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THE ANXIOUS MOUSE:

implications for preclinical research and animal welfare

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The anxious mouse:
implications for preclinical research
and animal welfare

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THE ANXIOUS MOUSE:

implications for preclinical research and animal welfare

De angstige muis:
implicaties voor preklinisch onderzoek en dierenwelzijn

(met een samenvatting in het Nederlands)

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Chapter

1

General Introduction

Biological relevance of anxiety

Anxiety is a fundamental emotion that is highly conserved during evolution and is present in animals (Livesey, 1986) and serves as a warning system that is elicited by real or potentially real threatening stimuli, which include for example novelty. An anxiety response is accompanied by behavioural, neurological and physiological responses which prepare the individual to react adequately to environmental challenges, such as improving attention and reducing reaction times (Humble, 1987). A moderate threat usually results in defensive responses, i.e. avoidance of the threat, hiding and risk assessment behaviours; while an extreme threat can lead to flight or fight behaviours representing an emotional state of extreme anxiety (Blanchard et al., 2001). In humans, anxiety responses can be associated with bodily discomfort and can be experienced as distressing and negative emotions (Beck et al., 1985). Despite of its emotionally negative nature, anxiety is a biologically useful and adaptive response. It allows the individual to learn about stimuli by identifying familiar ones and developing appropriate approaches, thereby increasing its survival.

In humans, dysfunctional anxiety responses are commonly observed. When anxiety responses become disproportionate to the potential threat or are elicited in the absence of a recognizable threat, a chronic state of anxiety, and eventually pathological anxiety, may develop (Lang et al., 1998). Anxiety disorders are among the most prevalent types of psychiatric disorders which affect about 10-17% of the world population (Somers et al., 2006) and present a major problem on an economical and social level in society (Greenberg et al., 1999). However, anxiety is not a unitary phenomenon and comprises more than just an emotional response to a threat. Different brain areas and transmitters are involved in different aspects of anxiety *[see Fig 1]*. First of all, fear differs from anxiety since fear is elicited by immediate and present danger, while anxiety is evoked by a potential threat and can be described as a future oriented mood (Barlow and Wincze, 1998). Both phenomena produce a completely different response mediated by different brain areas (Davis, 2006). Fear leads to flight or fight reactions mediated by the periaqueductal gray and the amygdala (Behbehani, 1995; Litvin et al., 2007).

However, approach or avoidance behaviour of a potential threat is thought to be mediated by the septo-hippocampal system and basolateral amygdala (Gray, 1982; Gray and McNaughton, 1983; McNaughton and Corr, 2004). Although, some suggest that fear is elicited by conditioned stimuli and anxiety by unconditioned (Walker and Davis, 1997), it is also found that animal tests that model fear and anxiety can be both conditioned and unconditioned, while the conditioned response is thought to be controlled by the central amygdala which results in freezing behaviour (Killcross et

al., 1997; Wilensky et al., 2006). Another distinction can be made between state and trait anxiety (Belzung and Griebel, 2001): 'state anxiety' can be described as situation evoked anxiety, whereas 'trait anxiety' is considered as an enduring feature of an individual. Notably, both phenomena cannot easily be separated from each other since an individual with high trait anxiety would probably also show high state anxiety. In fact, it has been suggested that pathological anxiety in general is a cognitive dysfunction in which the sufferer is unable to attune or integrate information on a higher cognitive level, which results in inappropriate emotional responses (McNaughton, 1997). Higher cortical brain areas, such as the prefrontal cortex, are believed to be involved when stimuli are more cognitive demanding and cognitive control is needed to some extent (Goldman-Rakic, 1995).

