.
- Elliot EE, White JM (2001): The acute effects of zolpidem compared to diazepam and lorazepam using radiotelemetry. *Neuropharmacology* 40:717-721.
- Elmqvist JK, Saper CB (1996): Activation of neurons projecting to the paraventricular hypothalamic nucleus by intravenous lipopolysaccharide. *J Comp Neurol* 374:315-331.
- Elmqvist JK, Scammell TE, Jacobson CD, Saper CB (1996): Distribution of Fos-like immunoreactivity in the rat brain following intravenous lipopolysaccharide administration. *J Comp Neurol* 371:85-103.
- Emmert MH, Herman JP (1999): Differential forebrain c-fos mRNA induction by ether inhalation and novelty: evidence for distinctive stress pathways. *Brain Res* 845:60-67.
- Engblom D, Saha S, Engstrom L, Westman M, Audoly LP, Jakobsson PJ, Blomqvist A (2003): Microsomal prostaglandin E synthase-1 is the central switch during immune-induced pyresis. *Nat Neurosci* 6:1137-1138.
- Ericsson A, Kovacs KJ, Sawchenko PE (1994): A functional anatomical analysis of central pathways subserving the effects of interleukin-1 on stress-related neuroendocrine neurons. *J Neurosci* 14:897-913.
- Esposito E (2006): Serotonin-dopamine interaction as a focus of novel antidepressant drugs. *Curr Drug Targets* 7:177-185.

**F**

- Fahey JM, Pritchard GA, Grassi JM, Pratt JS, Shader RI, Greenblatt DJ (1999): In situ hybridization histochemistry as a method to assess GABA<sub>(A)</sub> receptor subunit mRNA expression following chronic alprazolam administration. *J Psychopharmacol* 13:211-218.
- Fahey JM, Pritchard GA, Reddi JM, Pratt JS, Grassi JM, Shader RI, Greenblatt DJ (2006): The effect of chronic lorazepam administration in aging mice. *Brain Res* 1118:13-24.
- Farrant M, Nusser Z (2005): Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat Rev Neurosci* 6:215-229.
- Feldman S, Conforti N, Itzik A, Weidenfeld J (1994): Differential effect of amygdaloid lesions on CRF-41, ACTH and corticosterone responses following neural stimuli. *Brain Res* 658:21-26.
- Fernandes C, Arnot MI, Irvine EE, Bateson AN, Martin IL, File SE (1999): The effect of treatment regimen on the development of tolerance to the sedative and anxiolytic effects of diazepam. *Psychopharmacology (Berl)* 145:251-259.
- Fernandez-Guasti A, Ferreira A, Picazo O (2001): Diazepam, but not buspirone, induces similar anxiolytic-like actions in lactating and ovariectomized Wistar rats. *Pharmacol Biochem Behav* 70:85-93.
- Fernandez-Guasti A, Lopez-Rubalcava C (1998): Modification of the anxiolytic action of 5-HT<sub>1A</sub> compounds by GABA-benzodiazepine agents in rats. *Pharmacol Biochem Behav* 60:27-32.
- Fernandez-Guasti A, Picazo O (1990): The actions of diazepam and serotonergic anxiolytics vary according to the gender and the estrous cycle phase. *Pharmacol Biochem Behav* 37:77-81.

- Figueiredo HF, Bodie BL, Tauchi M, Dolgas CM, Herman JP (2003a): Stress integration after acute and chronic predator stress: differential activation of central stress circuitry and sensitization of the hypothalamo-pituitary-adrenocortical axis. *Endocrinology* 144:5249-5258.
- Figueiredo HF, Bruestle A, Bodie B, Dolgas CM, Herman JP (2003b): The medial prefrontal cortex differentially regulates stress-induced c-fos expression in the forebrain depending on type of stressor. *Eur J Neurosci* 18:2357-2364.
- File SE, Andrews N, Wu PY, Zharkovsky A, Zangrossi H, Jr. (1992): Modification of chlordiazepoxide's behavioral and neurochemical effects by handling and plus-maze experience. *Eur J Pharmacol* 218:9-14.
- File SE, Baldwin HA (1987): Effects of beta-carbolines in animal models of anxiety. *Brain Res Bull* 19:293-299.
- File SE, Mabbutt PS, Andrews N (1991a): Diazepam withdrawal responses measured in the social interaction test of anxiety and their reversal by baclofen. *Psychopharmacology (Berl)* 104:62-66.
- File SE, Zharkovsky A, Gulati K (1991b): Effects of baclofen and nitrendipine on ethanol withdrawal responses in the rat. *Neuropharmacology* 30:183-190.
- Fletcher A, Forster EA, Bill DJ, Brown G, Cliffe IA, Hartley JE, et al (1996): Electrophysiological, biochemical, neurohormonal and behavioral studies with WAY-100635, a potent, selective and silent 5-HT<sub>1A</sub> receptor antagonist. *Behav Brain Res* 73:337-353.
- Folkow B, Hallback-Nordlander M, Martner J, Nordborg C (1982): Influence of amygdala lesions on cardiovascular responses to alerting stimuli, on behavior and on blood pressure development in spontaneously hypertensive rats. *Acta Physiol Scand* 116:133-139.
- Follesa P, Biggio F, Talani G, Murru L, Serra M, Sanna E, Biggio G (2006): Neurosteroids, GABAA receptors, and ethanol dependence. *Psychopharmacology (Berl)* 186:267-280.
- Forchetti CM, Meek JL (1981): Evidence for a tonic GABAergic control of serotonin neurons in the median raphe nucleus. *Brain Res* 206:208-212.
- Fornal CA, Metzler CW, Gallegos RA, Veasey SC, McCreary AC, Jacobs BL (1996): WAY-100635, a potent and selective 5-hydroxytryptamine<sub>1A</sub> antagonist, increases serotonergic neuronal activity in behaving cats: comparison with (S)-WAY-100135. *J Pharmacol Exp Ther* 278:752-762.
- Forster C, Parkes J, Cox B (1980): Effects of olfactory bulbectomy and peripherally-induced anosmia on thermoregulation in the rat: susceptibility to antidepressant type drugs. *J Pharm Pharmacol* 32:630-634.
- Forster EA, Cliffe IA, Bill DJ, Dover GM, Jones D, Reilly Y, Fletcher A (1995): A pharmacological profile of the selective silent 5-HT<sub>1A</sub> receptor antagonist, WAY-100635. *Eur J Pharmacol* 281:81-88.
- Francis DD, Szegda K, Campbell G, Martin WD, Insel TR (2003): Epigenetic sources of behavioral differences in mice. *Nat Neurosci* 6:445-446.
- Frank SM, Kluger MJ, Kunkel SL (2000): Elevated thermostatic setpoint in postoperative patients. *Anesthesiology* 93:1426-1431.
- Franzini C, Lenzi P, Cianci T (1981): Interactions between temperature regulation and emotional arousal in the rabbit. *Exp Brain Res* 43:87-92.
- Freedman RR (2002): Core body temperature variation in symptomatic and asymptomatic postmenopausal women: brief report. *Menopause* 9:399-401.
- Freedman RR (2005): Hot flashes: behavioral treatments, mechanisms, and relation to sleep. *Am J Med* 118 Suppl 12B:124-130.
- Freeman EW, Sammel MD, Lin H, Gracia CR, Kapoor S, Ferdousi T (2005): The role of anxiety and hormonal changes in menopausal hot flashes. *Menopause* 12:258-266.
- Frenois F, Moreau M, O'Connor J, Lawson M, Micon C, Lestage J, et al (2007): Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining within the mouse extended amygdala,

- hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. *Psychoneuroendocrinology* 32:516-531.
- Fries E, Hellhammer DH, Hellhammer J (2006): Attenuation of the hypothalamic-pituitary-adrenal axis responsiveness to the Trier Social Stress Test by the benzodiazepine alprazolam. *Psychoneuroendocrinology* 31:1278-1288.
- Frosini M, Valoti M, Sgaragli G (2004): Changes in rectal temperature and ECoG spectral power of sensorimotor cortex elicited in conscious rabbits by i.c.v. injection of GABA, GABA<sub>(A)</sub> and GABA<sub>(B)</sub> agonists and antagonists. *Br J Pharmacol* 141:152-162.
- Fyer AJ, Hamilton SP, Durner M, Haghighi F, Heiman GA, Costa R, et al (2006): A third-pass genome scan in panic disorder: evidence for multiple susceptibility loci. *Biol Psychiatry* 60:388-401.
- ## G
- Gale CC (1973): Neuroendocrine aspects of thermoregulation. *Annu Rev Physiol* 35:391-430.
- Gao B, Fritschy JM, Benke D, Mohler H (1993): Neuron-specific expression of GABAA-receptor subtypes: differential association of the alpha 1- and alpha 3-subunits with serotonergic and GABAergic neurons. *Neuroscience* 54:881-892.
- Garner M, Mohler H, Stein DJ, Mueggler T, Baldwin DS (2009): Research in anxiety disorders: from the bench to the bedside. *Eur Neuropsychopharmacol* 19:381-390.
- Gartside SE, Johnson DA, Leitch MM, Troakes C, Ingram CD (2003): Early life adversity programs changes in central 5-HT neuronal function in adulthood. *Eur J Neurosci* 17:2401-2408.
- Gartside SE, Umbers V, Hajos M, Sharp T (1995): Interaction between a selective 5-HT<sub>1A</sub> receptor antagonist and an SSRI in vivo: effects on 5-HT cell firing and extracellular 5-HT. *Br J Pharmacol* 115:1064-1070.
- Gaspar P, Cases O, Maroteaux L (2003): The developmental role of serotonin: news from mouse molecular genetics. *Nat Rev Neurosci* 4:1002-1012.
- Gaykema RP, Chen CC, Goehler LE (2007): Organization of immune-responsive medullary projections to the bed nucleus of the stria terminalis, central amygdala, and paraventricular nucleus of the hypothalamus: evidence for parallel viscerosensory pathways in the rat brain. *Brain Res* 1130:130-145.
- Gervasoni D, Peyron C, Rampon C, Barbagli B, Chouvet G, Urbain N, et al (2000): Role and origin of the GABAergic innervation of dorsal raphe serotonergic neurons. *J Neurosci* 20:4217-4225.
- Giardina WJ, Radek RJ (1991): Effects of imipramine on the nocturnal behavior of bilateral olfactory bulbectomized rats. *Biol Psychiatry* 29:1200-1208.
- Gillespie NA, Kirk KM, Evans DM, Heath AC, Hickie IB, Martin NG (2004): Do the genetic or environmental determinants of anxiety and depression change with age? A longitudinal study of Australian twins. *Twin Res* 7:39-53.
- Gillman PK (2007): Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. *Br J Pharmacol* 151:737-748.
- Gingrich JA, Hen R (2001): Dissecting the role of the serotonin system in neuropsychiatric disorders using knockout mice. *Psychopharmacology (Berl)* 155:1-10.
- Gjoni T, Desrayaud S, Imobersteg S, Urwyler S (2006): The positive allosteric modulator GS39783 enhances GABA<sub>(B)</sub> receptor-mediated inhibition of cyclic AMP formation in rat striatum in vivo. *J Neurochem* 96:1416-1422.
- Gobbi G, Murphy DL, Lesch K, Blier P (2001): Modifications of the serotonergic system in mice lacking serotonin transporters: an in vivo electrophysiological study. *J Pharmacol Exp Ther* 296:987-995.
- Gold PW, Chrousos GP (2002): Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. *Mol Psychiatry* 7:254-275.
- Gonzalez-Pardo H, Conejo NM, Arias JL (2006): Oxidative metabolism of limbic structures after acute administration of diazepam, alprazolam and zolpidem. *Prog Neuropsychopharmacol Biol Psychiatry* 30:1020-1026.

- Gordon C, Rowsey P, Yang Y (2002): Effect of repeated nicotine exposure on core temperature and motor activity in male and female rats. *Journal of Thermal Biology* 27:485-492.
- Gotsev T, Ivanov A (1950): Psychogenic elevation of body temperature in healthy persons. *Acta Physiol Hung* 1:53-62.
- Gotsev T, Ivanov A (1954): Elevation of body temperature in students during examinations. *Acta Physiol Hung* 6:427-431.
- Graham NM, Burrell CJ, Douglas RM, DeBelle P, Davies L (1990): Adverse effects of aspirin, acetaminophen, and ibuprofen on immune function, viral shedding, and clinical status in rhinovirus-infected volunteers. *J Infect Dis* 162:1277-1282.
- Grailhe R, Waeber C, Dulawa SC, Hornung JP, Zhuang X, Brunner D, et al (1999): Increased exploratory activity and altered response to LSD in mice lacking the 5-HT<sub>5A</sub> receptor. *Neuron* 22:581-591.
- Grant BF, Hasin DS, Stinson FS, Dawson DA, June Ruan W, Goldstein RB, et al (2005): Prevalence, correlates, co-morbidity, and comparative disability of DSM-IV generalized anxiety disorder in the USA: results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Psychol Med* 35:1747-1759.
- Gray DA, Maloney SK, Kamerman PR (2008): Restraint increases afebrile body temperature but attenuates fever in Pekin ducks (*Anas platyrhynchos*). *Am J Physiol Regul Integr Comp Physiol* 294:R1666-1671.
- Gray TS (1993): Amygdaloid CRF pathways. Role in autonomic, neuroendocrine, and behavioral responses to stress. *Ann N Y Acad Sci* 697:53-60.
- Griebel G, Belzung C, Perrault G, Sanger DJ (2000a): Differences in anxiety-related behaviors and in sensitivity to diazepam in inbred and outbred strains of mice. *Psychopharmacology (Berl)* 148:164-170.
- Griebel G, Perrault G, Letang V, Granger P, Avenet P, Schoemaker H, Sanger DJ (1999): New evidence that the pharmacological effects of benzodiazepine receptor ligands can be associated with activities at different BZ (omega) receptor subtypes. *Psychopharmacology (Berl)* 146:205-213.
- Griebel G, Rodgers RJ, Perrault G, Sanger DJ (2000b): The effects of compounds varying in selectivity as 5-HT<sub>1A</sub> receptor antagonists in three rat models of anxiety. *Neuropharmacology* 39:1848-1857.
- Griebel G, Simiand J, Steinberg R, Jung M, Gully D, Roger P, et al (2002): 4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]-5-methyl-N-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor(1) receptor antagonist. II. Characterization in rodent models of stress-related disorders. *J Pharmacol Exp Ther* 301:333-345.
- Grimm VE, Jancourt A (1983): The effects of chronic diazepam treatment on body weight and food intake in rats. *Int J Neurosci* 18:127-135.
- Groenink L, Dirks A, Verdouw PM, Schipholt M, Veening JG, van der Gugten J, Olivier B (2002): HPA axis dysregulation in mice overexpressing corticotropin releasing hormone. *Biol Psychiatry* 51:875-881.
- Groenink L, Mos J, Van der Gugten J, Olivier B (1996a): The 5-HT<sub>1A</sub> receptor is not involved in emotional stress-induced rises in stress hormones. *Pharmacol Biochem Behav* 55:303-308.
- Groenink L, Pattij T, De Jongh R, Van der Gugten J, Oosting RS, Dirks A, Olivier B (2003a): 5-HT<sub>1A</sub> receptor knockout mice and mice overexpressing corticotropin-releasing hormone in models of anxiety. *Eur J Pharmacol* 463:185-197.
- Groenink L, van Bogaert MJ, van der Gugten J, Oosting RS, Olivier B (2003b): 5-HT<sub>1A</sub> receptor and 5-HT<sub>1B</sub> receptor knockout mice in stress and anxiety paradigms. *Behav Pharmacol* 14:369-383.
- Groenink L, van der Gugten J, Zethof T, van der Heyden J, Olivier B (1994): Stress-induced hyperthermia in mice: hormonal correlates. *Physiol Behav* 56:747-749.

- Groenink L, van der Gugten J, Zethof TJ, van der Heyden JA, Olivier B (1996b): Neuroendocrine effects of diazepam and flesinoxan in the stress-induced hyperthermia test in mice. *Pharmacol Biochem Behav* 54:249-254.
- Groenink L, Verdouw PM, van Oorschot R, Olivier B (2009): Models of anxiety: ultrasonic vocalizations of isolated rat pups. *Current protocols of pharmacology* S43:5.18.11-15.18.14.
- Groenink L, Vinkers CH, van Oorschot R, Olivier B (2009): Models of anxiety: Stress-Induced Hyperthermia (SIH) in singly housed mice. *Current protocols of pharmacology* S45:5.16.11 - 15.16.12.
- Gross C, Santarelli L, Brunner D, Zhuang X, Hen R (2000): Altered fear circuits in 5-HT<sub>(1A)</sub> receptor KO mice. *Biol Psychiatry* 48:1157-1163.
- Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, et al (2002): Serotonin1A receptor acts during development to establish normal anxiety-like behavior in the adult. *Nature* 416:396-400.
- Guscott M, Bristow LJ, Hadingham K, Rosahl TW, Beer MS, Stanton JA, et al (2005): Genetic knockout and pharmacological blockade studies of the 5-HT<sub>7</sub> receptor suggest therapeutic potential in depression. *Neuropharmacology* 48:492-502.
- Guscott MR, Egan E, Cook GP, Stanton JA, Beer MS, Rosahl TW, et al (2003): The hypothermic effect of 5-CT in mice is mediated through the 5-HT<sub>7</sub> receptor. *Neuropharmacology* 44:1031-1037.

## H

- Hackler EA, Turner GH, Gresch PJ, Sengupta S, Deutch AY, Avison MJ, et al (2007): 5-Hydroxytryptamine<sub>2C</sub> receptor contribution to m-chlorophenylpiperazine and N-methyl-beta-carboline-3-carboxamide-induced anxiety-like behavior and limbic brain activation. *J Pharmacol Exp Ther* 320:1023-1029.
- Haefely W, Martin JR, Schoch P (1990): Novel anxiolytics that act as partial agonists at benzodiazepine receptors. *Trends Pharmacol Sci* 11:452-456.
- Hagan JJ, Price GW, Jeffrey P, Deeks NJ, Stean T, Piper D, et al (2000): Characterization of SB-269970-A, a selective 5-HT<sub>(7)</sub> receptor antagonist. *Br J Pharmacol* 130:539-548.
- Hamson DK, Jones BA, Watson NV (2004): Distribution of androgen receptor immunoreactivity in the brainstem of male rats. *Neuroscience* 127:797-803.
- Han WW, Yakatan GJ, Maness DD (1976): Kinetics and mechanisms of hydrolysis of 1,4-benzodiazepines I: chlordiazepoxide and demoxepam. *J Pharm Sci* 65:1198-1204.
- Haney M, Comer SD, Fischman MW, Foltin RW (1997): Alprazolam increases food intake in humans. *Psychopharmacology (Berl)* 132:311-314.
- Harandi M, Aguera M, Gamrani H, Didier M, Maitre M, Calas A, Belin MF (1987): gamma-Aminobutyric acid and 5-hydroxytryptamine interrelationship in the rat nucleus raphe dorsalis: combination of radioautographic and immunocytochemical techniques at light and electron microscopy levels. *Neuroscience* 21:237-251.
- Hariri AR, Drabant EM, Munoz KE, Kolachana BS, Mattay VS, Egan MF, Weinberger DR (2005): A susceptibility gene for affective disorders and the response of the human amygdala. *Arch Gen Psychiatry* 62:146-152.
- Hariri AR, Mattay VS, Tessitore A, Kolachana B, Fera F, Goldman D, et al (2002): Serotonin transporter genetic variation and the response of the human amygdala. *Science* 297:400-403.
- Harper RM, Frysiner RC, Trelease RB, Marks JD (1984): State-dependent alteration of respiratory cycle timing by stimulation of the central nucleus of the amygdala. *Brain Res* 306:1-8.
- Harris RA (1999): Ethanol actions on multiple ion channels: which are important? *Alcohol Clin Exp Res* 23:1563-1570.
- Hauger RL, Risbrough V, Brauns O, Dautzenberg FM (2006): Corticotropin releasing factor (CRF) receptor signaling in the central nervous system: new molecular targets. *CNS Neurol Disord Drug Targets* 5:453-479.
- Heim C, Nemeroff CB (2001): The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* 49:1023-1039.