Fig. 1

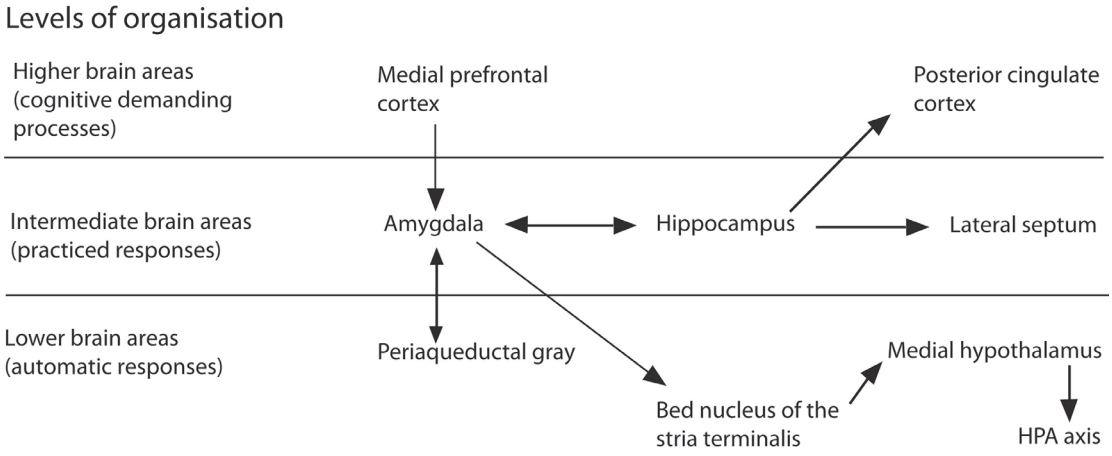

Figure 1: A simplified organization of the neural circuitry underlying anxiety adapted from Finn et al. (2003). Anxiety is thought to be organized in different levels. Higher cortical regions, such as the prefrontal cortex manage more cognitive demanding stimuli, thereby enhancing behavioural flexibility and integrating information to guide subsequent behaviour. A connection between the amygdala and prefrontal cortex is needed for extinction of conditioned fear responses and suppression of responses to anxiety related stimuli. The amygdala itself has extensive projections to the cortex and the brainstem and therefore plays an important role in response to danger. The hippocampus is thought to function as comparator system, which can distinguish novel from familiar stimuli. Lower brain areas such as the periaqueductal gray, mediate automatic responses, while the hypothalamus is a critical site for the integration of autonomic and endocrine responses to anxiety, such as activation of the hypothalamic-pituitary-adrenal axis.

At the biochemical level, several neurotransmitters are known to play a role in regulating anxiety. The neuromodulator corticotropin-releasing-factor (CRF) is often implicated as a causal factor in anxiety disorders (Arzt and Holsboer, 2006). CRF is a mediator of endocrine, autonomic and behavioural responses to stress and is found in the brain of all mammals (Takahashi et al., 2001). Moreover, CRF is localized in several brain areas involved in anxiety (Korosi et al., 2006). A variety of animal studies suggest a modulatory effect of CRF on anxiety responses. For example, central administration of CRF in mice resulted in anxiogenic (anxiety-enhancing) effects (Momose et al., 1999), while CRF knock-out mice showed an anxiolytic (anxiety-reducing) behavioural profile (Contarino et al., 1999; Smith et al., 1998). At a higher cortical level, CRF is a key mediator needed to adapt to stress. But on a lower brain level, other neurotransmitters are involved such as the main excitatory transmitter 'glutamate'. This most prevalent neurotransmitter helps maintaining brain homeostasis and alterations in its release have been found to exert effects that disrupt normal functioning (Kent et al., 2002; Mathew et al., 2001). Novel ligands that target glutamate receptors have been shown to induce anxiolytic effects in animals (Simon and Gorman, 2006; Spooren et al., 2000). It can thus be hypothesised that modulatory neurotransmitters, such as CRF exert their effects on generally available neurotransmitter systems, such as glutamate, working in a concerted action (Liu et al., 2004). Alterations in these neurotransmitter and neuromodulatory systems can affect normal functioning eventually leading to inappropriate emotional responses. Although many factors have been found to play a role in the aetiology of anxiety disorders, such as altered neurotransmission and the influence of environmental factors, the specific neurobiological mechanisms underlying pathological anxiety still have to be elucidated. An animal model for pathological anxiety would not only be of high use for translational research for human anxiety disorders, but also for the investigation of animal welfare aspects.
An appropriate animal model for pathological anxiety would lead to more understanding of underlying mechanisms in both humans and animals.