- Heinrichs SC, Lapsansky J, Lovenberg TW, De Souza EB, Chalmers DT (1997): Corticotropin-releasing factor CRF<sub>1</sub>, but not CRF<sub>2</sub>, receptors mediate anxiogenic-like behavior. *Regul Pept* 71:15-21.
- Heinrichs SC, Li DL, Iyengar S (2001): Corticotropin-releasing factor (CRF) or CRF binding-protein ligand inhibitor administration suppresses food intake in mice and elevates body temperature in rats. *Brain Res* 900:177-185.
- Heisler LK, Chu HM, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH (1998): Elevated anxiety and antidepressant-like responses in serotonin 5-HT<sub>1A</sub> receptor mutant mice. *Proc Natl Acad Sci U S A* 95:15049-15054.
- Heisler LK, Zhou L, Bajwa P, Hsu J, Tecott LH (2007): Serotonin 5-HT<sub>2C</sub> receptors regulate anxiety-like behavior. *Genes Brain Behav* 6:491-496.
- Heninger C, Saito N, Tallman JF, Garrett KM, Vitek MP, Duman RS, Gallager DW (1990): Effects of continuous diazepam administration on GABA<sub>A</sub> subunit mRNA in rat brain. *J Mol Neurosci* 2:101-107.
- Herdade KC, Strauss CV, Zangrossi Junior H, Viana MB (2006): Effects of medial amygdala inactivation on a panic-related behavior. *Behav Brain Res* 172:316-323.
- Herman JP, Cullinan WE (1997): Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. *Trends Neurosci* 20:78-84.
- Herman JP, Figueiredo H, Muellera NK, Ulrich-Lai Y, Ostrander MM, Choia DC, Cullinan WE (2003): Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Frontiers in Neuroendocrinology* 24:151-180.
- Herman JP, Mueller NK, Figueiredo H (2004): Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration. *Ann N Y Acad Sci* 1018:35-45.
- Herman JP, Ostrander MM, Mueller NK, Figueiredo H (2005): Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry* 29:1201-1213.
- Herman JP, Tasker JG, Ziegler DR, Cullinan WE (2002): Local circuit regulation of paraventricular nucleus stress integration: glutamate-GABA connections. *Pharmacol Biochem Behav* 71:457-468.
- Herrel A, James RS, Van Damme R (2007): Fight versus flight: physiological basis for temperature-dependent behavioral shifts in lizards. *J Exp Biol* 210:1762-1767.
- Hettema JM, Neale MC, Kendler KS (2001): A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am J Psychiatry* 158:1568-1578.
- Hinderer SR (1990): The supraspinal anxiolytic effect of baclofen for spasticity reduction. *Am J Phys Med Rehabil* 69:254-258.
- Hjorth S, Westlin D, Bengtsson HJ (1997): WAY100635-induced augmentation of the 5-HT-elevating action of citalopram: relative importance of the dose of the 5-HT<sub>1A</sub> (auto)receptor blocker versus that of the 5-HT reuptake inhibitor. *Neuropharmacology* 36:461-465.
- Ho YJ, Chang YC, Liu TM, Tai MY, Wong CS, Tsai YF (2000): Striatal glutamate release during novelty exposure-induced hyperactivity in olfactory bulbectomized rats. *Neurosci Lett* 287:117-120.
- Hoehn-Saric R, McLeod DR, Funderburk F, Kowalski P (2004): Somatic symptoms and physiologic responses in generalized anxiety disorder and panic disorder: an ambulatory monitor study. *Arch Gen Psychiatry* 61:913-921.
- Holmes A, Iles JP, Mayell SJ, Rodgers RJ (2001): Prior test experience compromises the anxiolytic efficacy of chlordiazepoxide in the mouse light/dark exploration test. *Behav Brain Res* 122:159-167.
- Holmes A, Lit Q, Murphy DL, Gold E, Crawley JN (2003a): Abnormal anxiety-related behavior in serotonin transporter null mutant mice: the influence of genetic background. *Genes Brain Behav* 2:365-380.

- Holmes A, Murphy DL, Crawley JN (2003b): Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. *Biol Psychiatry* 54:953-959.
- Holmes A, Yang RJ, Lesch KP, Crawley JN, Murphy DL (2003c): Mice lacking the serotonin transporter exhibit 5-HT<sub>1A</sub> receptor-mediated abnormalities in tests for anxiety-like behavior. *Neuropsychopharmacology* 28:2077-2088.
- Holsboer F (2000): The corticosteroid receptor hypothesis of depression. *Neuropsychopharmacology* 23:477-501.
- Holt RA, Bateson AN, Martin IL (1997): Chronic zolpidem treatment alters GABA<sub>A</sub> receptor mRNA levels in the rat cortex. *Eur J Pharmacol* 329:129-132.
- Homberg JR, De Boer SF, Raaso HS, Olivier JD, Verheul M, Ronken E, et al (2008): Adaptations in pre- and postsynaptic 5-HT<sub>1A</sub> receptor function and cocaine supersensitivity in serotonin transporter knockout rats. *Psychopharmacology (Berl)* 200:367-380.
- Hori T, Kiyohara T, Shibata M, Oomura Y, Nishino H, Aou S, Fujita I (1986): Responsiveness of monkey preoptic thermosensitive neurons to non-thermal emotional stimuli. *Brain Res Bull* 17:75-82.
- Horiuchi J, McDowall LM, Dampney RA (2006): Differential control of cardiac and sympathetic vasomotor activity from the dorsomedial hypothalamus. *Clin Exp Pharmacol Physiol* 33:1265-1268.
- Hosie AM, Clarke L, da Silva H, Smart TG (2009): Conserved site for neurosteroid modulation of GABA<sub>A</sub> receptors. *Neuropharmacology* 56:149-154.
- Hosie AM, Wilkins ME, Smart TG (2007): Neurosteroid binding sites on GABA<sub>A</sub> receptors. *Pharmacol Ther* 116:7-19.
- Hoyer D, Pazos A, Probst A, Palacios JM (1986): Serotonin receptors in the human brain. I. Characterization and autoradiographic localization of 5-HT<sub>1A</sub> recognition sites. Apparent absence of 5-HT<sub>1B</sub> recognition sites. *Brain Res* 376:85-96.
- Hrdina PD, Foy B, Hepner A, Summers RJ (1990): Antidepressant binding sites in brain: autoradiographic comparison of [3H]paroxetine and [3H]imipramine localization and relationship to serotonin transporter. *J Pharmacol Exp Ther* 252:410-418.
- Huang Q, He X, Ma C, Liu R, Yu S, Dayer CA, et al (2000): Pharmacophore/receptor models for GABA(A)/BzR subtypes (alpha1beta3gamma2, alpha5beta3gamma2, and alpha6beta3gamma2) via a comprehensive ligand-mapping approach. *J Med Chem* 43:71-95.
- Humphries AC, Gancia E, Gilligan MT, Goodacre S, Hallett D, Merchant KJ, Thomas SR (2006): 8-Fluoroimidazo[1,2-a]pyridine: synthesis, physicochemical properties and evaluation as a bioisosteric replacement for imidazo[1,2-a]pyrimidine in an allosteric modulator ligand of the GABA A receptor. *Bioorg Med Chem Lett* 16:1518-1522.
- Hunter WS (1997): Anteroventral third ventricle lesion suppresses fever, but not stress-induced hyperthermia in rats. *Ann N Y Acad Sci* 813:420-426.
- Huopaniemi L, Keist R, Randolph A, Certa U, Rudolph U (2004): Diazepam-induced adaptive plasticity revealed by alpha1 GABA<sub>A</sub> receptor-specific expression profiling. *J Neurochem* 88:1059-1067.
- Hutchinson MA, Smith PF, Darlington CL (1996): The behavioral and neuronal effects of the chronic administration of benzodiazepine anxiolytic and hypnotic drugs. *Prog Neurobiol* 49:73-97.
- I
- Iijima M, Shimazaki T, Ito A, Chaki S (2007): Effects of metabotropic glutamate 2/3 receptor antagonists in the stress-induced hyperthermia test in singly housed mice. *Psychopharmacology (Berl)* 190:233-239.
- Ikegaya Y, Abe K, Saito H, Nishiyama N (1995): Medial amygdala enhances synaptic transmission and synaptic plasticity in the dentate gyrus of rats in vivo. *J Neurophysiol* 74:2201-2203.

- Impagnatiello F, Pesold C, Longone P, Caruncho H, Fritschy JM, Costa E, Guidotti A (1996): Modifications of gamma-aminobutyric acid<sub>A</sub> receptor subunit expression in rat neocortex during tolerance to diazepam. *Mol Pharmacol* 49:822-831.
- Iyer RN, Bradberry CW (1996): Serotonin-mediated increase in prefrontal cortex dopamine release: pharmacological characterization. *J Pharmacol Exp Ther* 277:40-47.

**J**

- Jacobson LH, Cryan JF (2005): Differential sensitivity to the motor and hypothermic effects of the GABA<sub>B</sub> receptor agonist baclofen in various mouse strains. *Psychopharmacology (Berl)* 179:688-699.
- Jacobson LH, Cryan JF (2008): Evaluation of the anxiolytic-like profile of the GABA<sub>B</sub> receptor positive modulator CGP7930 in rodents. *Neuropharmacology* 54:854-862.
- Jacobson LH, Guery S, Froestl W, Gentsch C, Enz A, Cryan JF, Kaupmann K (2008): In vitro and in vivo characterization of a novel GABA<sub>B</sub> receptor positive allosteric modulator. *Program No 8245 Neuroscience meeting planner, Washington, DC, Society for Neuroscience.*
- Jia F, Chandra D, Homanics GE, Harrison NL (2008): Ethanol modulates synaptic and extrasynaptic GABA<sub>A</sub> receptors in the thalamus. *J Pharmacol Exp Ther* 326:475-482.
- Jia F, Pignataro L, Schofield CM, Yue M, Harrison NL, Goldstein PA (2005): An extrasynaptic GABA<sub>A</sub> receptor mediates tonic inhibition in thalamic VB neurons. *J Neurophysiol* 94:4491-4501.
- Jiang Q, Cross AS, Singh IS, Chen TT, Viscardi RM, Hasday JD (2000): Febrile core temperature is essential for optimal host defense in bacterial peritonitis. *Infect Immun* 68:1265-1270.
- Jiang X, Xing G, Yang C, Verma A, Zhang L, Li H (2009): Stress impairs 5-HT<sub>2A</sub> receptor-mediated serotonergic facilitation of GABA release in juvenile rat basolateral amygdala. *Neuropsychopharmacology* 34:410-423.
- Joels M, Baram TZ (2009): The neuro-symphony of stress. *Nat Rev Neurosci* 10:459-466.
- Joffe H, Cohen LS (1998): Estrogen, serotonin, and mood disturbance: where is the therapeutic bridge? *Biol Psychiatry* 44:798-811.
- Joffe H, Soares CN, Petrillo LF, Viguera AC, Somley BL, Koch JK, Cohen LS (2007): Treatment of depression and menopause-related symptoms with the serotonin-norepinephrine reuptake inhibitor duloxetine. *J Clin Psychiatry* 68:943-950.
- Jones BJ, Costall B, Domeney AM, Kelly ME, Naylor RJ, Oakley NR, Tyers MB (1988): The potential anxiolytic activity of GR38032F, a 5-HT<sub>3</sub>-receptor antagonist. *Br J Pharmacol* 93:985-993.

**K**

- Kahn RS, Wetzler S, Asnis GM, Kling MA, Suckow RF, van Praag HM (1990): Effects of m-chlorophenylpiperazine in normal subjects: a dose-response study. *Psychopharmacology (Berl)* 100:339-344.
- Kaiyala KJ, Vincow ES, Sexton TJ, Neumaier JF (2003): 5-HT<sub>1B</sub> receptor mRNA levels in dorsal raphe nucleus: inverse association with anxiety behavior in the elevated plus maze. *Pharmacol Biochem Behav* 75:769-776.
- Kajantie E, Phillips DI (2006): The effects of sex and hormonal status on the physiological response to acute psychosocial stress. *Psychoneuroendocrinology* 31:151-178.
- Kakizaki Y, Watanobe H, Kohsaka A, Suda T (1999): Temporal profiles of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in the plasma and hypothalamic paraventricular nucleus after intravenous or intraperitoneal administration of lipopolysaccharide in the rat: estimation by push-pull perfusion. *Endocr J* 46:487-496.
- Kalueff AV, Nutt DJ (2007): Role of GABA in anxiety and depression. *Depress Anxiety* 24:495-517.
- Kang I, Miller LG (1991): Decreased GABA<sub>A</sub> receptor subunit mRNA concentrations following chronic lorazepam administration. *Br J Pharmacol* 103:1285-1287.
- Kang N, Baum MJ, Cherry JA (2009): A direct main olfactory bulb projection to the 'vomeronasal' amygdala in female mice selectively responds to volatile pheromones from males. *Eur J Neurosci* 29:624-634.

- Karamanakos PN, Pappas P, Marselos M (2004): Involvement of the brain serotonergic system in the locomotor stimulant effects of chlorpheniramine in Wistar rats: implication of postsynaptic 5-HT<sub>1A</sub> receptors. *Behav Brain Res* 148:199-208.
- Kas MJ, de Mooij-van Malsen AJ, Olivier B, Spruijt BM, van Ree JM (2008): Differential genetic regulation of motor activity and anxiety-related behaviors in mice using an automated home cage task. *Behav Neurosci* 122:769-776.
- Kas MJ, de Mooij-van Malsen JG, de Krom M, van Gassen KL, van Lith HA, Olivier B, et al (2009a): High-resolution genetic mapping of mammalian motor activity levels in mice. *Genes Brain Behav* 8:13-22.
- Kas MJ, Gelegen C, van Nieuwerburgh F, Westenberg HG, Deforce D, Denys D (2009b): Compulsivity in mouse strains homologous with chromosomes 7p and 15q linked to obsessive-compulsive disorder. *Am J Med Genet B Neuropsychiatr Genet*.
- Kaschka W, Feistel H, Ebert D (1995): Reduced benzodiazepine receptor binding in panic disorders measured by iomazenil SPECT. *J Psychiatr Res* 29:427-434.
- Kash TL, Winder DG (2006): Neuropeptide Y and corticotropin-releasing factor bi-directionally modulate inhibitory synaptic transmission in the bed nucleus of the stria terminalis. *Neuropharmacology* 51:1013-1022.
- Kawasaki H, Watanabe S, Ueki S (1980): Changes in blood pressure and heart rate following bilateral olfactory bulbectomy in rats. *Physiol Behav* 24:51-56.
- Kawashima N, Karasawa J, Shimazaki T, Chaki S, Okuyama S, Yasuhara A, Nakazato A (2005): Neuropharmacological profiles of antagonists of group II metabotropic glutamate receptors. *Neurosci Lett* 378:131-134.
- Kelly JP, Wrynn AS, Leonard BE (1997): The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol Ther* 74:299-316.
- Kennett GA, Bailey F, Piper DC, Blackburn TP (1995): Effect of SB 200646A, a 5-HT<sub>2C</sub>/5-HT<sub>2B</sub> receptor antagonist, in two conflict models of anxiety. *Psychopharmacology (Berl)* 118:178-182.
- Kennett GA, Bright F, Trail B, Blackburn TP, Sanger GJ (1997a): Anxiolytic-like actions of the selective 5-HT<sub>4</sub> receptor antagonists SB 204070A and SB 207266A in rats. *Neuropharmacology* 36:707-712.
- Kennett GA, Whitton P, Shah K, Curzon G (1989): Anxiogenic-like effects of mCPP and TFMP in animal models are opposed by 5-HT<sub>1C</sub> receptor antagonists. *Eur J Pharmacol* 164:445-454.
- Kennett GA, Wood MD, Bright F, Trail B, Riley G, Holland V, et al (1997b): SB 242084, a selective and brain penetrant 5-HT<sub>2C</sub> receptor antagonist. *Neuropharmacology* 36:609-620.
- Kerman IA (2008): Organization of brain somatomotor-sympathetic circuits. *Exp Brain Res* 187:1-16.
- Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE (2005): Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. *Arch Gen Psychiatry* 62:617-627.
- Kevetter GA, Winans SS (1981): Connections of the corticomedial amygdala in the golden hamster. I. Efferents of the "vomeronasal amygdala". *J Comp Neurol* 197:81-98.
- Kimura H, Kuriyama K (1975): Distribution of gamma-aminobutyric acid (GABA) in the rat hypothalamus: functional correlates of GABA with activities of appetite controlling mechanisms. *J Neurochem* 24:903-907.
- Kinney GG, Taber MT, Gribkoff VK (2000): The augmentation hypothesis for improvement of antidepressant therapy: is pindolol a suitable candidate for testing the ability of 5HT<sub>1A</sub> receptor antagonists to enhance SSRI efficacy and onset latency? *Mol Neurobiol* 21:137-152.
- Kirby LG, Freeman-Daniels E, Lemos JC, Nunan JD, Lamy C, Akanwa A, Beck SG (2008): Corticotropin-releasing factor increases GABA synaptic activity and induces inward current in 5-hydroxytryptamine dorsal raphe neurons. *J Neurosci* 28:12927-12937.
- Kirschbaum C, Pirke KM, Hellhammer DH (1993): The 'Trier Social Stress Test'--a tool for investigating psychobiological stress responses in a laboratory setting. *Neuropsychobiology* 28:76-81.

- Kishimoto K, Koyama S, Akaike N (2000): Presynaptic modulation of synaptic gamma-aminobutyric acid transmission by tandospirone in rat basolateral amygdala. *Eur J Pharmacol* 407:257-265.
- Klein E, Zohar J, Geraci MF, Murphy DL, Uhde TW (1991): Anxiogenic effects of m-CPP in patients with panic disorder: comparison to caffeine's anxiogenic effects. *Biol Psychiatry* 30:973-984.
- Kleitman N, Jackson DP (1950): Body temperature and performance under different routines. *J Appl Physiol* 3:309-328.
- Kluger MJ (1991): Fever: role of pyrogens and cryogens. *Physiol Rev* 71:93-127.
- Kluger MJ, O'Reilly B, Shope TR, Vander AJ (1987): Further evidence that stress hyperthermia is a fever. *Physiol Behav* 39:763-766.
- Koch M (1999): The neurobiology of startle. *Prog Neurobiol* 59:107-128.
- Kollack-Walker S, Watson SJ, Akil H (1997): Social stress in hamsters: defeat activates specific neurocircuits within the brain. *J Neurosci* 17:8842-8855.
- Komesaroff PA, Esler MD, Sudhir K (1999): Estrogen supplementation attenuates glucocorticoid and catecholamine responses to mental stress in perimenopausal women. *J Clin Endocrinol Metab* 84:606-610.
- Korosi A, Veening JG, Kozicz T, Henckens M, Dederen J, Groenink L, et al (2006): Distribution and expression of CRF receptor 1 and 2 mRNAs in the CRF over-expressing mouse brain. *Brain Res* 1072:46-54.
- Korpi ER, Grunder G, Luddens H (2002): Drug interactions at GABA<sub>A</sub> receptors. *Prog Neurobiol* 67:113-159.
- Korte SM, Koolhaas JM, Schuurman T, Traber J, Bohus B (1990): Anxiolytics and stress-induced behavioral and cardiac responses: a study of diazepam and ipsapirone (TVX Q 7821). *Eur J Pharmacol* 179:393-401.
- Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005): The Darwinian concept of stress: benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. *Neurosci Biobehav Rev* 29:3-38.
- Kubo T, Okatani H, Nishigori Y, Hagiwara Y, Fukumori R, Goshima Y (2004): Involvement of the medial amygdaloid nucleus in restraint stress-induced pressor responses in rats. *Neurosci Lett* 354:84-86.
- Kudielka BM, Schommer NC, Hellhammer DH, Kirschbaum C (2004): Acute HPA axis responses, heart rate, and mood changes to psychosocial stress (TSST) in humans at different times of day. *Psychoneuroendocrinology* 29:983-992.
- Kumar S, Kralic JE, O'Buckley TK, Grobin AC, Morrow AL (2003): Chronic ethanol consumption enhances internalization of alpha1 subunit-containing GABA<sub>A</sub> receptors in cerebral cortex. *J Neurochem* 86:700-708.
- Kung MP, Zhuang ZP, Frederick D, Kung HF (1994): In vivo binding of [123I]4-(2'-methoxyphenyl)-1-[2'-(N-2"-pyridinyl)- p-iodobenzamido-]ethyl-piperazine, p-MPPI, to 5-HT<sub>1A</sub> receptors in rat brain. *Synapse* 18:359-366.
- Kuwaki T, Zhang W, Nakamura A, Deng BS (2008): Emotional and state-dependent modification of cardiorespiratory function: role of orexinergic neurons. *Auton Neurosci* 142:11-16.

**L**

- Laarakker MC, Ohl F, van Lith HA (2008): Chromosomal assignment of quantitative trait loci influencing modified hole board behavior in laboratory mice using consomic strains, with special reference to anxiety-related behavior and mouse chromosome 19. *Behav Genet* 38:159-184.
- Laarakker MJ, Ohl F, Lith van HJ (2006): Reducing the number of animals used in behavioral genetic experiments using chromosome substitution strains. *Animal Welfare* 15:49-54.
- Lack LC, Gradisar M, Van Someren EJ, Wright HR, Lushington K (2008): The relationship between insomnia and body temperatures. *Sleep Med Rev* 12:307-317.

- Lacroix S, Rivest S (1997): Functional circuitry in the brain of immune-challenged rats: partial involvement of prostaglandins. *J Comp Neurol* 387:307-324.
- Lacroix S, Vallieres L, Rivest S (1996): C-fos mRNA pattern and corticotropin-releasing factor neuronal activity throughout the brain of rats injected centrally with a prostaglandin of E<sub>2</sub> type. *J Neuroimmunol* 70:163-179.
- Lancel M, Langebartels A (2000): gamma-aminobutyric Acid(A) (GABA<sub>(A)</sub>) agonist 4,5,6, 7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol persistently increases sleep maintenance and intensity during chronic administration to rats. *J Pharmacol Exp Ther* 293:1084-1090.
- Langen B, Dietze S, Fink H (2002): Acute effect of ethanol on anxiety and 5-HT in the prefrontal cortex of rats. *Alcohol* 27:135-141.
- Lau CE, Ma F, Wang Y, Smith C (1996): Pharmacokinetics and bioavailability of midazolam after intravenous, subcutaneous, intraperitoneal and oral administration under a chronic food-limited regimen: relating DRL performance to pharmacokinetics. *Psychopharmacology (Berl)* 126:241-248.
- Lecci A, Borsini F, Mancinelli A, D'Aranno V, Stasi MA, Volterra G, Meli A (1990a): Effect of serotoninergic drugs on stress-induced hyperthermia (SIH) in mice. *J Neural Transm Gen Sect* 82:219-230.
- Lecci A, Borsini F, Volterra G, Meli A (1990b): Pharmacological validation of a novel animal model of anticipatory anxiety in mice. *Psychopharmacology (Berl)* 101:255-261.
- Leclercq Y (2007): Widespread underrecognition and undertreatment of anxiety and mood disorders: results from 3 European studies. *J Clin Psychiatry* 68 Suppl 2:36-41.
- LeDoux JE (2000): Emotion circuits in the brain. *Annu Rev Neurosci* 23:155-184.
- Leonard BE, Song C (1999): Stress, depression, and the role of cytokines. *Adv Exp Med Biol* 461:251-265.
- Lepicard EM, Joubert C, Hagneau I, Perez-Diaz F, Chapouthier G (2000): Differences in anxiety-related behavior and response to diazepam in BALB/cByJ and C57BL/6J strains of mice. *Pharmacol Biochem Behav* 67:739-748.
- Lerer B, Gelfin Y, Gorfine M, Allolio B, Lesch KP, Newman ME (1999): 5-HT<sub>1A</sub> receptor function in normal subjects on clinical doses of fluoxetine: blunted temperature and hormone responses to ipsapirone challenge. *Neuropsychopharmacology* 20:628-639.
- Lesch KP, Hoh A, Schulte HM, Osterheider M, Muller T (1991): Long-term fluoxetine treatment decreases 5-HT<sub>1A</sub> receptor responsivity in obsessive-compulsive disorder. *Psychopharmacology (Berl)* 105:415-420.
- Lesscher HM, Kas MJ, van der Elst S, van Lith HA, Vanderschuren LJ (2009): A grandparent-influenced locus for alcohol preference on mouse chromosome 2. *Pharmacogenet Genomics*.
- Leventopoulos M, Russig H, Feldon J, Pryce CR, Opacka-Juffry J (2009): Early deprivation leads to long-term reductions in motivation for reward and 5-HT<sub>1A</sub> binding and both effects are reversed by fluoxetine. *Neuropharmacology* 56:692-701.
- Levinson DF (2006): The genetics of depression: a review. *Biol Psychiatry* 60:84-92.
- Li CI, Magliano TL, Takahashi LK (2004): Medial amygdala modulation of predator odor-induced unconditioned fear in the rat. *Behav Neurosci* 118:324-332.
- Li Q, Wichems C, Heils A, Van De Kar LD, Lesch KP, Murphy DL (1999): Reduction of 5-hydroxytryptamine (5-HT)<sub>(1A)</sub>-mediated temperature and neuroendocrine responses and 5-HT<sub>(1A)</sub> binding sites in 5-HT transporter knockout mice. *J Pharmacol Exp Ther* 291:999-1007.
- Licht CM, de Geus EJ, van Dyck R, Penninx BW (2009): Association between anxiety disorders and heart rate variability in The Netherlands Study of Depression and Anxiety (NESDA). *Psychosom Med* 71:508-518.
- Lin D, Parsons LH (2002): Anxiogenic-like effect of serotonin<sub>(1B)</sub> receptor stimulation in the rat elevated plus-maze. *Pharmacol Biochem Behav* 71:581-587.

- Lin KM, Friedel RO (1979): Relationship of plasma levels of chlordiazepoxide and metabolites to clinical response. *Am J Psychiatry* 136:18-23.
- Lindheim SR, Legro RS, Bernstein L, Stanczyk FZ, Vijod MA, Presser SC, Lobo RA (1992): Behavioral stress responses in premenopausal and postmenopausal women and the effects of estrogen. *Am J Obstet Gynecol* 167:1831-1836.
- Linthorst AC, Penalva RG, Flachskamm C, Holsboer F, Reul JM (2002): Forced swim stress activates rat hippocampal serotonergic neurotransmission involving a corticotropin-releasing hormone receptor-dependent mechanism. *Eur J Neurosci* 16:2441-2452.
- Lippa A, Czobor P, Stark J, Beer B, Kostakis E, Gravielle M, et al (2005): Selective anxiolysis produced by ocinaplon, a GABA<sub>(A)</sub> receptor modulator. *Proc Natl Acad Sci U S A* 102:7380-7385.
- Lissek S, Powers AS, McClure EB, Phelps EA, Woldehawariat G, Grillon C, Pine DS (2005): Classical fear conditioning in the anxiety disorders: a meta-analysis. *Behav Res Ther* 43:1391-1424.
- Lista A, Blier P, De Montigny C (1989): In vivo presynaptic modulation of serotonergic neurotransmission in the rat hippocampus by diazepam. *Eur J Pharmacol* 171:229-231.
- Liu J, Yu B, Neugebauer V, Grigoriadis DE, Rivier J, Vale WW, et al (2004): Corticotropin-releasing factor and Urocortin I modulate excitatory glutamatergic synaptic transmission. *J Neurosci* 24:4020-4029.
- Lo Iacono L, Gross C (2008): Alpha-Ca2+/calmodulin-dependent protein kinase II contributes to the developmental programming of anxiety in serotonin receptor 1A knock-out mice. *J Neurosci* 28:6250-6257.
- Long NC, Morimoto A, Nakamori T, Yamashiro O, Murakami N (1991): Intraperitoneal injections of prostaglandin E<sub>2</sub> attenuate hyperthermia induced by restraint or interleukin-1 in rats. *J Physiol* 444:363-373.
- Long NC, Vander AJ, Kluger MJ (1990a): Stress-induced rise of body temperature in rats is the same in warm and cool environments. *Physiol Behav* 47:773-775.
- Long NC, Vander AJ, Kunkel SL, Kluger MJ (1990b): Antiserum against tumor necrosis factor increases stress hyperthermia in rats. *Am J Physiol* 258:R591-595.
- Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, et al (2000): Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* 290:131-134.
- Lowther S, De Paermentier F, Cheetham SC, Crompton MR, Katona CL, Horton RW (1997a): 5-HT<sub>1A</sub> receptor binding sites in post-mortem brain samples from depressed suicides and controls. *J Affect Disord* 42:199-207.
- Lowther S, Katona CL, Crompton MR, Horton RW (1997b): Brain [3H]cAMP binding sites are unaltered in depressed suicides, but decreased by antidepressants. *Brain Res* 758:223-228.
- Lowy MT, Meltzer HY (1988): Stimulation of serum cortisol and prolactin secretion in humans by MK-212, a centrally active serotonin agonist. *Biol Psychiatry* 23:818-828.
- Lucki I, Dalvi A, Mayorga AJ (2001): Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. *Psychopharmacology (Berl)* 155:315-322.
- Ludewig K, Geyer MA, Etzensberger M, Vollenweider FX (2002): Stability of the acoustic startle reflex, prepulse inhibition, and habituation in schizophrenia. *Schizophr Res* 55:129-137.
- Lukkes JL, Forster GL, Renner KJ, Summers CH (2008): Corticotropin-releasing factor 1 and 2 receptors in the dorsal raphe differentially affect serotonin release in the nucleus accumbens. *Eur J Pharmacol* 578:185-193.

## M

- Ma S, Morilak DA (2005): Norepinephrine release in medial amygdala facilitates activation of the hypothalamic-pituitary-adrenal axis in response to acute immobilisation stress. *J Neuroendocrinol* 17:22-28.

- Mackowiak P (1997): Normal 'body' temperature. In *Fever Basic Mechanisms and Management* (Mackowiak PA ed) Philadelphia, NY: Lippincott-Raven:207-213.
- MacNeil G, Sela Y, McIntosh J, Zacharko RM (1997): Anxiogenic behavior in the light-dark paradigm following intraventricular administration of cholecystokinin-8S, restraint stress, or uncontrollable footshock in the CD-1 mouse. *Pharmacol Biochem Behav* 58:737-746.
- Madden CJ, Morrison SF (2003): Excitatory amino acid receptor activation in the raphe pallidus area mediates prostaglandin-evoked thermogenesis. *Neuroscience* 122:5-15.
- Madden CJ, Morrison SF (2004): Excitatory amino acid receptors in the dorsomedial hypothalamus mediate prostaglandin-evoked thermogenesis in brown adipose tissue. *Am J Physiol Regul Integr Comp Physiol* 286:R320-325.
- Madjid N, Tottie EE, Lutgen M, Meister B, Sandin J, Kuzmin A, et al (2006): 5-Hydroxytryptamine <sub>1A</sub> receptor blockade facilitates aversive learning in mice: interactions with cholinergic and glutamatergic mechanisms. *J Pharmacol Exp Ther* 316:581-591.
- Malherbe P, Masciadri R, Norcross RD, Knoflach F, Kratzeisen C, Zenner MT, et al (2008): Characterization of (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one as a positive allosteric modulator of GABA<sub>B</sub> receptors. *Br J Pharmacol* 154:797-811.
- Malhotra AK, Murphy GM, Jr., Kennedy JL (2004): Pharmacogenetics of psychotropic drug response. *Am J Psychiatry* 161:780-796.
- Mandema JW, Tukker E, Danhof M (1991): Pharmacokinetic-pharmacodynamic modelling of the EEG effects of midazolam in individual rats: influence of rate and route of administration. *Br J Pharmacol* 102:663-668.
- Mannoury la Cour C, Boni C, Hanoun N, Lesch KP, Hamon M, Lanfumey L (2001): Functional consequences of 5-HT transporter gene disruption on 5-HT(1a) receptor-mediated regulation of dorsal raphe and hippocampal cell activity. *J Neurosci* 21:2178-2185.
- Marazziti D, Di Muro A, Castrogiovanni P (1992): Psychological stress and body temperature changes in humans. *Physiol Behav* 52:393-395.
- Marcilhac A, Maurel D, Anglade G, Ixart G, Mekaouche M, Hery F, Siaud P (1997): Effects of bilateral olfactory bulbectomy on circadian rhythms of ACTH, corticosterone, motor activity and body temperature in male rats. *Arch Physiol Biochem* 105:552-559.
- Marcondes FK, Miguel KJ, Melo LL, Spadari-Bratfisch RC (2001): Estrous cycle influences the response of female rats in the elevated plus-maze test. *Physiol Behav* 74:435-440.
- Marieb E, Hoehn K (2007): *Human anatomy & physiology*, seventh ed. San Francisco: Pearson International Edition.
- Markham CM, Huhman KL (2008): Is the medial amygdala part of the neural circuit modulating conditioned defeat in Syrian hamsters? *Learn Mem* 15:6-12.
- Marowsky A, Fritschy JM, Vogt KE (2004): Functional mapping of GABA A receptor subtypes in the amygdala. *Eur J Neurosci* 20:1281-1289.
- Martinez V, Wang L, Rivier J, Grigoriadis D, Tache Y (2004): Central CRF, urocortins and stress increase colonic transit via CRF<sub>1</sub> receptors while activation of CRF<sub>2</sub> receptors delays gastric transit in mice. *J Physiol* 556:221-234.
- Masaoka Y, Homma I (2004): Amygdala and emotional breathing in humans. *Adv Exp Med Biol* 551:9-14.
- Masini CV, Sasse SK, Garcia RJ, Nyhuis TJ, Day HE, Campeau S (2009): Disruption of neuroendocrine stress responses to acute ferret odor by medial, but not central amygdala lesions in rats. *Brain Res*.
- Mastorakos G, Ilias I (2006): Interleukin-6: a cytokine and/or a major modulator of the response to somatic stress. *Ann N Y Acad Sci* 1088:373-381.
- Mathis C, Neumann PE, Gershenfeld H, Paul SM, Crawley JN (1995): Genetic analysis of anxiety-related behaviors and responses to benzodiazepine-related drugs in AXB and BXA recombinant inbred mouse strains. *Behav Genet* 25:557-568.

- Matsubara S, Arora RC, Meltzer HY (1991): Serotonergic measures in suicide brain: 5-HT<sub>1A</sub> binding sites in frontal cortex of suicide victims. *J Neural Transm Gen Sect* 85:181-194.
- Matsuzaki I, Takamatsu Y, Moroji T (1989): The effects of intracerebroventricularly injected corticotropin-releasing factor (CRF) on the central nervous system: behavioral and biochemical studies. *Neuropeptides* 13:147-155.
- Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, et al (2007): Guidelines on nicotine dose selection for in vivo research. *Psychopharmacology (Berl)* 190:269-319.
- Matuszewich L, Yamamoto BK (2003): Long-lasting effects of chronic stress on DOI-induced hyperthermia in male rats. *Psychopharmacology (Berl)* 169:169-175.
- Maurel JL, Autin JM, Funes P, Newman-Tancredi A, Colpaert F, Vacher B (2007): High-efficacy 5-HT<sub>1A</sub> agonists for antidepressant treatment: a renewed opportunity. *J Med Chem* 50:5024-5033.
- McClellan JL, Klir JJ, Morrow LE, Kluger MJ (1994): Central effects of glucocorticoid receptor antagonist RU-38486 on lipopolysaccharide and stress-induced fever. *Am J Physiol* 267:R705-711.
- McEwen BS, Alves SE (1999): Estrogen actions in the central nervous system. *Endocr Rev* 20:279-307.
- McFarland R (1985): Relationship of skin temperature changes to the emotions accompanying music. *Biofeedback and Self-Regulation* 10:255-267.
- McFarland RA, Kennison R (1989): Asymmetry in the relationship between finger temperature changes and emotional state in males. *Biofeedback Self Regul* 14:281-290.
- McGregor A, Herbert J (1992): Differential effects of excitotoxic basolateral and corticomедial lesions of the amygdala on the behavioral and endocrine responses to either sexual or aggression-promoting stimuli in the male rat. *Brain Res* 574:9-20.
- McIlwain KL, Merriweather MY, Yuva-Paylor LA, Paylor R (2001): The use of behavioral test batteries: effects of training history. *Physiol Behav* 73:705-717.
- McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, et al (2000): Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA<sub>A</sub> receptor alpha<sub>1</sub> subtype. *Nat Neurosci* 3:587-592.
- McKernan RM, Whiting PJ (1996): Which GABAA-receptor subtypes really occur in the brain? *Trends Neurosci* 19:139-143.
- McKinney WT, Jr., Bunney WE, Jr. (1969): Animal model of depression. I. Review of evidence: implications for research. *Arch Gen Psychiatry* 21:240-248.
- McNamara RK, Skelton RW (1997): Tolerance develops to the spatial learning deficit produced by diazepam in rats. *Pharmacol Biochem Behav* 56:383-389.
- McNish KA, Davis M (1997): Olfactory bulbectomy enhances sensitization of the acoustic startle reflex produced by acute or repeated stress. *Behav Neurosci* 111:80-91.
- McQueen JK, Wilson H, Fink G (1997): Estradiol-17 beta increases serotonin transporter (SERT) mRNA levels and the density of SERT-binding sites in female rat brain. *Brain Res Mol Brain Res* 45:13-23.
- Meera P, Olsen RW, Otis TS, Wallner M (2009): Etomidate, propofol and the neurosteroid THDOC increase the GABA efficacy of recombinant alpha4beta3delta and alpha4beta3 GABA<sub>A</sub> receptors expressed in HEK cells. *Neuropharmacology* 56:155-160.
- Melke J, Westberg L, Nilsson S, Landen M, Soderstrom H, Baghaei F, et al (2003): A polymorphism in the serotonin receptor 3A (HTR3A) gene and its association with harm avoidance in women. *Arch Gen Psychiatry* 60:1017-1023.
- Meltzer HY (1990): Role of serotonin in depression. *Ann N Y Acad Sci* 600:486-499; discussion 499-500.
- Merikangas KR, Ames M, Cui L, Stang PE, Ustun TB, Von Korff M, Kessler RC (2007): The impact of comorbidity of mental and physical conditions on role disability in the US adult household population. *Arch Gen Psychiatry* 64:1180-1188.