Identification of pathological anxiety in mice

The assessment of anxiety-related behaviour in rodents is based on the assumption that anxiety in animals is comparable to anxiety in humans (Hall, 1936). Although we cannot prove that they experience anxiety similarly to humans, distinct behavioural and physiological reactions in rodents and humans are comparable. For example, anxiety-related behaviour in rodents is affected by anxiolytic and anxiogenic drugs known to have similar effects in humans (Blanchard et al., 2001; Leonardo and Hen, 2006). This supports the idea that the neural circuitry as well as several neurotransmitter

systems are highly conserved between humans and rodents (Chrousos, 1998). Therefore animal models for anxiety can help understand the difference as well as the overlap, in neural circuitry that underlies normal and pathological anxiety in both humans and animals. One potential drawback however, is that commonly used mouse models are based on normal (i.e. adaptive) behaviour.
In humans, pathological anxiety is accompanied by excessive worrying (apprehensive expectations about a number of events or activities, DSM IV 2000) and anxiousness and diagnosis of a psychiatric disorder relies heavily on a patient's self-report.
Since it can hardly be proven that animals can experience 'worry', other behavioural indicators are necessary to identify pathological anxiety in rodents.
Pathological anxiety can be described as an excessive anxiety response, or even as a different state than normal anxiety.
Since anxiety in animals is already poorly defined and is primarily based on human characteristics, we use the definition of pathological anxiety proposed by Ohl et al. (2008) which is the only available definition of pathological anxiety in animals and states:

'Pathological anxiety is a persistent, uncontrollable, excessive, inappropriate and generalized dysfunctional and aversive emotion, triggering physiological and behavioural responses lacking adaptive value. Pathological anxiety-related behaviour is a response to the exaggerated anticipation or perception of threats, which is incommensurate with the actual situation.'

In this definition the authors do not try to translate the different sorts of anxiety disorders present in humans to differentiate between anxiety states in animals, rather, they aim at differentiating between 'normal' and 'non-adaptive' anxiety, which helps researchers to identify pathological anxiety in rodents. In this thesis, we use this definition to identify pathological anxiety in mice.

Testing for anxiety

In order to test for 'non-adaptive' anxiety in mice, a novel approach is needed, since most animal models of anxiety are based on acute exposure to a novel environment where anxiety would be an adaptive i.e. normal response. In contrast, an appropriate animal model for pathological anxiety should mirror a biologically non-adaptive, i.e. pathological response. Adaptation in animals can be assessed for example by changes in behavioural responses over time, i.e. habituation. Correspondingly, pathological i.e. non-adaptive anxiety behaviour would be mirrored by a lack of habituation to an anxiogenic stimulus. Notably, adaptation refers to the ability of an individual to change in order to 'adapt' to its environment during its lifetime while habituation is classically defined as the waning of a

response elicited by repeated exposure to a novel stimulus which is not accompanied by any biological relevant consequence (Eisenstein et al., 2001; Eisenstein and Eisenstein, 2006; Thompson and Spencer, 1966). One form of habituation is the change in behavioural response seen in rodents to continued or repeated exposure to a test environment. According to the cognitive map theory by O'Keefe (O'Keefe, 1999), an animal will begin to form a new recollection of the environment, a spatial map as an internal representation of the new encountered environment. Once the animal has learned about its spatial surroundings, exploration and locomotion will decrease i.e. habituation takes place. The level of habituation can be assessed in a number of ways, though most studies have focussed on habituation in locomotor or exploratory activity e.g. total distance travelled (Bolivar, 2009). The change in behavioural response over time can be measured within a single test session (intrasession habituation) but also across several test sessions (intersession habituation). It is assumed that intrasession habituation reflects adaptability to the environment, while intersession habituation reflects memory of the previous exposure (Muller et al., 1994). *[Fig 2]* illustrates four types of situations. An animal might display both intra and intersession habituation (a), only intersession habituation (b), only intrasession habituation (c) or no habituation at all (d).