- Merikangas KR, Angst J, Eaton W, Canino G, Rubio-Stipec M, Wacker H, et al (1996): Comorbidity and boundaries of affective disorders with anxiety disorders and substance misuse: results of an international task force. *Br J Psychiatry Suppl*:58-67.
- Meyer LC, Fick L, Matthee A, Mitchell D, Fuller A (2008): Hyperthermia in captured impala (*Aepyceros melampus*): a fright not flight response. *J Wildl Dis* 44:404-416.
- Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, et al (1999): Attenuated sensitivity to neuroactive steroids in gamma-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci U S A* 96:12905-12910.
- Millan MJ (2003): The neurobiology and control of anxious states. *Prog Neurobiol* 70:83-244.
- Millan MJ, Brocco M, Gobert A, Dorey G, Casara P, Dekeyne A (2001): Anxiolytic properties of the selective, non-peptidergic CRF<sub>1</sub> antagonists, CP154,526 and DMP695: a comparison to other classes of anxiolytic agent. *Neuropsychopharmacology* 25:585-600.
- Minano FJ, Meneres Sancho MS, Sancibrian M, Salinas P, Myers RD (1992): GABA<sub>A</sub> receptors in the amygdala: role in feeding in fasted and satiated rats. *Brain Res* 586:104-110.
- Mirescu C, Peters JD, Gould E (2004): Early life experience alters response of adult neurogenesis to stress. *Nat Neurosci* 7:841-846.
- Mirza NR, Larsen JS, Mathiasen C, Jacobsen TA, Munro G, Erichsen HK, et al (2008): NS11394 [3'-(5-(1-hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl)-biphenyl-2-carbonitrile], a unique subtype-selective GABA<sub>A</sub> receptor positive allosteric modulator: in vitro actions, pharmacokinetic properties and in vivo anxiolytic efficacy. *J Pharmacol Exp Ther* 327:954-968.
- Misane I, Ogren SO (2003): Selective 5-HT<sub>1A</sub> antagonists WAY 100635 and NAD-299 attenuate the impairment of passive avoidance caused by scopolamine in the rat. *Neuropsychopharmacology* 28:253-264.
- Mittelman B, Wolff H (1939): Affective states and skin temperature: experimental study of subjects with "cold hands" and Raynaud's syndrome. *Psychosomatic medicine* 1:271-292.
- Miyata M, Okada D, Hashimoto K, Kano M, Ito M (1999): Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. *Neuron* 22:763-775.
- Moffat A, Osselton M, Widdop B (2004): Clarke's Analysis of Drugs and Poisons. *Pharmaceutical Press*.
- Moffitt JA, Grippo AJ, Holmes PV, Johnson AK (2002): Olfactory bulbectomy attenuates cardiovascular sympathoexcitatory reflexes in rats. *Am J Physiol Heart Circ Physiol* 283:H2575-2583.
- Mogenson GJ, Jones DL, Yim CY (1980): From motivation to action: functional interface between the limbic system and the motor system. *Prog Neurobiol* 14:69-97.
- Mombereau C, Kaupmann K, Froestl W, Sansig G, van der Putten H, Cryan JF (2004): Genetic and pharmacological evidence of a role for GABA(B) receptors in the modulation of anxiety- and antidepressant-like behavior. *Neuropsychopharmacology* 29:1050-1062.
- Mombereau C, Kaupmann K, Gassmann M, Bettler B, van der Putten H, Cryan JF (2005): Altered anxiety and depression-related behavior in mice lacking GABA<sub>B(2)</sub> receptor subunits. *Neuroreport* 16:307-310.
- Mora S, Dussaubat N, Diaz-Velaz G (1996): Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. *Psychoneuroendocrinology* 21:609-620.
- Moret C, Briley M (1997): 5-HT autoreceptors in the regulation of 5-HT release from guinea pig raphe nucleus and hypothalamus. *Neuropharmacology* 36:1713-1723.
- Moret C, Briley M (2000): The possible role of 5-HT(1B/D) receptors in psychiatric disorders and their potential as a target for therapy. *Eur J Pharmacol* 404:1-12.
- Morikawa H, Manzoni OJ, Crabbe JC, Williams JT (2000): Regulation of central synaptic transmission by 5-HT(1B) auto- and heteroreceptors. *Mol Pharmacol* 58:1271-1278.
- Morimoto A, Watanabe T, Morimoto K, Nakamori T, Murakami N (1991): Possible involvement of prostaglandins in psychomotor stress-induced responses in rats. *J Physiol* 443:421-429.
- Morrow LE, McClellan JL, Conn CA, Kluger MJ (1993): Glucocorticoids alter fever and IL-6 responses to psychological stress and to lipopolysaccharide. *Am J Physiol* 264:R1010-1016.

- Morrow LE, McClellan JL, Klir JJ, Kluger MJ (1996): The CNS site of glucocorticoid negative feedback during LPS- and psychological stress-induced fevers. *Am J Physiol* 271:R732-737.
- Munday MK, Fletcher A, Marsden CA (1996): Effects of 8-OHDPAT and 5-HT<sub>1A</sub> antagonists WAY100135 and WAY100635, on guinea-pig behavior and dorsal raphe 5-HT neurone firing. *Br J Pharmacol* 117:750-756.
- Murphy DL, Andrews AM, Wichems CH, Li Q, Tohda M, Greenberg B (1998): Brain serotonin neurotransmission: an overview and update with an emphasis on serotonin subsystem heterogeneity, multiple receptors, interactions with other neurotransmitter systems, and consequent implications for understanding the actions of serotonergic drugs. *J Clin Psychiatry* 59 Suppl 15:4-12.
- Murray CJ, Lopez AD (1997): Alternative projections of mortality and disability by cause 1990-2020: Global Burden of Disease Study. *Lancet* 349:1498-1504.
- Myint AM, O'Mahony S, Kubera M, Kim YK, Kenny C, Kaim-Basta A, et al (2007): Role of paroxetine in interferon- $\alpha$ -induced immune and behavioral changes in male Wistar rats. *J Psychopharmacol* 21:843-850.

**N**

- Nadeau JH, Singer JB, Matin A, Lander ES (2000): Analysing complex genetic traits with chromosome substitution strains. *Nat Genet* 24:221-225.
- Nagashima K, Nakai S, Tanaka M, Kanosue K (2000): Neuronal circuitries involved in thermoregulation. *Auton Neurosci* 85:18-25.
- Nakamori T, Morimoto A, Murakami N (1993): Effect of a central CRF antagonist on cardiovascular and thermoregulatory responses induced by stress or IL-1 beta. *Am J Physiol* 265:R834-839.
- Nakamura K, Kaneko T, Yamashita Y, Hasegawa H, Katoh H, Ichikawa A, Negishi M (1999): Immunocytochemical localization of prostaglandin EP<sub>3</sub> receptor in the rat hypothalamus. *Neurosci Lett* 260:117-120.
- Nakamura K, Matsumura K, Hubschle T, Nakamura Y, Hioki H, Fujiyama F, et al (2004): Identification of sympathetic premotor neurons in medullary raphe regions mediating fever and other thermoregulatory functions. *J Neurosci* 24:5370-5380.
- Nakamura K, Matsumura K, Kaneko T, Kobayashi S, Katoh H, Negishi M (2002): The rostral raphe pallidus nucleus mediates pyrogenic transmission from the preoptic area. *J Neurosci* 22:4600-4610.
- Nakamura K, Matsumura K, Kobayashi S, Kaneko T (2005a): Sympathetic premotor neurons mediating thermoregulatory functions. *Neurosci Res* 51:1-8.
- Nakamura Y, Nakamura K, Matsumura K, Kobayashi S, Kaneko T, Morrison SF (2005b): Direct pyrogenic input from prostaglandin EP<sub>3</sub> receptor-expressing preoptic neurons to the dorsomedial hypothalamus. *Eur J Neurosci* 22:3137-3146.
- Nakayama K, Goto S, Kuraoka K, Nakamura K (2005): Decrease in nasal temperature of rhesus monkeys (*Macaca mulatta*) in negative emotional state. *Physiol Behav* 84:783-790.
- Nalivaiko E, Ootsuka Y, Blessing WW (2005): Activation of 5-HT<sub>1A</sub> receptors in the medullary raphe reduces cardiovascular changes elicited by acute psychological and inflammatory stresses in rabbits. *Am J Physiol Regul Integr Comp Physiol* 289:R596-R604.
- Nastiti K, Benton D, Brain PF (1991): The effects of compounds acting at the benzodiazepine receptor complex on the ultrasonic calling of mouse pups. *Behav Pharmacol* 2:121-128.
- Navines R, Martin-Santos R, Gomez-Gil E, Martinez de Osaba MJ, Imaz ML, Gasto C (2007): Effects of citalopram treatment on hypothalamic and hormonal responses to the 5-HT<sub>1A</sub> receptor agonist buspirone in patients with major depression and therapeutic response. *Psychoneuroendocrinology* 32:411-416.
- Nedved ML, Habibi-Goudarzi S, Ganem B, Henion JD (1996): Characterization of benzodiazepine "combinatorial" chemical libraries by on-line immunoaffinity extraction, coupled column HPLC-ion spray mass spectrometry-tandem mass spectrometry. *Anal Chem* 68:4228-4236.

- Nemeroff CB (2003): The role of GABA in the pathophysiology and treatment of anxiety disorders. *Psychopharmacol Bull* 37:133-146.
- Neumaier JF, Edwards E, Plotsky PM (2002): 5-HT<sub>(1B)</sub> mRNA regulation in two animal models of altered stress reactivity. *Biol Psychiatry* 51:902-908.
- Nichols DE, Nichols CD (2008): Serotonin receptors. *Chem Rev* 108:1614-1641.
- Nie Z, Schweitzer P, Roberts AJ, Madamba SG, Moore SD, Siggins GR (2004): Ethanol augments GABAergic transmission in the central amygdala via CRF<sub>1</sub> receptors. *Science* 303:1512-1514.
- Nisijima K, Yoshino T, Yui K, Katoh S (2001): Potent serotonin (5-HT)<sub>(2A)</sub> receptor antagonists completely prevent the development of hyperthermia in an animal model of the 5-HT syndrome. *Brain Res* 890:23-31.
- Nordquist RE, Durkin S, Jaeschke G, Spooren W (2007): Stress-induced hyperthermia: effects of acute and repeated dosing of MPEP. *Eur J Pharmacol* 568:199-202.
- Nusser Z, Sieghart W, Somogyi P (1998): Segregation of different GABA<sub>A</sub> receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* 18:1693-1703.
- Nutt DJ (2005): Overview of diagnosis and drug treatments of anxiety disorders. *CNS Spectr* 10:49-56.
- Nutt DJ, Malizia AL (2001): New insights into the role of the GABA<sub>(A)</sub>-benzodiazepine receptor in psychiatric disorder. *Br J Psychiatry* 179:390-396.
- O**
- Oka T, Oka K, Hori T (2001): Mechanisms and mediators of psychological stress-induced rise in core temperature. *Psychosom Med* 63:476-486.
- Oka T, Oka K, Kobayashi T, Sugimoto Y, Ichikawa A, Ushikubi F, et al (2003): Characteristics of thermoregulatory and febrile responses in mice deficient in prostaglandin EP<sub>1</sub> and EP<sub>3</sub> receptors. *J Physiol* 551:945-954.
- Olivier B, Bouwknecht JA, Pattij T, Leahy C, van Oorschot R, Zethof TJ (2002): GABA<sub>A</sub>-benzodiazepine receptor complex ligands and stress-induced hyperthermia in singly housed mice. *Pharmacol Biochem Behav* 72:179-188.
- Olivier B, Mos J, van Oorschot R, Hen R (1995): Serotonin receptors and animal models of aggressive behavior. *Pharmacopsychiatry* 28 Suppl 2:80-90.
- Olivier B, Pattij T, Wood SJ, Oosting R, Sarnyai Z, Toth M (2001): The 5-HT<sub>(1A)</sub> receptor knockout mouse and anxiety. *Behav Pharmacol* 12:439-450.
- Olivier B, van Bogaert, M., van Oorschot, R., Oosting, R., Groenink, L. (2005): Stress-induced hyperthermia. *Handbook of Stress and the Brain, Steckler, T, Kalin, NH, Reul, JM (Eds), vol 15 Elsevier, Amsterdam:pp. 135-155.*
- Olivier B, van Oorschot R (2005): 5-HT<sub>1B</sub> receptors and aggression: a review. *Eur J Pharmacol* 526:207-217.
- Olivier B, Zethof T, Pattij T, van Boogaert M, van Oorschot R, Leahy C, et al (2003): Stress-induced hyperthermia and anxiety: pharmacological validation. *Eur J Pharmacol* 463:117-132.
- Olivier B, Zethof TJ, Ronken E, van der Heyden JA (1998): Anxiolytic effects of flesinoxan in the stress-induced hyperthermia paradigm in singly-housed mice are 5-HT<sub>1A</sub> receptor mediated. *Eur J Pharmacol* 342:177-182.
- Olivier JD, Cools AR, Olivier B, Homberg JR, Cuppen E, Ellenbroek BA (2008a): Stress-induced hyperthermia and basal body temperature are mediated by different 5-HT<sub>(1A)</sub> receptor populations: a study in SERT knockout rats. *Eur J Pharmacol* 590:190-197.
- Olivier JD, Van Der Hart MG, Van Swelm RP, Dederen PJ, Homberg JR, Cremers T, et al (2008b): A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. *Neuroscience* 152:573-584.
- Onaivi ES, Martin BR (1989): Neuropharmacological and physiological validation of a computer-controlled two-compartment black and white box for the assessment of anxiety. *Prog Neuropsychopharmacol Biol Psychiatry* 13:963-976.

- Ootsuka Y, Blessing WW (2003): 5-Hydroxytryptamine  $1A$  receptors inhibit cold-induced sympathetically mediated cutaneous vasoconstriction in rabbits. *J Physiol* 552:303-314.
- Ootsuka Y, Blessing WW (2006a): Activation of 5-HT $1A$  receptors in rostral medullary raphe inhibits cutaneous vasoconstriction elicited by cold exposure in rabbits. *Brain Res* 1073-1074:252-261.
- Ootsuka Y, Blessing WW (2006b): Thermogenesis in brown adipose tissue: increase by 5-HT $2A$  receptor activation and decrease by 5-HT $1A$  receptor activation in conscious rats. *Neurosci Lett* 395:170-174.
- Oswald I, Adam K (1980): Benzodiazepines cause small loss of body weight. *Br Med J* 281:1039-1040.
- Owens MJ, Nemeroff CB (1991): Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol Rev* 43:425-473.
- Owens MJ, Nemeroff CB (1998): The serotonin transporter and depression. *Depress Anxiety* 8 Suppl 1:5-12.
- P**
- Panagiotaropoulos T, Pondiki S, Papaioannou A, Alikaridis F, Stamatakis A, Gerozissis K, Stylianopoulou F (2004): Neonatal handling and gender modulate brain monoamines and plasma corticosterone levels following repeated stressors in adulthood. *Neuroendocrinology* 80:181-191.
- Pandey GN, Pandey SC, Dwivedi Y, Sharma RP, Janicak PG, Davis JM (1995): Platelet serotonin $2A$  receptors: a potential biological marker for suicidal behavior. *Am J Psychiatry* 152:850-855.
- Pandey GN, Pandey SC, Janicak PG, Marks RC, Davis JM (1990): Platelet serotonin-2 receptor binding sites in depression and suicide. *Biol Psychiatry* 28:215-222.
- Pardon MC, Kendall DA, Perez-Diaz F, Duxon MS, Marsden CA (2004): Repeated sensory contact with aggressive mice rapidly leads to an anticipatory increase in core body temperature and physical activity that precedes the onset of aversive responding. *Eur J Neurosci* 20:1033-1050.
- Parks CL, Robinson PS, Sibille E, Shenk T, Toth M (1998): Increased anxiety of mice lacking the serotonin $1A$  receptor. *Proc Natl Acad Sci U S A* 95:10734-10739.
- Parr L, Hopkins W (2000a): Brain temperature asymmetries and emotional perception in chimpanzees, *Pan troglodytes*. *Physiology & behavior* 71:363-371.
- Parr LA, Hopkins WD (2000b): Brain temperature asymmetries and emotional perception in chimpanzees, *Pan troglodytes*. *Physiol Behav* 71:363-371.
- Parrott RF, DM LL (1995): Restraint, but not frustration, induces prostaglandin-mediated hyperthermia in pigs. *Physiol Behav* 57:1051-1055.
- Parsey RV, Oquendo MA, Ogden RT, Olvet DM, Simpson N, Huang YY, et al (2006): Altered serotonin  $1A$  binding in major depression: a [carbonyl-C-11]WAY100635 positron emission tomography study. *Biol Psychiatry* 59:106-113.
- Paterson NE, Vlachou S, Guery S, Kaupmann K, Froestl W, Markou A (2008): Positive modulation of GABA(B) receptors decreased nicotine self-administration and counteracted nicotine-induced enhancement of brain reward function in rats. *J Pharmacol Exp Ther* 326:306-314.
- Pattij T, Groenink L, Hijzen TH, Oosting RS, Maes RA, van der Gugten J, Olivier B (2002a): Autonomic changes associated with enhanced anxiety in 5-HT(1A) receptor knockout mice. *Neuropsychopharmacology* 27:380-390.
- Pattij T, Groenink L, Oosting RS, van der Gugten J, Maes RA, Olivier B (2002b): GABA $(A)$ -benzodiazepine receptor complex sensitivity in 5-HT(1A) receptor knockout mice on a 129/Sv background. *Eur J Pharmacol* 447:67-74.
- Pattij T, Hijzen TH, Groenink L, Oosting RS, van der Gugten J, Maes RA, et al (2001): Stress-induced hyperthermia in the 5-HT $(1A)$  receptor knockout mouse is normal. *Biol Psychiatry* 49:569-574.
- Paxinos G, Watson C (1986): *The rat brain in stereotaxic coordinates*, Second ed. San Diego: Academic Press Inc.

- Pehek EA, Nocjar C, Roth BL, Byrd TA, Mabrouk OS (2006): Evidence for the preferential involvement of 5-HT<sub>2A</sub> serotonin receptors in stress- and drug-induced dopamine release in the rat medial prefrontal cortex. *Neuropsychopharmacology* 31:265-277.
- Peloso E, Wachulec M, Satinoff E (2002): Stress-induced hyperthermia depends on both time of day and light condition. *J Biol Rhythms* 17:164-170.
- Peloso ED, Florez-Duquet M, Buchanan JB, Satinoff E (2003): LPS fever in old rats depends on the ambient temperature. *Physiol Behav* 78:651-654.
- Penington NJ, Kelly JS, Fox AP (1993): Whole-cell recordings of inwardly rectifying K<sup>+</sup> currents activated by 5-HT<sub>1A</sub> receptors on dorsal raphe neurones of the adult rat. *J Physiol* 469:387-405.
- Pesold C, Caruncho HJ, Impagnatiello F, Berg MJ, Fritschy JM, Guidotti A, Costa E (1997): Tolerance to diazepam and changes in GABA<sub>(A)</sub> receptor subunit expression in rat neocortical areas. *Neuroscience* 79:477-487.
- Petersen DR, Norris KJ, Thompson JA (1984): A comparative study of the disposition of nicotine and its metabolites in three inbred strains of mice. *Drug Metab Dispos* 12:725-731.
- Petroski RE, Pomeroy JE, Das R, Bowman H, Yang W, Chen AP, Foster AC (2006): Indiplon is a high-affinity positive allosteric modulator with selectivity for alpha1 subunit-containing GABA<sub>A</sub> receptors. *J Pharmacol Exp Ther* 317:369-377.
- Petryshen TL, Kirby A, Hammer RP, Jr., Purcell S, O'Leary SB, Singer JB, et al (2005): Two quantitative trait loci for prepulse inhibition of startle identified on mouse chromosome 16 using chromosome substitution strains. *Genetics* 171:1895-1904.
- Pezzone MA, Lee WS, Hoffman GE, Rabin BS (1992): Induction of c-Fos immunoreactivity in the rat forebrain by conditioned and unconditioned aversive stimuli. *Brain Res* 597:41-50.
- Phelps EA, LeDoux JE (2005): Contributions of the amygdala to emotion processing: from animal models to human behavior. *Neuron* 48:175-187.
- Picciotto MR, Brunzell DH, Caldarone BJ (2002): Effect of nicotine and nicotinic receptors on anxiety and depression. *Neuroreport* 13:1097-1106.
- Pieper DR, Loboocki CA (1991): Olfactory bulbectomy lengthens circadian period of locomotor activity in golden hamsters. *Am J Physiol* 261:R973-978.
- Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000): GABA<sub>(A)</sub> receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* 101:815-850.
- Pitchot W, Wauthy J, Hansenne M, Pinto E, Fuchs S, Reggers J, et al (2002): Hormonal and temperature responses to the 5-HT<sub>1A</sub> receptor agonist flesinoxan in normal volunteers. *Psychopharmacology (Berl)* 164:27-32.
- Pitchot W, Wauthy J, Legros JJ, Ansseau M (2004): Hormonal and temperature responses to flesinoxan in normal volunteers: an antagonist study. *Eur Neuropsychopharmacol* 14:151-155.
- Pompeiano M, Palacios JM, Mengod G (1992): Distribution and cellular localization of mRNA coding for 5-HT<sub>1A</sub> receptor in the rat brain: correlation with receptor binding. *J Neurosci* 12:440-453.
- Popik P, Kostakis E, Krawczyk M, Nowak G, Szewczyk B, Krieter P, et al (2006): The anxiolytic agent 7-(2-chloropyridin-4-yl)pyrazolo-[1,5-a]-pyrimidin-3-yl(pyridin-2-yl)meth anone (DOV 51892) is more efficacious than diazepam at enhancing GABA-gated currents at alpha1 subunit-containing GABA<sub>A</sub> receptors. *J Pharmacol Exp Ther* 319:1244-1252.
- Possidente B, Lumia AR, McGinnis MY, Rapp M, McEldowney S (1996): Effects of fluoxetine and olfactory bulbectomy on mouse circadian activity rhythms. *Brain Res* 713:108-113.
- Possidente B, Lumia AR, McGinnis MY, Teicher MH, deLemos E, Sterner L, Deros L (1990): Olfactory bulb control of circadian activity rhythm in mice. *Brain Res* 513:325-328.

- Potier MC, Prado de Carvalho L, Venault P, Chapouthier G, Rossier J (1988): Demonstration of the partial agonist profiles of Ro 16-6028 and Ro 17-1812 in mice in vivo. *Eur J Pharmacol* 156:169-172.
- Pratt J, Jenner P, Reynolds EH, Marsden CD (1979): Clonazepam induces decreased serotonergic activity in the mouse brain. *Neuropharmacology* 18:791-799.
- Price ML, Curtis AL, Kirby LG, Valentino RJ, Lucki I (1998): Effects of corticotropin-releasing factor on brain serotonergic activity. *Neuropsychopharmacology* 18:492-502.
- Primeaux SD, Holmes PV (1999): Role of aversively motivated behavior in the olfactory bulbectomy syndrome. *Physiol Behav* 67:41-47.
- Primus RJ, Yu J, Xu J, Hartnett C, Meyyappan M, Kostas C, et al (1996): Allosteric uncoupling after chronic benzodiazepine exposure of recombinant gamma-aminobutyric acid<sub>(A)</sub> receptors expressed in Sf9 cells: ligand efficacy and subtype selectivity. *J Pharmacol Exp Ther* 276:882-890.
- Pritchett DB, Luddens H, Seeburg PH (1989): Type I and type II GABA<sub>A</sub>-benzodiazepine receptors produced in transfected cells. *Science* 245:1389-1392.
- Pritchett DB, Seeburg PH (1990): Gamma-aminobutyric acid<sub>A</sub> receptor alpha<sub>5</sub>-subunit creates novel type II benzodiazepine receptor pharmacology. *J Neurochem* 54:1802-1804.
- Pucadyil TJ, Kalipatnapu S, Chattopadhyay A (2005): The serotonin<sub>1A</sub> receptor: a representative member of the serotonin receptor family. *Cell Mol Neurobiol* 25:553-580.
- Puder JJ, Freda PU, Goland RS, Wardlaw SL (2001): Estrogen modulates the hypothalamic-pituitary-adrenal and inflammatory cytokine responses to endotoxin in women. *J Clin Endocrinol Metab* 86:2403-2408.
- R**
- Rahman M, Lindblad C, Johansson IM, Backstrom T, Wang MD (2006): Neurosteroid modulation of recombinant rat alpha5beta2gamma2L and alpha1beta2gamma2L GABA<sub>(A)</sub> receptors in *Xenopus* oocyte. *Eur J Pharmacol* 547:37-44.
- Rainnie DG (1999): Serotonergic modulation of neurotransmission in the rat basolateral amygdala. *J Neurophysiol* 82:69-85.
- Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, Shekhar A (2004): Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. *J Neurosci* 24:3471-3479.
- Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, et al (1998): Serotonin receptor<sub>1A</sub> knockout: an animal model of anxiety-related disorder. *Proc Natl Acad Sci U S A* 95:14476-14481.
- Ramsey-Williams VA, Carter DB (1996): Chronic triazolam and its withdrawal alters GABA<sub>A</sub> receptor subunit mRNA levels: an in situ hybridization study. *Brain Res Mol Brain Res* 43:132-140.
- Rapkin AJ (2007): Vasomotor symptoms in menopause: physiologic condition and central nervous system approaches to treatment. *Am J Obstet Gynecol* 196:97-106.
- Ravishankar A, Carnwath T (1998): Zolpidem tolerance and dependence--two case reports. *J Psychopharmacol* 12:103-104.
- Reddy DS, Kulkarni SK (1999): Sex and estrous cycle-dependent changes in neurosteroid and benzodiazepine effects on food consumption and plus-maze learning behaviors in rats. *Pharmacol Biochem Behav* 62:53-60.
- Refinetti R (1999): Relationship between the daily rhythms of locomotor activity and body temperature in eight mammalian species. *Am J Physiol* 277:R1493-1500.
- Renbourn ET (1960): Body temperature and pulse rate in boys and young men prior to sporting contests. A study of emotional hyperthermia: with a review of the literature. *J Psychosom Res* 4:149-175.
- Ressler KJ, Nemeroff CB (2000): Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. *Depress Anxiety* 12 Suppl 1:2-19.

- Reul JM, Holsboer F (2002): Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol* 2:23-33.
- Reves JG, Fragen RJ, Vinik HR, Greenblatt DJ (1985): Midazolam: pharmacology and uses. *Anesthesiology* 62:310-324.
- Risbrough VB, Stein MB (2006): Role of corticotropin releasing factor in anxiety disorders: a translational research perspective. *Horm Behav* 50:550-561.
- Risch N, Herrell R, Lehner T, Liang KY, Eaves L, Hoh J, et al (2009): Interaction between the serotonin transporter gene (5-HTTLPR), stressful life events, and risk of depression: a meta-analysis. *Jama* 301:2462-2471.
- Risoli A, Cheng JB, Verkerk UH, Zhao J, Ragno G, Hopkinson AC, Siu KW (2007): Gas-phase fragmentation of protonated benzodiazepines. *Rapid Commun Mass Spectrom* 21:2273-2281.
- Ritter MJ, Ellis M, Anderson DB, Curtis SE, Keffaber KK, Killefer J, et al (2009): Effects of multiple concurrent stressors on rectal temperature, blood acid-base status, and longissimus muscle glycolytic potential in market-weight pigs. *J Anim Sci* 87:351-362.
- Rivest S, Laflamme N (1995): Neuronal activity and neuropeptide gene transcription in the brains of immune-challenged rats. *J Neuroendocrinol* 7:501-525.
- Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR (2003): Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. *Proc Natl Acad Sci U S A* 100:2053-2058.
- Roberts AJ, Krucker T, Levy CL, Slanina KA, Sutcliffe JG, Hedlund PB (2004a): Mice lacking 5-HT receptors show specific impairments in contextual learning. *Eur J Neurosci* 19:1913-1922.
- Roberts C, Thomas DR, Bate ST, Kew JN (2004b): GABAergic modulation of 5-HT<sub>7</sub> receptor-mediated effects on 5-HT efflux in the guinea-pig dorsal raphe nucleus. *Neuropharmacology* 46:935-941.
- Roche M, Harkin A, Kelly JP (2007): Chronic fluoxetine treatment attenuates stressor-induced changes in temperature, heart rate, and neuronal activation in the olfactory bulbectomized rat. *Neuropsychopharmacology* 32:1312-1320.
- Rodgers RJ, Boullier E, Chatzimichalaki P, Cooper GD, Shorten A (2002a): Contrasting phenotypes of C57BL/6JOLA<sup>Hsd</sup>, 129S2/SvHsd and 129/SvEv mice in two exploration-based tests of anxiety-related behavior. *Physiol Behav* 77:301-310.
- Rodgers RJ, Davies B, Shore R (2002b): Absence of anxiolytic response to chlordiazepoxide in two common background strains exposed to the elevated plus-maze: importance and implications of behavioral baseline. *Genes Brain Behav* 1:242-251.
- Roland BL, Sawchenko PE (1993): Local origins of some GABAergic projections to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. *J Comp Neurol* 332:123-143.
- Rollins BL, Stines SG, McGuire HB, King BM (2001): Effects of amygdala lesions on body weight, conditioned taste aversion, and neophobia. *Physiol Behav* 72:735-742.
- Romanovsky AA, Ivanov AI, Szekely M (2000): Neural route of pyrogen signaling to the brain. *Clin Infect Dis* 31 Suppl 5:S162-167.
- Rooszendaal B, Koolhaas JM, Bohus B (1991): Central amygdala lesions affect behavioral and autonomic balance during stress in rats. *Physiol Behav* 50:777-781.
- Rooszendaal B, McEwen BS, Chattarji S (2009): Stress, memory and the amygdala. *Nat Rev Neurosci* 10:423-433.
- Rorick-Kehn LM, Hart JC, McKinzie DL (2005): Pharmacological characterization of stress-induced hyperthermia in DBA/2 mice using metabotropic and ionotropic glutamate receptor ligands. *Psychopharmacology (Berl)* 183:226-240.
- Rosenfeld P, Wetmore JB, Levine S (1992): Effects of repeated maternal separations on the adrenocortical response to stress of preweanling rats. *Physiol Behav* 52:787-791.

- Rotondo D, Abul HT, Milton AS, Davidson J (1988): Pyrogenic immunomodulators increase the level of prostaglandin E2 in the blood simultaneously with the onset of fever. *Eur J Pharmacol* 154:145-152.
- Rowlett JK, Platt DM, Lelas S, Attack JR, Dawson GR (2005): Different GABA<sub>A</sub> receptor subtypes mediate the anxiolytic, abuse-related, and motor effects of benzodiazepine-like drugs in primates. *Proc Natl Acad Sci U S A* 102:915-920.
- Roy-Byrne P, Wingerson DK, Radant A, Greenblatt DJ, Cowley DS (1996): Reduced benzodiazepine sensitivity in patients with panic disorder: comparison with patients with obsessive-compulsive disorder and normal subjects. *Am J Psychiatry* 153:1444-1449.
- Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, et al (1999): Benzodiazepine actions mediated by specific gamma-aminobutyric acid<sub>(A)</sub> receptor subtypes. *Nature* 401:796-800.
- Rudolph U, Mohler H (2004): Analysis of GABA<sub>A</sub> receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. *Annu Rev Pharmacol Toxicol* 44:475-498.
- Rudolph U, Mohler H (2006): GABA-based therapeutic approaches: GABA<sub>A</sub> receptor subtype functions. *Curr Opin Pharmacol* 6:18-23.
- Russek SJ (1999): Evolution of GABA<sub>(A)</sub> receptor diversity in the human genome. *Gene* 227:213-222.
- Rusyniak DE, Zaretskaia MV, Zaretsky DV, DiMicco JA (2007): 3,4-Methylenedioxymethamphetamine- and 8-hydroxy-2-di-n-propylamino-tetralin-induced hypothermia: role and location of 5-hydroxytryptamine 1A receptors. *J Pharmacol Exp Ther* 323:477-487.
- Rybaczyk LA, Bashaw MJ, Pathak DR, Moody SM, Gilders RM, Holzschu DL (2005): An overlooked connection: serotonergic mediation of estrogen-related physiology and pathology. *BMC Womens Health* 5:12.
- S**
- Saarelainen KS, Ranna M, Rabe H, Sinkkonen ST, Moykkynen T, Uusi-Oukari M, et al (2008): Enhanced behavioral sensitivity to the competitive GABA agonist, gaboxadol, in transgenic mice over-expressing hippocampal extrasynaptic alpha6beta GABA(A) receptors. *J Neurochem* 105:338-350.
- Saenz del Burgo L, Cortes R, Mengod G, Zarate J, Echevarria E, Salles J (2008): Distribution and neurochemical characterization of neurons expressing GIRK channels in the rat brain. *J Comp Neurol* 510:581-606.
- Sah P, Faber ES, Lopez De Armentia M, Power J (2003): The amygdaloid complex: anatomy and physiology. *Physiol Rev* 83:803-834.
- Saha S, Engstrom L, Mackerlova L, Jakobsson PJ, Blomqvist A (2005): Impaired febrile responses to immune challenge in mice deficient in microsomal prostaglandin E synthase-1. *Am J Physiol Regul Integr Comp Physiol* 288:R1100-1107.
- Sakaue M, Ago Y, Murakami C, Sowa C, Sakamoto Y, Koyama Y, et al (2001): Involvement of benzodiazepine binding sites in an antiaggressive effect by 5-HT<sub>(1A)</sub> receptor activation in isolated mice. *Eur J Pharmacol* 432:163-166.
- Saleh MC, Connell BJ, Saleh TM (2000): Autonomic and cardiovascular reflex responses to central estrogen injection in ovariectomized female rats. *Brain Res* 879:105-114.
- Saleh TM, Connell BJ (2003): Estrogen-induced autonomic effects are mediated by NMDA and GABA<sub>A</sub> receptors in the parabrachial nucleus. *Brain Res* 973:161-170.
- Salomon L, Lanteri C, Glowinski J, Tassin JP (2006): Behavioral sensitization to amphetamine results from an uncoupling between noradrenergic and serotonergic neurons. *Proc Natl Acad Sci U S A* 103:7476-7481.
- Sanchez MM, Aguado F, Sanchez-Toscano F, Saphier D (1995): Effects of prolonged social isolation on responses of neurons in the bed nucleus of the stria terminalis, preoptic area, and hypothalamic paraventricular nucleus to stimulation of the medial amygdala. *Psychoneuroendocrinology* 20:525-541.

- Sargent P, Williamson DJ, Pearson G, Odontiadis J, Cowen PJ (1997): Effect of paroxetine and nefazodone on 5-HT<sub>1A</sub> receptor sensitivity. *Psychopharmacology (Berl)* 132:296-302.
- Sarnyai Z, Shaham Y, Heinrichs SC (2001): The role of corticotropin-releasing factor in drug addiction. *Pharmacol Rev* 53:209-243.
- Satinoff E (1972): Salicylate: action on normal body temperature in rats. *Science* 176:532-533.
- Satow A, Maehara S, Ise S, Hikichi H, Fukushima M, Suzuki G, et al (2008): Pharmacological effects of the metabotropic glutamate receptor 1 antagonist compared with those of the metabotropic glutamate receptor 5 antagonist and metabotropic glutamate receptor 2/3 agonist in rodents: detailed investigations with a selective allosteric metabotropic glutamate receptor 1 antagonist, FTDC [4-[1-(2-fluoropyridine-3-yl)-5-methyl-1H-1,2,3-triazol-4-yl]-N-isopropyl- N-methyl-3,6-dihydropyridine-1(2H)-carboxamide]. *J Pharmacol Exp Ther* 326:577-586.
- Saudou F, Amara DA, Dierich A, LeMeur M, Ramboz S, Segu L, et al (1994): Enhanced aggressive behavior in mice lacking 5-HT<sub>1B</sub> receptor. *Science* 265:1875-1878.
- Savic MM, Huang S, Furtmuller R, Clayton T, Huck S, Obradovic DI, et al (2008): Are GABA<sub>A</sub> receptors containing alpha5 subunits contributing to the sedative properties of benzodiazepine site agonists? *Neuropsychopharmacology* 33:332-339.
- Savic MM, Obradovic DI, Ugresic ND, Cook JM, Yin W, Bokonjic DR (2004): Bidirectional effects of benzodiazepine binding site ligands in the elevated plus-maze: differential antagonism by flumazenil and beta-CCT. *Pharmacol Biochem Behav* 79:279-290.
- Savic MM, Obradovic DI, Ugresic ND, Cook JM, Yin W, Van Linn M, Bokonjic DR (2006): Benzodiazepine site inverse agonists and locomotor activity in rats: bimodal and biphasic influence. *Pharmacol Biochem Behav* 84:35-42.
- Savitz J, Lucki I, Drevets W (2009): 5-HT<sub>1A</sub> receptor function in major depressive disorder. *Progress in Neurobiology* 88:17-31.
- Sawchenko PE, Brown ER, Chan RK, Ericsson A, Li HY, Roland BL, Kovacs KJ (1996): The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress. *Prog Brain Res* 107:201-222.
- Sawchenko PE, Li HY, Ericsson A (2000): Circuits and mechanisms governing hypothalamic responses to stress: a tale of two paradigms. *Prog Brain Res* 122:61-78.
- Scalia F, Winans SS (1975): The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. *J Comp Neurol* 161:31-55.
- Scammell TE, Elmquist JK, Griffin JD, Saper CB (1996): Ventromedial preoptic prostaglandin E2 activates fever-producing autonomic pathways. *J Neurosci* 16:6246-6254.
- Scearce-Levie K, Chen JP, Gardner E, Hen R (1999): 5-HT receptor knockout mice: pharmacological tools or models of psychiatric disorders. *Ann N Y Acad Sci* 868:701-715.
- Schierloh A, Deussing J, Wurst W, Zieglansberger W, Rammes G (2007): Corticotropin-releasing factor (CRF) receptor type 1-dependent modulation of synaptic plasticity. *Neurosci Lett* 416:82-86.
- Schneiderman N, Ironson G, Siegel SD (2005): Stress and health: psychological, behavioral, and biological determinants. *Annu Rev Clin Psychol* 1:607-628.
- Schwartz MA, Postma E, Gaut Z (1971): Biological half-life of chlordiazepoxide and its metabolite, demoxepam, in man. *J Pharm Sci* 60:1500-1503.
- Scott-Stevens P, Atack JR, Sohail B, Worboys P (2005): Rodent pharmacokinetics and receptor occupancy of the GABA<sub>A</sub> receptor subtype selective benzodiazepine site ligand L-838417. *Biopharm Drug Dispos* 26:13-20.
- Seggie J (1987): Differential responsiveness of corticosterone and prolactin to stress following lesions of the septum or amygdala: implications for psychoneuroendocrinology. *Prog Neuropsychopharmacol Biol Psychiatry* 11:315-324.
- Shaban H, Humeau Y, Herry C, Cassasus G, Shigemoto R, Ciocchi S, et al (2006): Generalization of amygdala LTP and conditioned fear in the absence of presynaptic inhibition. *Nat Neurosci*.