Fig. 2

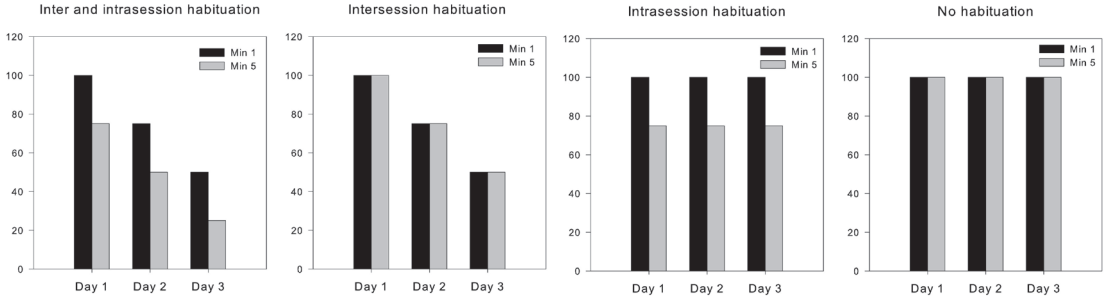

Figure 2: theoretical examples of inter and intrasession habituation. Figure adapted from Leussis et al. (2006). Graphs represent locomotor activity over a 5 min trial each day over 3 consecutive days. Activity is presented in arbitrary units. The black bars represent the first minute and the grey bars the last minute of the 5 min trial.

Generally, rodents will display a decrease in activity over time when repeatedly exposed to a test environment. However, several lines of evidence suggest that habituation may differ across sex, strain and age (Bolivar et al., 2000). Additionally, other factors may play a confounding role on the level of habituation achieved.
For example, initial exploratory levels can influence the level of habituation, especially across sessions. An animal that did not explore the novel environment in the preset test time may not be able to complete the cognitive map of the environment. Upon re-exposure to the test environment, the animal might show no habituation, i.e. decrease in exploratory or locomotor activity, which would not result from memory deficits, but from incomplete exploration of the test environment. This situation may occur when an animal is particularly anxious when initially exposed to the test environment. Thus, anxiety profiles may influence habituation patterns and this is indeed found in several mouse and rat strains. For example, CRF overexpressing mice were characterized by a high anxious phenotype, and show abnormal behavioural habituation after repeated exposure to a test environment (Kasahara et al., 2007); whereas C57BL/6 mice who were initially less anxious, show almost no further habituation during repeated test exposures (Ohl et al., 2001b). Remarkably, DBA/2 mice (Ohl et al., 2003) and selectively-bred rats with high anxiety-related behaviour (Ohl et al., 2002) show rapid habituation after repeated exposure to the same test set-up. However, not much is known about the interaction between emotionality and habituation, especially in the context of pathological anxiety.
Several inbred strains show differences in innate emotionality (Bothe et al., 2004, 2005), it would therefore seem interesting to screen these mouse strains for their habituation capacity.
This approach has several advantages. First, a number of inbred strains have already been reported to show a high anxious phenotype (Belzung and Griebel, 2001). These strains can be investigated for their habituation ability to determine whether their anxious phenotype extends over a longer period of time or that these strains in fact show initially high anxiety which later becomes adaptive. Thereby, inbred mouse strains might show elevated anxiety because it is an enduring feature of a strain or individual which makes it possible to study the complex mechanisms behind anxiety. Moreover, pathological anxiety in commonly used inbred strains would not only imply deficits in emotional functioning in these strains, but would also affect and possibly compromise their welfare.

Aim and outline of the thesis

The main aim of this thesis was to evaluate behavioural habituation as an indicator of non-adaptive, i.e. pathological anxiety in mice. In addition to behavioural performance, stress hormones were measured and the immediate early gene c-Fos, as a marker for neural activity was investigated in brain areas related to anxiety.