- Shanks N, Anisman H (1989): Strain-specific effects of antidepressants on escape deficits induced by inescapable shock. *Psychopharmacology (Berl)* 99:122-128.
- Sharp T, Umbers V, Gartside SE (1997): Effect of a selective 5-HT reuptake inhibitor in combination with 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> receptor antagonists on extracellular 5-HT in rat frontal cortex in vivo. *Br J Pharmacol* 121:941-946.
- Shephard RA, Buxton DA, Broadhurst PL (1982): Drug interactions do not support reduction in serotonin turnover as the mechanism of action of benzodiazepines. *Neuropharmacology* 21:1027-1032.
- Shephard RA, Wedlock P, Wilson NE (1992): Direct evidence for mediation of an anticonflict effect of baclofen by GABA<sub>B</sub> receptors. *Pharmacol Biochem Behav* 41:651-653.
- Shibata H, Nagasaka T (1982): Contribution of nonshivering thermogenesis to stress-induced hyperthermia in rats. *Jpn J Physiol* 32:991-995.
- Shields J, King JA (2008): The role of 5-HT<sub>1A</sub> receptors in the behavioral responses associated with innate fear. *Behav Neurosci* 122:611-617.
- Shiloh R, Kushnir T, Gilat Y, Gross-Isseroff R, Hermesh H, Munitz H, et al (2008): In vivo occipital-frontal temperature-gradient in schizophrenia patients and its possible association with psychopathology: a magnetic resonance spectroscopy study. *Eur Neuropsychopharmacol* 18:557-564.
- Shiloh R, Munitz H, Portuguese S, Gross-Isseroff R, Sigler M, Bodinger L, et al (2005): Corneal temperature in schizophrenia patients. *Int J Neuropsychopharmacol* 8:537-547.
- Shiloh R, Munitz H, Stryjer R, Weizman A (2007): A significant correlation between ward temperature and the severity of symptoms in schizophrenia inpatients--a longitudinal study. *Eur Neuropsychopharmacol* 17:478-482.
- Shiloh R, Schapir L, Bar-Ziv D, Stryjer R, Konas S, Louis R, et al (2009a): Association between corneal temperature and mental status of treatment-resistant schizophrenia inpatients. *Eur Neuropsychopharmacol*.
- Shiloh R, Weizman A, Stryjer R, Kahan N, Waitman DA (2009b): Altered thermoregulation in ambulatory schizophrenia patients: a naturalistic study. *World J Biol Psychiatry* 10:163-170.
- Shoemaker JM, Pitcher L, Noh HR, Swerdlow NR (2003): Quetiapine produces a prolonged reversal of the sensorimotor gating-disruptive effects of basolateral amygdala lesions in rats. *Behavioral neuroscience* 117:136-143.
- Shughrue PJ, Lane MV, Merchenthaler I (1997): Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. *J Comp Neurol* 388:507-525.
- Sibille E, Pavlides C, Benke D, Toth M (2000): Genetic inactivation of the Serotonin<sub>1A</sub> receptor in mice results in downregulation of major GABA<sub>A</sub> receptor alpha subunits, reduction of GABA<sub>A</sub> receptor binding, and benzodiazepine-resistant anxiety. *J Neurosci* 20:2758-2765.
- Sieghart W (1995): Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. *Pharmacol Rev* 47:181-234.
- Sieghart W, Sperk G (2002): Subunit composition, distribution and function of GABA<sub>A</sub> receptor subtypes. *Curr Top Med Chem* 2:795-816.
- Siemiakowski M, Sienkiewicz-Jaros H, Czlonkowska AI, Bidzinski A, Plaznik A (2000): Effects of buspirone, diazepam, and zolpidem on open field behavior, and brain [3H]muscimol binding after buspirone pretreatment. *Pharmacol Biochem Behav* 66:645-651.
- Singer JB, Hill AE, Burrage LC, Olszens KR, Song J, Justice M, et al (2004): Genetic dissection of complex traits with chromosome substitution strains of mice. *Science* 304:445-448.
- Singer R, Harker CT, Vander AJ, Kluger MJ (1986): Hyperthermia induced by open-field stress is blocked by salicylate. *Physiol Behav* 36:1179-1182.
- Skolnick P, Popik P, Janowsky A, Beer B, Lippa AS (2003): "Broad spectrum" antidepressants: is more better for the treatment of depression? *Life Sci* 73:3175-3179.

- Smyth WF, McClean S, Ramachandran VN (2000): A study of the electrospray ionisation of pharmacologically significant 1,4-benzodiazepines and their subsequent fragmentation using an ion-trap mass spectrometer. *Rapid Commun Mass Spectrom* 14:2061-2069.
- Song C, Leonard BE (2005): The olfactory bulbectomised rat as a model of depression. *Neurosci Biobehav Rev* 29:627-647.
- Soszynski D (2001): The inhibition of nitric oxide synthase suppresses LPS- and psychological-stress-induced fever in rats. *Physiol Behav* 72:65-72.
- Soszynski D, Chelminiak M (2007): Intracerebroventricular injection of neuronal and inducible nitric oxide synthase inhibitors attenuates fever due to LPS in rats. *J Physiol Pharmacol* 58:551-561.
- Soszynski D, Kozak W, Kluger MJ (1998): Endotoxin tolerance does not alter open field-induced fever in rats. *Physiol Behav* 63:689-692.
- Souetre E, Salvati E, Belougou JL, Pringuey D, Candito M, Krebs B, et al (1989): Circadian rhythms in depression and recovery: evidence for blunted amplitude as the main chronobiological abnormality. *Psychiatr Res* 28:263-278.
- Spielberger C (1989): State-Trait Anxiety Inventory: v A Comprehensive Bibliography. Palo Alto, Consulting Psychologists Press.
- Spooren WP, Schoeffer P, Gasparini F, Kuhn R, Gentsch C (2002): Pharmacological and endocrinological characterisation of stress-induced hyperthermia in singly housed mice using classical and candidate anxiolytics (LY314582, MPEP and NKP608). *Eur J Pharmacol* 435:161-170.
- Stanhope KJ, Dourish CT (1996): Effects of 5-HT<sub>1A</sub> receptor agonists, partial agonists and a silent antagonist on the performance of the conditioned emotional response test in the rat. *Psychopharmacology (Berl)* 128:293-303.
- Stein L, Belluzzi JD, Wise CD (1977): Benzodiazepines: behavioral and neurochemical mechanisms. *Am J Psychiatry* 134:665-669.
- Stein PK, Carney RM, Freedland KE, Skala JA, Jaffe AS, Kleiger RE, Rottman JN (2000): Severe depression is associated with markedly reduced heart rate variability in patients with stable coronary heart disease. *J Psychosom Res* 48:493-500.
- Steinbach JH, Akk G (2001): Modulation of GABA(A) receptor channel gating by pentobarbital. *J Physiol* 537:715-733.
- Stemmelin J, Cohen C, Terranova JP, Lopez-Grancha M, Pichat P, Bergis O, et al (2008): Stimulation of the beta3-Adrenoceptor as a novel treatment strategy for anxiety and depressive disorders. *Neuropsychopharmacology* 33:574-587.
- Stenzel-Poore MP, Duncan JE, Rittenberg MB, Bakke AC, Heinrichs SC (1996): CRH overproduction in transgenic mice: behavioral and immune system modulation. *Ann N Y Acad Sci* 780:36-48.
- Stevenson CW, Gratton A (2004): Role of basolateral amygdala dopamine in modulating prepulse inhibition and latent inhibition in the rat. *Psychopharmacology* 176:139-145.
- Stewart SH, Westra HA (2002): Benzodiazepine side-effects: from the bench to the clinic. *Curr Pharm Des* 8:1-3.
- Stitt JT (1986): Prostaglandin E as the neural mediator of the febrile response. *Yale J Biol Med* 59:137-149.
- Stockmeier CA, Dilley GE, Shapiro LA, Overholser JC, Thompson PA, Meltzer HY (1997): Serotonin receptors in suicide victims with major depression. *Neuropsychopharmacology* 16:162-173.
- Stockmeier CA, Howley E, Shi X, Sobanska A, Clarke G, Friedman L, Rajkowska G (2009): Antagonist but not agonist labeling of serotonin-1A receptors is decreased in major depressive disorder. *J Psychiatr Res* 43:887-894.
- Sturdee DW, Wilson KA, Pipili E, Crocker AD (1978): Physiological aspects of menopausal hot flush. *Br Med J* 2:79-80.

- Sund-Levander M, Forsberg C, Wahren LK (2002): Normal oral, rectal, tympanic and axillary body temperature in adult men and women: a systematic literature review. *Scand J Caring Sci* 16:122-128.
- Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W (1982): Corticotropin releasing factor produces behavioral activation in rats. *Nature* 297:331-333.
- Swanson LW (2003): The amygdala and its place in the cerebral hemisphere. *Ann NY Acad Sci* 985:174-184.
- Swanson LW, Petrovich GD (1998): What is the amygdala? *Trends Neurosci* 21:323-331.
- Swartzman LC, Edelberg R, Kemmann E (1990): Impact of stress on objectively recorded menopausal hot flushes and on flush report bias. *Health Psychol* 9:529-545.
- Swerdlow NR, Geyer MA, Braff DL (2001): Neural circuit regulation of prepulse inhibition of startle in the rat: current knowledge and future challenges. *Psychopharmacology (Berl)* 156:194-215.
- T**
- Tache Y, Million M, Nelson AG, Lamy C, Wang L (2005): Role of corticotropin-releasing factor pathways in stress-related alterations of colonic motor function and viscerosensitivity in female rodents. *Gen Med* 2:146-154.
- Takakusaki K, Saitoh K, Harada H, Kashiwayanagi M (2004): Role of basal ganglia-brainstem pathways in the control of motor behaviors. *Neurosci Res* 50:137-151.
- Takeno S, Hirano Y, Kitamura A, Sakai T (1993): Comparative developmental toxicity and metabolism of nitrazepam in rats and mice. *Toxicol Appl Pharmacol* 121:233-238.
- Tang X, Xiao J, Parris BS, Fang J, Sanford LD (2005): Differential effects of two types of environmental novelty on activity and sleep in BALB/cJ and C57BL/6J mice. *Physiol Behav* 85:419-429.
- Tatsumi M, Groshan K, Blakely RD, Richelson E (1997): Pharmacological profile of antidepressants and related compounds at human monoamine transporters. *Eur J Pharmacol* 340:249-258.
- ter Horst GJ, Luiten PG (1986): The projections of the dorsomedial hypothalamic nucleus in the rat. *Brain Res Bull* 16:231-248.
- Thiebot MH (1986): Are serotonergic neurons involved in the control of anxiety and in the anxiolytic activity of benzodiazepines? *Pharmacol Biochem Behav* 24:1471-1477.
- Thiebot MH, Soubrie P, Hamon M, Simon P (1984): Evidence against the involvement of serotonergic neurons in the anti-punishment activity of diazepam in the rat. *Psychopharmacology (Berl)* 82:355-359.
- Thompson CI, Brannon AJ, Heck AL (2003): Emotional fever after habituation to the temperature-recording procedure. *Physiol Behav* 80:103-108.
- Thompson RH, Canteras NS, Swanson LW (1996): Organization of projections from the dorsomedial nucleus of the hypothalamus: a PHA-L study in the rat. *J Comp Neurol* 376:143-173.
- Thompson RH, Swanson LW (1998): Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. *Brain Res Brain Res Rev* 27:89-118.
- Thrivikraman KV, Su Y, Plotsky PM (1997): Patterns of Fos-Immunoreactivity in the CNS Induced by Repeated Hemorrhage in Conscious Rats: Correlations with Pituitary-Adrenal Axis Activity. *Stress* 2:145-158.
- Tietz EI, Huang X, Chen S, Ferencak WF, 3rd (1999): Temporal and regional regulation of alpha<sub>1</sub>, beta<sub>2</sub> and beta<sub>3</sub>, but not alpha<sub>2</sub>, alpha<sub>4</sub>, alpha<sub>5</sub>, alpha<sub>6</sub>, beta<sub>1</sub> or gamma<sub>2</sub> GABA<sub>(A)</sub> receptor subunit messenger RNAs following one-week oral flurazepam administration. *Neuroscience* 91:327-341.
- Tietz EI, Huang X, Weng X, Rosenberg HC, Chiu TH (1993): Expression of alpha<sub>1</sub>, alpha<sub>5</sub>, and gamma<sub>2</sub> GABAA receptor subunit mRNAs measured in situ in rat hippocampus and cortex following chronic flurazepam administration. *J Mol Neurosci* 4:277-292.
- Toth M (2003): 5-HT<sub>1A</sub> receptor knockout mouse as a genetic model of anxiety. *Eur J Pharmacol* 463:177-184.

- Trulson ME, Preussler DW, Howell GA, Frederickson CJ (1982): Raphe unit activity in freely moving cats: effects of benzodiazepines. *Neuropharmacology* 21:1045-1050.
- Tsetsenis T, Ma XH, Lo Iacono L, Beck SG, Gross C (2007): Suppression of conditioning to ambiguous cues by pharmacogenetic inhibition of the dentate gyrus. *Nat Neurosci* 10:896-902.
- Turri MG, Datta SR, DeFries J, Henderson ND, Flint J (2001): QTL analysis identifies multiple behavioral dimensions in ethological tests of anxiety in laboratory mice. *Curr Biol* 11:725-734.

## U

- Ulrich-Lai YM, Herman JP (2009): Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci* 10:397-409.
- Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A (2003): Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. *Neuron* 39:401-407.
- Urwyler S, Mosbacher J, Lingenhoehl K, Heid J, Hofstetter K, Froestl W, et al (2001): Positive allosteric modulation of native and recombinant gamma-aminobutyric acid(B) receptors by 2,6-Di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930) and its aldehyde analog CGP13501. *Mol Pharmacol* 60:963-971.
- Urwyler S, Pozza MF, Lingenhoehl K, Mosbacher J, Lampert C, Froestl W, et al (2003): N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and structurally related compounds: novel allosteric enhancers of gamma-aminobutyric acidB receptor function. *J Pharmacol Exp Ther* 307:322-330.

## V

- Valdizan EM, Gutierrez O, Pazos A (2003): Adenylate cyclase activity in postmortem brain of suicide subjects: reduced response to beta-adrenergic stimulation. *Biol Psychiatry* 54:1457-1464.
- Vale W, Spiess J, Rivier C, Rivier J (1981): Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 213:1394-1397.
- Van Bogaert M, Oosting R, Toth M, Groenink L, van Oorschot R, Olivier B (2006a): Effects of genetic background and null mutation of 5-HT<sub>1A</sub> receptors on basal and stress-induced body temperature: modulation by serotonergic and GABA<sub>A</sub>-ergic drugs. *Eur J Pharmacol* 550:84-90.
- van Bogaert MJ, Groenink L, Oosting RS, Westphal KG, van der Gugten J, Olivier B (2006b): Mouse strain differences in autonomic responses to stress. *Genes Brain Behav* 5:139-149.
- van den Buuse M, Van Acker SA, Fluttert M, De Kloet ER (2001): Blood pressure, heart rate, and behavioral responses to psychological "novelty" stress in freely moving rats. *Psychophysiology* 38:490-499.
- Van der Heyden JA, Zethof TJ, Olivier B (1997): Stress-induced hyperthermia in singly housed mice. *Physiol Behav* 62:463-470.
- van der Zwaal EM, Luijendijk MC, Adan RA, la Fleur SE (2008): Olanzapine-induced weight gain: chronic infusion using osmotic minipumps does not result in stable plasma levels due to degradation of olanzapine in solution. *Eur J Pharmacol* 585:130-136.
- Van Diest I, Thayer JF, Vandeputte B, Van de Woestijne KP, Van den Bergh O (2006): Anxiety and respiratory variability. *Physiol Behav* 89:189-195.
- van Marle HJ, Hermans EJ, Qin S, Fernandez G (2009): From Specificity to Sensitivity: How Acute Stress Affects Amygdala Processing of Biologically Salient Stimuli. *Biol Psychiatry*.
- van Marum RJ, Wegewijs MA, Loonen AJ, Beers E (2007): Hypothermia following antipsychotic drug use. *Eur J Clin Pharmacol* 63:627-631.
- Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, et al (2000): Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* 428:191-212.

- van Praag HM (2004): Can stress cause depression? *Prog Neuropsychopharmacol Biol Psychiatry* 28:891-907.
- van Riezen H, Leonard BE (1990): Effects of psychotropic drugs on the behavior and neurochemistry of olfactory bulbectomized rats. *Pharmacol Ther* 47:21-34.
- van Riezen H, Schnieden H, Wren AF (1977): Olfactory bulb ablation in the rat: behavioral changes and their reversal by antidepressant drugs. *Br J Pharmacol* 60:521-528.
- van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, et al (2004): Requirement of alpha<sub>5</sub>-GABA<sub>A</sub> receptors for the development of tolerance to the sedative action of diazepam in mice. *J Neurosci* 24:6785-6790.
- van Steveninck AL, Gieschke R, Schoemaker RC, Roncari G, Tuk B, Pieters MS, et al (1996): Pharmacokinetic and pharmacodynamic interactions of bretazenil and diazepam with alcohol. *Br J Clin Pharmacol* 41:565-573.
- Varga V, Szekely AD, Csillag A, Sharp T, Hajos M (2001): Evidence for a role of GABA interneurons in the cortical modulation of midbrain 5-hydroxytryptamine neurones. *Neuroscience* 106:783-792.
- Vaswani M, Linda FK, Ramesh S (2003): Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review. *Prog Neuropsychopharmacol Biol Psychiatry* 27:85-102.
- Vazquez DM, Eskandari R, Zimmer CA, Levine S, Lopez JF (2002): Brain 5-HT receptor system in the stressed infant rat: implications for vulnerability to substance abuse. *Psychoneuroendocrinology* 27:245-272.
- Veening JG, Bocker KB, Verdouw PM, Olivier B, de Jongh R, Groenink L (2009): Activation of the septohippocampal system differentiates anxiety from fear in startle paradigms. *Neuroscience*.
- Veening JG, Bouwknecht JA, Joosten HJ, Dederen PJ, Zethof TJ, Groenink L, et al (2004): Stress-induced hyperthermia in the mouse: c-fos expression, corticosterone and temperature changes. *Prog Neuropsychopharmacol Biol Psychiatry* 28:699-707.
- Veening JG, Coolen LM, de Jong TR, Joosten HW, de Boer SF, Koolhaas JM, Olivier B (2005): Do similar neural systems subserve aggressive and sexual behavior in male rats? Insights from c-Fos and pharmacological studies. *Eur J Pharmacol* 526:226-239.
- Vellucci SV, File SE (1979): Chlordiazepoxide loses its anxiolytic action with long-term treatment. *Psychopharmacology (Berl)* 62:61-65.
- Vellucci SV, Parrott RF (1995): Prostaglandin-dependent c-Fos expression in the median preoptic nucleus of pigs subjected to restraint: correlation with hyperthermia. *Neurosci Lett* 198:49-51.
- Verge D, Daval G, Marcinkiewicz M, Patey A, el Mestikawy S, Gozlan H, Hamon M (1986): Quantitative autoradiography of multiple 5-HT<sub>1</sub> receptor subtypes in the brain of control or 5,7-dihydroxytryptamine-treated rats. *J Neurosci* 6:3474-3482.
- Verheyen S, Blaton N, Kinget R, Van den Mooter G (2002): Mechanism of increased dissolution of diazepam and temazepam from polyethylene glycol 6000 solid dispersions. *Int J Pharm* 249:45-58.
- Vicentic A, Francis D, Moffett M, Lakatos A, Rogge G, Hubert GW, et al (2006): Maternal separation alters serotonergic transporter densities and serotonergic 1A receptors in rat brain. *Neuroscience* 140:355-365.
- Villafuerte S, Burmeister M (2003): Untangling genetic networks of panic, phobia, fear and anxiety. *Genome Biol* 4:224.
- Vinkers C, Oosting R, Bogaert Mv, Olivier B, Groenink L (2009a): Early-life blockade of 5-HT<sub>1A</sub> receptors alters adult anxiety behavior and benzodiazepine sensitivity. *Biol Psychiatry* In press.
- Vinkers CH, Breuer ME, Westphal KG, Korte SM, Oosting RS, Olivier B, Groenink L (2009b): Olfactory bulbectomy induces rapid and stable changes in basal and stress-induced locomotor

- activity, heart rate and body temperature responses in the home cage. *Neuroscience* 159:39-46.
- Vinkers CH, de Jong NM, Kalkman CJ, Westphal KG, van Oorschot R, Olivier B, et al (2009c): Stress-induced hyperthermia is reduced by rapid-acting anxiolytic drugs independent of injection stress in rats. *Pharmacol Biochem Behav* 93:413-418.
- Vinkers CH, Groenink L, van Bogaert MJ, Westphal KG, Kalkman CJ, van Oorschot R, et al (2009d): Stress-induced hyperthermia and infection-induced fever: two of a kind? *Physiol Behav* 98:37-43.
- Vinkers CH, Klanker M, Groenink L, Korte SM, Cook JM, Van Linn ML, et al (2009e): Dissociating anxiolytic and sedative effects of GABA<sub>A</sub>ergic drugs using temperature and locomotor responses to acute stress. *Psychopharmacology (Berl)*.
- Vinkers CH, Klanker M, Groenink L, Korte SM, Cook JM, Van Linn ML, et al (2009f): Dissociating anxiolytic and sedative effects of GABA<sub>A</sub>ergic drugs using temperature and locomotor responses to acute stress. *Psychopharmacology (Berl)* 204:299-311.
- Vinkers CH, Oorschot Rv, Olivier B, Groenink L (2009g): Stress-Induced Hyperthermia in the Mouse. *Mood and Anxiety Related Phenotypes in Mice: characterization Using Behavioral Tests; Series: Neuromethods Gould, Todd D (Ed) Vol. 42*
- Vinkers CH, van Bogaert MJ, Klanker M, Korte SM, Oosting R, Hanania T, et al (2008): Translational aspects of pharmacological research into anxiety disorders: the stress-induced hyperthermia (SIH) paradigm. *Eur J Pharmacol* 585:407-425.
- Vochtelloo JD, Koolhaas JM (1987): Medial amygdala lesions in male rats reduce aggressive behavior: interference with experience. *Physiol Behav* 41:99-102.
- Voss J, Sanchez C, Michelsen S, Ebert B (2003): Rotarod studies in the rat of the GABA<sub>A</sub> receptor agonist gaboxadol: lack of ethanol potentiation and benzodiazepine cross-tolerance. *Eur J Pharmacol* 482:215-222.
- W**
- Wafford KA (2005): GABA<sub>A</sub> receptor subtypes: any clues to the mechanism of benzodiazepine dependence? *Curr Opin Pharmacol* 5:47-52.
- Wafford KA, Ebert B (2006): Gaboxadol—a new awakening in sleep. *Curr Opin Pharmacol* 6:30-36.
- Wafford KA, Whiting PJ, Kemp JA (1993): Differences in affinity and efficacy of benzodiazepine receptor ligands at recombinant gamma-aminobutyric acidA receptor subtypes. *Mol Pharmacol* 43:240-244.
- Wahlsten D, Metten P, Phillips TJ, Boehm SL, 2nd, Burkhart-Kasch S, Dorow J, et al (2003): Different data from different labs: lessons from studies of gene-environment interaction. *J Neurobiol* 54:283-311.
- Walker DL, Davis M (1997): Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. *J Neurosci* 17:9375-9383.
- Walker DL, Miles LA, Davis M (2009): Selective participation of the bed nucleus of the stria terminalis and CRF in sustained anxiety-like versus phasic fear-like responses. *Prog Neuropsychopharmacol Biol Psychiatry*.
- Walker DL, Paschall GY, Davis M (2005): Glutamate receptor antagonist infusions into the basolateral and medial amygdala reveal differential contributions to olfactory vs. context fear conditioning and expression. *Learn Mem* 12:120-129.
- Walker DL, Toufexis DJ, Davis M (2003): Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *Eur J Pharmacol* 463:199-216.
- Wallner M, Hanchar HJ, Olsen RW (2003): Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci U S A* 100:15218-15223.

- Wallner M, Olsen RW (2008): Physiology and pharmacology of alcohol: the imidazobenzodiazepine alcohol antagonist site on subtypes of GABA<sub>A</sub> receptors as an opportunity for drug development? *Br J Pharmacol* 154:288-298.
- Wan FJ, Swerdlow NR (1997): The basolateral amygdala regulates sensorimotor gating of acoustic startle in the rat. *Neuroscience* 76:715-724.
- Weidenfeld J, Itzik A, Goshen I, Yirmiya R, Ben-Hur T (2005): Role of the central amygdala in modulating the pituitary-adrenocortical and clinical responses in experimental herpes simplex virus-1 encephalitis. *Neuroendocrinology* 81:267-272.
- Weisstaub NV, Zhou M, Lira A, Lambe E, Gonzalez-Maeso J, Hornung JP, et al (2006): Cortical 5-HT<sub>2A</sub> receptor signaling modulates anxiety-like behaviors in mice. *Science* 313:536-540.
- Werka T, Skar J, Ursin H (1978): Exploration and avoidance in rats with lesions in amygdala and piriform cortex. *J Comp Physiol Psychol* 92:672-681.
- Wesolowska A (2008): The anxiolytic-like effect of the selective 5-HT<sub>6</sub> receptor antagonist SB-399885: the impact of benzodiazepine receptors. *Eur J Pharmacol* 580:355-360.
- Wesolowska A, Nikiforuk A (2007): Effects of the brain-penetrant and selective 5-HT<sub>6</sub> receptor antagonist SB-399885 in animal models of anxiety and depression. *Neuropharmacology* 52:1274-1283.
- Wesolowska A, Nikiforuk A, Stachowicz K, Tatarczynska E (2006): Effect of the selective 5-HT<sub>7</sub> receptor antagonist SB 269970 in animal models of anxiety and depression. *Neuropharmacology* 51:578-586.
- Wesolowska A, Paluchowska M, Chojnacka-Wojcik E (2003): Involvement of presynaptic 5-HT(1A) and benzodiazepine receptors in the anticonflict activity of 5-HT(1A) receptor antagonists. *Eur J Pharmacol* 471:27-34.
- West CH, Weiss JM (2005): A selective test for antidepressant treatments using rats bred for stress-induced reduction of motor activity in the swim test. *Psychopharmacology (Berl)* 182:9-23.
- Whiting PJ (2006): GABA-A receptors: a viable target for novel anxiolytics? *Curr Opin Pharmacol* 6:24-29.
- Whyte DG, Johnson AK (2007): Lesions of the anteroventral third ventricle region exaggerate neuroendocrine and thermogenic but not behavioral responses to a novel environment. *Am J Physiol Regul Integr Comp Physiol* 292:R137-142.
- Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992): The distribution of 13 GABA<sub>A</sub> receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J Neurosci* 12:1040-1062.
- Wood MD, Reavill C, Trail B, Wilson A, Stean T, Kennett GA, et al (2001): SB-243213; a selective 5-HT<sub>2C</sub> receptor inverse agonist with improved anxiolytic profile: lack of tolerance and withdrawal anxiety. *Neuropharmacology* 41:186-199.
- Woolley ML, Marsden CA, Fone KC (2004): 5-HT<sub>6</sub> receptors. *Curr Drug Targets CNS Neurol Disord* 3:59-79.
- Wright IK, Upton N, Marsden CA (1992): Effect of established and putative anxiolytics on extracellular 5-HT and 5-HIAA in the ventral hippocampus of rats during behavior on the elevated X-maze. *Psychopharmacology (Berl)* 109:338-346.
- Wrynn AS, Sebens JB, Koch T, Leonard BE, Korf J (2000): Prolonged c-Jun expression in the basolateral amygdala following bulbectomy: possible implications for antidepressant activity and time of onset. *Brain Res Mol Brain Res* 76:7-17.
- Wu Y, Rosenberg HC, Chiu TH, Zhao TJ (1994): Subunit- and brain region-specific reduction of GABA<sub>A</sub> receptor subunit mRNAs during chronic treatment of rats with diazepam. *J Mol Neurosci* 5:105-120.

**X**

Xu Y, Day TA, Buller KM (1999): The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta administration. *Neuroscience* 94:175-183.

**Y**

Yamada J, Sugimoto Y, Ohkura M, Inoue K (2001): Effects of the 5-HT<sub>2</sub> receptor antagonist, ritanserin on hyperthermia and depletion of 5-HT in frontal cortex induced by a 5-HT releasing drug, p-chloroamphetamine (PCA) in mice. *Biol Pharm Bull* 24:1195-1197.

Yeragani VK, Balon R, Pohl R, Ramesh C, Glitz D, Weinberg P, Merlos B (1990): Decreased R-R variance in panic disorder patients. *Acta Psychiatr Scand* 81:554-559.

Young SN (1996): Behavioral effects of dietary neurotransmitter precursors: basic and clinical aspects. *Neurosci Biobehav Rev* 20:313-323.

Yu B, Shinnick-Gallagher P (1998): Corticotropin-releasing factor increases dihydropyridine- and neurotoxin-resistant calcium currents in neurons of the central amygdala. *J Pharmacol Exp Ther* 284:170-179.

Yu H, Lewander T (1997): Pharmacokinetic and pharmacodynamic studies of (R)-8-hydroxy-2-(di-n-propylamino)tetralin in the rat. *Eur Neuropsychopharmacol* 7:165-172.

**Z**

Zaleski MJ, Nunes Filho JR, Lemos T, Morato GS (2001): GABA<sub>(B)</sub> receptors play a role in the development of tolerance to ethanol in mice. *Psychopharmacology (Berl)* 153:415-424.

Zammit G (2009): Comparative tolerability of newer agents for insomnia. *Drug Saf* 32:735-748.

Zaretskaia MV, Zaretsky DV, Shekhar A, DiMicco JA (2002): Chemical stimulation of the dorsomedial hypothalamus evokes non-shivering thermogenesis in anesthetized rats. *Brain Res* 928:113-125.

Zethof TJ, Van der Heyden JA, Tolboom JT, Olivier B (1994): Stress-induced hyperthermia in mice: a methodological study. *Physiol Behav* 55:109-115.

Zethof TJ, Van der Heyden JA, Tolboom JT, Olivier B (1995): Stress-induced hyperthermia as a putative anxiety model. *Eur J Pharmacol* 294:125-135.

Zhang J, Rivest S (2000): A functional analysis of EP4 receptor-expressing neurons in mediating the action of prostaglandin E<sub>2</sub> within specific nuclei of the brain in response to circulating interleukin-1beta. *J Neurochem* 74:2134-2145.

Zhang YH, Lu J, Elmquist JK, Saper CB (2000): Lipopolysaccharide activates specific populations of hypothalamic and brainstem neurons that project to the spinal cord. *J Neurosci* 20:6578-6586.

Zhao TJ, Chiu TH, Rosenberg HC (1994): Reduced expression of gamma-aminobutyric acid type A/benzodiazepine receptor gamma<sub>2</sub> and alpha<sub>5</sub> subunit mRNAs in brain regions of flurazepam-treated rats. *Mol Pharmacol* 45:657-663.

Zhuang X, Gross C, Santarelli L, Compan V, Trillat AC, Hen R (1999): Altered emotional states in knockout mice lacking 5-HT<sub>1A</sub> or 5-HT<sub>1B</sub> receptors. *Neuropsychopharmacology* 21:52S-60S.

Ziabreva I, Poeggel G, Schnabel R, Braun K (2003a): Separation-induced receptor changes in the hippocampus and amygdala of Octodon degus: influence of maternal vocalizations. *J Neurosci* 23:5329-5336.

Ziabreva I, Schnabel R, Poeggel G, Braun K (2003b): Mother's voice "buffers" separation-induced receptor changes in the prefrontal cortex of octodon degus. *Neuroscience* 119:433-441.

Zohar J, Westenberg HG (2000): Anxiety disorders: a review of tricyclic antidepressants and selective serotonin reuptake inhibitors. *Acta Psychiatr Scand Suppl* 403:39-49.



The background features a faded image of a person with their arms raised in a celebratory gesture. Overlaid on this is a faint line graph with several data points and connecting lines, suggesting a business or performance context.

**Summary**  
**Samenvatting**

Su

## Summary

The research described in this thesis shows that the change in body temperature in response to stress (stress-induced hyperthermia (SIH)) can be employed to study stress in its broadest sense with a wide variety of applications. In this thesis, we used the SIH paradigm to examine the pharmacological, genetic and mechanistic backgrounds of stress and their possible consequences at the receptor level.

An important finding of the current research is that the SIH paradigm is suitable to assess the acute and chronic effects of  $\alpha$  subunit-selective GABA<sub>A</sub> receptor agonists. Using novel selective ligands, we show that the SIH paradigm can be used to dissect the contributions of the different  $\alpha$  subunits (chapter 3 and 5). In particular, we confirm putative anxiolytic effects for GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonists (chapter 3) which do not result in the development of tolerance after chronic treatment (chapter 5). Further, our results indicate that the  $\alpha_1$  subunit is involved in hypothermia and that the  $\alpha_5$  subunit is not directly involved in the anxiolytic or hypothermic effects of benzodiazepines (chapter 3 and 5; for a review on the GABA<sub>A</sub> and GABA<sub>B</sub> receptor and the SIH paradigm see part II of chapter 15). Another important finding is that rapid tolerance did not occur after chronic activation of the  $\alpha_1$ ,  $\alpha_{2/3}$  or  $\alpha_5$  subunit, whereas this effect did occur after treatment with the non subunit-selective benzodiazepine diazepam (chapter 5). Together, these data indicate that selective GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonists constitute a promising class of novel anxiolytics.

In addition to the GABA<sub>A</sub> receptor studies, we confirm and extend the findings that a variety of putative anxiolytic ligands including corticotropin-releasing factor type 1 (CRF<sub>1</sub>), nicotinic, serotonergic and glutamatergic receptor ligands reduce the SIH response (chapter 8 and 10). In contrast, the SIH response is not reduced in response to non-steroidal anti-inflammatory drugs (NSAIDs), indicating that the SIH is distinct from infection-induced fever (chapter 7). Moreover, we provide evidence that the SIH response can be used to investigate the functional interactions of different neurotransmitter systems in response to stress (chapter 4 and 6). This is important since a proper functioning and cooperation of multiple neurotransmitter systems is necessary to generate a coordinated stress response, and may prevent the development of stress-related disorders. In this thesis, we show that high levels of CRF in the central nervous system modulate the GABA<sub>A</sub> and metabotropic glutamate receptor system (chapter 10). Chromosome substitution in mice affected benzodiazepine and serotonin receptor sensitivity (chapter 11). Moreover, the SIH paradigm is sufficiently sensitive to establish adult benzodiazepine insensitivity which is the result of a transient early-life disruption of the serotonin system (chapter 6; for a review on the role of the 5-HT system in the SIH paradigm see part III of chapter 15). Also, we found that the medial amygdala and the olfactory system are both closely involved in the SIH response (chapter 12 and 13). Of particular interest, we found that the medial amygdala is not only directly involved in the SIH response, but also alters sensorimotor gating and light-enhanced startle.

Our finding that exposure to psychological stress affects core and peripheral body temperature in healthy human volunteers indicates that the SIH paradigm may possess translational potential (chapter 14). This indicates that the preclinical results obtained with SIH in animals could be translated to - and could therefore be relevant for - research into human stress-related disorders. Moreover, a decrease in core temperature correlated with self-reported subjective stress in healthy human volunteers, suggesting that stress-induced core intestinal temperature changes may constitute a valid read out parameter (for a review on the translational potential of the SIH paradigm see part IV of chapter 15).

To summarize, the research described in this thesis shows that the SIH paradigm can be employed in preclinical and possibly clinical setups and provides a tool to examine the pharmacological, genetic and mechanistic background of stress.

## Samenvatting in het Nederlands

Stress is een uiterst complex begrip dat wordt geassocieerd met ziekte. Deze associatie is deels niet terecht omdat een stress reactie (de fysiologische en gedragsmatige respons van een organisme op een stressvolle situatie) een essentieel mechanisme vormt waarmee mensen zich kunnen handhaven in een veranderende omgeving. Chronische of extreme stress kan echter leiden tot een negatieve spiraal die uiteindelijk geassocieerd is met het ontstaan van verschillende ziektes. Hoe stress precies leidt tot een (psychiatrische) stoornis, en waarom de stressgevoeligheid sterk verschilt per individu is nog grotendeels onbekend.

In dit proefschrift wordt gebruik gemaakt van het gegeven dat blootstelling aan stress leidt tot temperatuursveranderingen (stress-geïnduceerde hyperthermie, SIH). Deze SIH reactie komt zowel voor in mens als dier en kan op veel verschillende manieren gebruikt worden om stress te bestuderen (hoofdstuk 1 en 2). In dit proefschrift hebben we de SIH gebruikt om de farmacologische effecten, de genetische achtergrond en de functie van bepaalde hersengebieden op de stressreactie te bestuderen.

De essentie van farmacologisch SIH-onderzoek bestaat eruit dat angstremmende stoffen de door stress veroorzaakte temperatuursstijging kunnen voorkómen. Als deze temperatuursstijging voorkomen kan worden, zou dit een indicatie kunnen zijn dat ook andere gevolgen van stress verminderd zouden kunnen zijn. Een belangrijke bevinding van ons farmacologisch onderzoek is dat met de SIH de acute en chronische effecten van de selectieve GABA<sub>A</sub> receptor agonisten te bestuderen zijn. Dit is van belang omdat niet-selectieve GABA<sub>A</sub> receptor agonisten (benzodiazepines) angstremmend zijn maar veel bijwerkingen kennen. Met het SIH paradigma bevestigen we dat de  $\alpha_{2/3}$  subunit van de GABA<sub>A</sub> receptor betrokken is bij de angstremmende effecten van benzodiazepines (hoofdstuk 3). Van belang is ook dat chronische blootstelling aan een stof die de GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit activeert er niet toe leidt dat steeds meer van deze stof nodig is om het gewenste effect te hebben (tolerantie), in tegenstelling tot de niet-selectieve benzodiazepine diazepam (hoofdstuk 5). Ook laten we zien dat de  $\alpha_1$  subunit betrokken is bij hypothermie (een daling van de lichaamstemperatuur) en dat de  $\alpha_5$  subunit niet direct betrokken is bij de angstremmende en bij de temperatuursverlagende effecten van benzodiazepines (hoofdstuk 3 en 5; voor een complete bespreking van de literatuur over de effecten van GABAerge stoffen: zie deel II van hoofdstuk 15). Daarmee lijken selectieve GABA<sub>A</sub> receptor  $\alpha_{2/3}$  subunit agonisten een veelbelovende nieuwe klasse angstremmende stoffen.