Chapter 2 is an introductory chapter and reviews anxiety in relation to animal welfare. We claim that detailed knowledge about the emotional phenotype of the experimental animals used is necessary to reach a balance between reliability of experimental results and the welfare of laboratory animals.
Chapter 3 investigates behavioural and neurological effects of repeated test exposure in male BALB/c and 129P3 mice differing in anxiety-related behaviour. Repeated testing showed that the initially highly anxious BALB/c mice reveal habituation over time, while the initially lower anxious 129P3 mice were characterized by a lack of habituation. To investigate a possible cognitive deficiency, the object recognition test was performed, but no difference in object memory performance was found between the mouse strains. c-Fos expression differences between BALB/c and 129P3 mice were found in the prelimbic cortex and lateral septum, brain areas known to be involved in the integration of information to guide subsequent behaviour. From these results we concluded that BALB/c mice show an adaptive phenotype whereas 129P3 mice are characterized by a non-adaptive phenotype.
To further validate the phenotype of 129P3 mice, we investigated whether their behavioural phenotype was susceptible to the effects of chronic mild stress in **Chapter 4**. We found that 129P3 mice were susceptible to the effects of repeated exposure to stress, which was indicated by an intensified lack of habituation after chronic mild stress. In **Chapter 5** as a first step in generalizing our results, we investigated the effects of repeated testing conditions in female 129P3 and BALB/c mice. We showed that the strain characteristics with respect to behavioural habituation as shown in male mice were also present in the female BALB/c and 129P3 mice. Extending the results found in males, female 129P3/J mice revealed higher post-testing plasma corticosterone levels and higher neural activity in brain areas related to emotional processing than females of the BALB/c strain. These results indicated that the 129P3 mouse strain could be an interesting model for investigating pathological anxiety. In the following step, we aimed at pharmacologically validating the anxiety phenotype in 129P3 mice. We hypothesized that (putative) anxiolytic treatment would ameliorate habituation in 129P3 mice. In **Chapter 6**, we addressed this hypothesis and studied the effects of the putative anxiolytics diazepam and MPEP on within-trial (intrassesion habituation) in the open field,

sensitization in the elevated plus maze and cognition in an object recognition test in male BALB/c and 129P3 mice. Results confirm an anxiety-related phenotype since the compound MPEP facilitated habituation in 129P3 mice without affecting cognitive performance. These results were accompanied by decreased stress-induced plasma corticosterone levels and decreased c-Fos expression in brain areas primarily involved in emotional processing, whereas increased c-Fos expression was found in brain areas primarily involved in higher cognitive processes. We hypothesized that the non-adaptive phenotype of 129P3 mice arises through enhanced glutamatergic transmission especially in higher cortical areas which affects downstream emotional targets. This hypothesis was further investigated in **Chapter 7**, where we used in situ hybridization to measure mRNA levels of the glutamate receptor 5 and the stress-related CRFR1 in the prelimbic cortex and amygdala. We showed that habituation is associated with a decrease in CRFR1 mRNA expression in the prelimbic cortex. Finally, in **Chapter 8** the experimental results from the preceding chapters are discussed in relation to the aim of this thesis and future prospects are addressed.

Chapter

2

Anxiety in relation to animal welfare and environment

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Abstract

Negative emotions do not compromise welfare, as long as they do not exceed the individual's adaptive capabilities.

Anxiety, though a negative emotion, is highly conserved during evolution, and essential for enabling an individual to both escape from dangerous situations and to avoid them in the future, i.e. to adapt to environmental challenges. However, the interactions between anxiety and environment are highly dynamic and can result in non-adaptive anxiety responses. Non-adaptive anxiety responses not only compromise the animal's welfare, but may be substantially detrimental to experimental results even in non-behavioural studies by dramatically reducing the reliability of the study results obtained.

Detailed knowledge about the emotional phenotype of experimental animals used is necessary to reach a balance between reliability of experimental research and the welfare of laboratory animals.

Aversive emotions in welfare

Definitions of welfare vary widely, but scientists and society agree that welfare is about more than just good physical health (Mills, 2008). Today it is generally accepted that animals perceive a variety of emotions and, consequently, the individual's emotional state has to be taken into account when welfare is considered. One broadly used welfare concept is the five degrees of freedom by Brambell (Brambell, 1965), who states that the absence of aversive states, such as hunger, distress and pain, as well as the possibility to express normal behaviour can be used as safeguards for welfare. This concept has been criticized because it neglects positive emotions as important part of intact welfare. However, it can be questioned whether negative emotions, such as fear and anxiety, necessarily compromise welfare. Broom states that welfare is *"the animal's state in relation to its ability to cope with its environment"* (Broom, 1986). This definition allows negative as well as positive emotions –as long as the individual can adequately cope with them. *"Thus, the most widely accepted definition of animal welfare is that it comprises the state of the animal's body and mind, and the extent to which its nature (genetic traits manifest in breed and temperament) is satisfied"* (Hewson, 2003).

Taking into consideration that an individual's needs depend on its biological nature, the absence of aversive experiences/emotions might not be sufficient to guarantee the individual's welfare. The animal may need positive experiences as well, and the experience of aversive experiences/emotions need not be detrimental to the animal's welfare if the individual is capable of coping with those negative experiences. Based on the example of anxiety, we will outline in the following that negative emotions do not necessarily compromise welfare, as long as they do not exceed adaptive capabilities.