Ook andere mogelijk angstremmende geneesmiddelklassen kunnen de SIH respons verminderen, waaronder corticotropin-releasing factor (CRF)<sub>1</sub>, nicotine, serotonerge en glutamaterge stoffen (hoofdstuk 8 en 10). Dit in tegenstelling tot ontstekingsremmende geneesmiddelen zoals acetylsalicylzuur die de SIH reactie niet beïnvloeden (hoofdstuk 7). Deze resultaten suggereren dat aan de temperatuursstijging door stress (SIH) een compleet ander mechanisme ten grondslag ligt dan aan de temperatuursstijging door een infectie (koorts). Daarnaast tonen we aan dat hoge concentraties van CRF in het brein leiden tot veranderingen in de GABA<sub>A</sub> en metabotrope glutamaat receptor systemen

(hoofdstuk 10). Ook bestudeerden we de invloed van verschillende chromosomen op de gevoeligheid voor GABAerge en serotonerge farmaca in het SIH paradigma (hoofdstuk 11).

Ons onderzoek toont dat het SIH paradigma inzicht kan geven in de neurobiologische mechanismen die met stress geassocieerd zijn. Zo laten we zien dat de SIH gebruikt kan worden om functionele interacties tussen verschillende neurotransmitter systemen bij stress bloot te leggen (met name tussen de GABA<sub>A</sub> en serotonine receptorsystemen) (hoofdstuk 4 en 6). Dit is van belang aangezien verschillende neurotransmitters nauw samenwerken om een gecoördineerde stress reactie te organiseren, en verandering in het functioneren van meerdere neurotransmitter systemen bijdragen aan de ontwikkeling van stress-gerelateerde aandoeningen.

Ook wijzen onze resultaten uit dat SIH gebruikt kan worden bij het identificeren van gevoeligheid van factoren die de stress respons beïnvloeden. SIH blijkt een sensitief instrument om ongevoeligheid voor benzodiazepines op volwassen leeftijd op te sporen, die het gevolg is van verstoring van het serotonine systeem tijdens de vroege levensjaren (hoofdstuk 6 ; voor een compleet overzicht over de rol van serotonine systeem in het SIH paradigma zie deel III van hoofdstuk 15). Daarnaast vonden we aanwijzingen dat de mediale amygdala en het olfactoire systeem beide bij SIH betrokken zijn (hoofdstuk 12 en 13).

Tot slot kan het in dit proefschrift vastgelegde onderzoek relevant zijn voor de klinische praktijk. De relatie tussen stress en temperatuur is namelijk niet alleen op dieren van toepassing, deze relatie vonden wij ook bij onderzoek in mensen. Wij tonen dat de centrale en perifere lichaamstemperatuur verandert in gezonde vrijwilligers na blootstelling aan (psychologische) stress, waarmee we bevestigen dat SIH translationeel potentieel bezit (hoofdstuk 14). Vooral het feit dat de centrale temperatuursdaling na stress correleert met de subjectieve ervaring van stresstoename suggereert dat lichaamstemperatuur een mogelijk gevoelige uitleesmaat voor het subjectieve stressniveau kan zijn. Verder onderzoek zal moeten uitwijzen in hoeverre het SIH paradigma direct toepasbaar is in klinisch onderzoek.

Met dit proefschrift laten we zien dat het SIH paradigma gebruikt kan worden bij zowel dieren als mensen. Hiermee vormt het mogelijkwijs een instrument waarmee stressprocessen bestudeerd kunnen worden, in het bijzonder de effecten van geneesmiddelen, de invloeden van de genetische achtergrond en de betrokkenheid van hersengebieden bij de stress reactie.



**List of Publications**  
**Author affiliations**  
**Acknowledgements**  
**About the author**

**A**

## List of publications

### Publications resulting from this thesis

1. Vinkers CH, van Bogaert MJ, Klanker M, Korte SM, Oosting R, Hanania T, et al (2008): Translational aspects of pharmacological research into anxiety disorders: the stress-induced hyperthermia (SIH) paradigm. *Eur J Pharmacol* 585:407-425.
2. Vinkers CH, Oorschot R v, Olivier B, Groenink L (2009): Stress-Induced Hyperthermia in the Mouse. Mood and Anxiety Related Phenotypes in Mice: characterization Using Behavioral Tests; Series: Neuromethods Gould, Todd D (Ed) Vol. 42.
3. Groenink L, Vinkers CH, van Oorschot R, Olivier B (2009): Models of anxiety: Stress-Induced Hyperthermia (SIH) in singly housed mice. *Current protocols of pharmacology* S45:5.16.11 - 15.16.12.
4. Vinkers CH, Klanker M, Groenink L, Korte SM, Cook JM, Van Linn ML, et al (2009): Dissociating anxiolytic and sedative effects of GABAergic drugs using temperature and locomotor responses to acute stress. *Psychopharmacology (Berl)* 204:299-311.
5. Vinkers CH, Oosting RS, Bogaert MJ van, Olivier B and Groenink L (2009), Early-life blockade of 5-HT<sub>1A</sub> receptors alters adult anxiety behavior and benzodiazepine sensitivity, *Biological Psychiatry, In Press*.
6. Vinkers CH, Groenink L, van Bogaert MJ, Westphal KG, Kalkman CJ, van Oorschot R, et al (2009): Stress-induced hyperthermia and infection-induced fever: two of a kind? *Physiol Behav* 98:37-43.
7. Vinkers CH, de Jong NM, Kalkman CJ, Westphal KG, van Oorschot R, Olivier B, et al (2009): Stress-induced hyperthermia is reduced by rapid-acting anxiolytic drugs independent of injection stress in rats. *Pharmacol Biochem Behav* 93:413-418.
8. Vinkers CH, Breuer ME, Westphal KG, Korte SM, Oosting RS, Olivier B, Groenink L (2009): Olfactory bulbectomy induces rapid and stable changes in basal and stress-induced locomotor activity, heart rate and body temperature responses in the home cage. *Neuroscience* 159:39-46.
9. Vinkers CH, Cryan JF, Olivier B and Groenink L (2009), Elucidating GABA<sub>A</sub> and GABA<sub>B</sub> receptor functions in anxiety using the stress-induced hyperthermia model: a review, *The Open Pharmacology Journal, In Press*.
10. Vinkers CH, Olivier B, Bouwknecht JA, Groenink L and Olivier JD (2009), Stress-induced hyperthermia, the serotonin system and anxiety, *The Open Pharmacology Journal, In Press*.

11. Vinkers CH, Penning R, Ebbens MM, Hellhammer J, Verster JC, Kalkman CJ and Olivier B (2009), Stress-induced hyperthermia in translational stress research, *The Open Pharmacology Journal*, *In Press*.

### **Other publications**

1. Korte SM, Prins J, Vinkers CH, Olivier B (2009), *The Veterinary Journal*, On the Origin of Allostasis and Stress-induced Pathology in Farm Animals: celebrating Darwin's Legacy, *The Veterinary Journal*, *In Press*.
2. Vinkers CH, Risbrough VB, Geyer MA, Caldwell S, Low MJ, Hauger RL (2007): Role of dopamine D1 and D2 receptors in CRF-induced disruption of sensorimotor gating. *Pharmacol Biochem Behav* 86:550-558.
3. Fabius AM, Cheung KC, Rijcken CJ, Vinkers CH, Talsma H (2004): Direct-to-consumer communication on prescription only medicines via the internet in the Netherlands, a pilot study. Opinion of the pharmaceutical industry, patient associations and support groups. *Pharm World Sci* 26:169-172.

## Author Affiliations

- Philip K. Ahring** Dept. of Pharmacology, NeuroSearch A/S, Ballerup, Denmark
- Elisabeth Y. Bijlsma** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
- Meg J. van Bogaert** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, The Netherlands
- Gerdien A.H. Bouws-Korte** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
- Adriaan J. Bouwknecht** Dept. Neuroscience, Pharmaceutical Research & Development, Johnson and Johnson, Beerse, Belgium
- Megan E. Breuer** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
- Una Campbell** Sepracor Inc., Marlborough, MA 01752, USA
- Terry Clayton Sr.** Dept. of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA
- James M. Cook** Dept. of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA

---

<b>John F. Cryan</b>	School of Pharmacy, Dept. of Pharmacology & Therapeutics, Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland
<b>Marieke M. Ebbens</b>	Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
<b>Lucianne Groenink</b>	Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
<b>Taleen Hanania</b>	PsychoGenics Inc. , Tarrytown, NY 10591, USA
<b>Henrik H. Hansen</b>	Dept. of Pharmacology, NeuroSearch A/S, Ballerup, Denmark
<b>Juliane Hellhammer</b>	DAaCRO Clinical Research Organization, Trier, Germany
<b>Hendrikus Hendriksen</b>	Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
<b>Seth C. Hopkins</b>	Sepracor Inc., Marlborough, MA 01752, USA
<b>Lotte C. Houtepen</b>	Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
<b>Shengming Huang</b>	Dept. of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA

- Gerhardus J. de Jong** Dept. of Biomedical Analysis, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands
- Noëlle M. de Jong** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
- Cor J. Kalkman** Dept. of Anesthesiology, Division of Perioperative & Emergency Medicine, University Medical Centre Utrecht, Utrecht, the Netherlands
- Martien J.H. Kas** Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Centre Utrecht, Utrecht, the Netherlands
- Marianne Klanker** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
- S. Mechiel Korte** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands
- Mike van Linn** Dept. of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA
- Mark J. Millan** Institute de Reserche Servier, Croissy sur Seine, France
- Naheed R. Mirza** Dept. of Pharmacology, NeuroSearch A/S, Ballerup, Denmark
- Elsebet Ø. Nielsen** Dept. of Pharmacology, NeuroSearch A/S, Ballerup, Denmark
- Ronald S. Oosting** Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute

of Neurosciences, Utrecht University, Utrecht, The Netherlands

**Berend Olivier**

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

Dept. of Psychiatry, Yale University School of Medicine, New Haven, CT, USA

**Hugo Oppelaar**

Department of Neuroscience and Pharmacology, Rudolf Magnus Institute of Neuroscience, University Medical Centre Utrecht, Utrecht, the Netherlands

**Jocelien D.A. Olivier**

Dept. of Molecular Animal Physiology, Radboud University and Donders Institute for Brain, Cognition and Behavior: Dept. for Neuroscience, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

**Ruud van Oorschot**

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

**Renske Penning**

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

**Daniëlle Peterse**

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

**Sundari Rallapalli**

Dept. of Chemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA

**Javier Sastre Torano**

Dept. of Biomedical Analysis, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands

**Jan G. Veening**

Dept. of Anatomy, UMC St Radboud, University of Nijmegen, Nijmegen, The Netherlands

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

**Joris C. Verster**

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

**Koen G.C. Westphal**

Dept. of Psychopharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neurosciences, Utrecht University, Utrecht, The Netherlands

## Acknowledgements / dankwoord

Allereerst wil ik Ruud van Oorschot en Koen Westphal bedanken voor hun essentiële en niet aflatende hulp om de dierexperimentele studies in dit proefschrift tot een succes te maken. Zonder jullie was het proefschrift in deze vorm er zeker niet geweest.

Berend Olivier en Cor Kalkman, mijn promotoren, wil ik hartelijk danken voor het in mij gestelde vertrouwen en de open en vrije sfeer waarin het onderzoek kon plaatsvinden. Berend, jouw enthousiasme en voortvarendheid om het onderzoeksgebied van de stressgeïnduceerde hyperthermie verder te verkennen werkten voor mij als een katalysator. Cor, jouw eigenschap om in zeer korte tijd tot de (wetenschappelijke) essentie door te dringen hoop ik ooit nog te mogen verwerven.

Lucianne Groenink en Mechiel Korte, mijn co-promotoren, wil ik danken voor de steun in woord en daad bij het uitvoeren van het onderzoek. Lucianne, je hebt een scherpe geest met daarbij een geweldig gevoel voor humor. Mechiel, jouw relativeringsvermogen en de aansporingen om mijn onderzoek in een groter verband te zien heb ik zeker in mijn oren geknoopt.

Ik wil ook alle collega's en studenten bij de afdeling Psychofarmacologie bedanken voor de uitstekende sfeer waarin ik de afgelopen jaren heb gewerkt en voor alle (spontane) hulp die ik heb mogen ontvangen: Marga (schaakkampioene in de dop), Monika (CRF<sub>OE</sub> studie), Jan V (laesiestudie), Gerdien (CDP studie), Joris (TEMP studie), Erik (mRNA bepalingen en het monitoren van de afdeling), Esther V (TEMP studie), Ronald (early-life studie), Jolanda (chronische benzostudie), Eelke, Johnny, Trynke, Meg B (OBX studie), Meg v B (meerdere studies), Liesbeth (laesiestudie), Tessa, Koen B, Ed, Monique (TEMP studie), Rudi en Martje (TEMP studie).

Ook wil ik de studenten bedanken die met grote inzet en zelfstandigheid in hoge mate hebben bijgedragen aan de totstandkoming van dit proefschrift: Marianne, Noëlle, Lotte, Daniëlle, Renske en Marieke. Jullie hulp is onmisbaar geweest!

Seth and Una, I would like to thank you both for our discussions on the SIH paradigm and your help in obtaining various compounds. I have also greatly enjoyed our discussions on subunit-selective GABAergic drugs.

Professor Jim Cook, thank you for the never-ending supply of novel compounds that made many studies possible. Also, thanks to all the other people who are closely involved in the development and synthesis of these compounds: Mike, Sundari, Shengming and Terry.

Max, thank you for your kind hospitality at Neurosearch as well as for your wonderful open mind which made our collaboration possible. I am looking forward to the continuation! Furthermore, I am grateful to all the people at Neurosearch directly involved in our collaboration: Elsebet, Henrik, Janus and Philip.

Professor Ad de Jong en Javier Sastre Toraño, bedankt voor de goede samenwerking. Het is geweldig om te zien dat een complementaire samenwerking op farmaceutisch gebied tot mooie resultaten kan leiden.

Juliane, I would like to thank you for your kind hospitality in Trier, and, moreover, for the great collaboration on making the human SIH study a success. Hopefully, our collaboration will extend into the future!

Martien en Hugo, dank voor de vruchtbare samenwerking die het afgelopen jaar tot stand kwam.

Hans en alle andere dierverzorgers, dank voor de goede zorgen voor alle proefdieren en de ongedwongen sfeer waarin alles mocht verlopen. Fred Poelma en Harry Blom, de proefdierdeskundigen, wil ik beide bedanken voor het met raad en daad terzijde staan tijdens de verschillende studies. Ik wil jullie complimenteren met de door jullie gekozen balans waarin zowel welzijn als praktische werkbaarheid van groot belang zijn. Paul Henricks wil ik bedanken voor de hulp bij het mogelijk maken van het importeren van proefdieren.

Mark Geyer and Vickie Risbrough, I would like to express my gratitude for your guidance and help in introducing me into the wonderful world of science.

Dr. Kees Braun en Dr. Geert-Jan Biessels wil ik bedanken voor hun hulp om het in dit proefschrift vastgelegde onderzoek te kunnen combineren met mijn opleiding tot arts.

Het fundament voor het humane onderzoek werd mede gelegd door Dr. Wim Bouma met wiens hulp ik mijn eerste auriculare temperatuursmetingen verrichtte.

Jocelien en Adriaan wil ik bedanken voor de goede interactie om de serotonine review zoals die in hoofdstuk 15 staat tot een goed einde te brengen. John, thanks for your swift and comprehensive contribution to our GABA review.

Ik wil ook graag een aantal vrienden en (oud)-huisgenoten bedanken voor de ontspanning de afgelopen jaren. Niels (E) en Martijn (P), ik beleef nog steeds erg veel plezier aan onze reguliere samenkomsten. Les/Brainz/Os, onze hatseflatsavonden zijn legendarisch! Koningen (Joost, Ome, Hassan, I, Dordt, Paul, Sjoerd, Rob, Niels, Maesch en de Natte) onze uitstapjes leiden altijd tot veel gekkigheid. EH-broeders en aanwas (Anton, Billum, Floris, Jacob, Martijn, Michiel en Persijn), het doet me deugd jullie nog steeds te zien. De editors-in-chief van de Journal of Handsome Scientists (Nelis/Rolf), hulde voor jullie prestatie! Ook wil ik een aantal mensen van de SUMMA opleiding bedanken met wie ik een geweldige tijd heb gehad: hopelijk blijven we elkaar nog vaak zien (Barbara, Benoit, Jesper, Joppe, Roeland, Roos, Tim, en last but not least Ula en Wendele)! Familie Meulenaar (Jelte/Albertien), jullie aanwezigheid (vooral in combinatie) is een niet te missen ervaring! To my foreign friends that I met through EPSA (Benigne, Timo, Riccardo, Sergio and all the others I forgot to mention): having you around is a great thing! Femke (nu collega), Jeroen (de toekomst van de farmacie – thans in duplo) en Rubi (ik denk met

plezier terug aan onze mooie tijd in San Diego), ons bestuursjaar vormde een solide basis voor vele mooie avonturen!

Joost en Roeland, ik ben vereerd dat jullie als paranimf willen optreden. De afgelopen jaren heb ik veel plezier beleefd aan onze serieuze en minder serieuze discussies!

Mijn familie wil ik vanaf deze plek bedanken voor alle steun maar nog meer het plezier dat ik samen met jullie heb. Marieke wil ik daarnaast extra danken voor het ontwerp van de omslag en het overzichtsfiguur. Speciaal wil ik mijn vader bedanken – ik ben misschien nog trotser op jou dan andersom!

Lieve Sanne, je begrijpt me volkomen binnen maar ook buiten de wetenschap. Je bent mijn lieve nerdje!

## About the author

The author of this thesis was born on September 6<sup>th</sup>, 1980 in Wageningen, The Netherlands. In 1998, he passed his VWO exam at the Revis Lyceum in Doorn. He subsequently studied pharmacy (MSc 2004), medicine (Selective Medical Master Utrecht, MD 2009) and law (LLB with a minor philosophy (2007), and LLM in public law (2009)) at Utrecht University, Utrecht, the Netherlands. During his studies, he carried out research at the University of California, San Diego on the central dopaminergic effects of corticotropin-releasing factor under supervision of Dr. V.B. Risbrough and Prof. Dr. M.A. Geyer (2004) and followed a clinical internship in internal medicine in Paris, France (2006). His master thesis in public law concerned the public background of mental health care under supervision of Prof. R. Widdershoven. The research described in this thesis was initiated in 2006. In September 2009, he started his specialization in psychiatry at the University Medical Center in Utrecht, the Netherlands.

*Habe nun, ach! Philosophie,  
Juristerei und Medizin,  
Und leider auch Theologie!  
Durchaus studiert, mit heißem Bemühn.  
Da steh ich nun, ich armer Thor!  
Und bin so klug als wie zuvor.*

J.W. von Goethe, Faust I, 354-359 (1808)