Homeostasis versus allostasis

When an animal is confronted with environmental challenges, it responds with behavioural and physiological mechanisms to maintain a constant internal milieu, a mechanism called homeostasis (Bernard, 1865; Cannon, 1932). Homeostasis requires that physiological parameters are maintained at a certain level, termed the set point. For distinct physiological systems, such as body temperature and blood pH level, maintenance of homeostasis at a fixed set point is crucial for survival. Consequently, these systems will be modulated when challenged in a time limited manner and turn back to normal as fast as possible. Other systems, such as central nervous receptor systems, in contrast may show long-lasting changes, i.e. shifts of their set point, due to external challenges.

These shifts characterize the adaptation of the organism in a changing environment. When applying the concept of homeostasis to animal welfare, it means that any environmental challenge will compromise

welfare. Another concept, allostasis, which was first introduced by Sterling (Sterling and Eyer, 1988), implies that an organism must be able to vary its internal milieu in order to appropriately adjust to changing environmental demands (Koob and Le, 2001; Koolhaas et al., 1999; McEwen, 2000). Allostasis thus refers to maintaining stability through change, i.e. by adaptive processes, contrasting the idea of a fixed, context-independent homeostatic level (Koob and Le, 2001; McEwen, 2000). The costs of the body for adapting to environmental challenges, called allostatic load, correlates to the magnitude of environmental stimulation (McEwen and Stellar, 1993). Following this concept, animal welfare can be described as the physiological and behavioural ability of an animal to maintain allostasis, i.e. to adapt to environmental challenges (Korte et al., 2007)

The impact of the environment

The individuals' adaptive capability depends upon the interaction of internal and external factors. The genetic makeup of the individual provides it with a distinct susceptibility to the impact of any environmental stimulation. Nevertheless, the behavioural expression of the individuals' genetic background can be modulated by environmental factors (Paylor et al., 1992). Parameters measuring alterations in brain, behaviour and physiology related to emotions such as anxiety are thought to be highly vulnerable to environmental factors (Pryce et al., 2002) but literature from research in laboratory rodents also shows an inconsistency in the effects of distinct environmental conditions on anxiety-related behaviour. Early aversive experiences can induce long-lasting changes with respect to responses to aversive events in later life (Barros et al., 2006; Chorpita and Barlow, 1998), but it has not been investigated whether this response probability indicates good or poor adaptive abilities. Dimitsantos (Dimitsantos et al., 2007) found anxiety-related behaviour of Sprague-Dawley rats in an elevated plus maze (EPM) to be affected by pre-weaning litter size. In contrast, increased cage size had no impact on reproductive behaviour in C57BL/6Tac mice or behaviour of offspring (Whitaker et al., 2007) and 2 weeks of enrichment by a nest box revealed no effects on anxiety-indicating EPM behaviour in BALB/c mice (Okva et al., 2007). Cage enrichment was also found to lead to an increased ability to adapt to novel situations in mice (Baumans, 1997; Tuli et al., 1995; van de Weerd and Baumans, 1995) and to result in a broader behavioural repertoire as well as less sensitivity to stressful experiences (Van de Weerd et al., 1997). Morphology, physiology and chemistry of the CNS and the psychic abilities of animals are affected by the complexity of housing conditions (Rosenzweig, 1998). Notably, environmental enrichment (interpreted as positive stimulation) can stimulate dendritic growth, cortical thickness, levels of nerve growth factor, increased brain weight (Winocur, 1998),

hippocampal neurogenesis and behavioural and cognitive flexibility (Kempermann et al., 1997). Furthermore, Soffié (Soffie et al., 1999) found that an enriched environment positively affects plasticity of e.g. hippocampus and cerebral cortex and facilitates e.g. the performance of complex cognitive functions. Environmental enrichment however, does not per se increase the emotional response of animals. Several studies found decreased levels of emotional responses due to environmental enrichment as well (Chamove, 1989; Chapillon et al., 2002; Pham et al., 1999).

Findings on the influence of an important environmental factor, the social environment, are also inconsistent. In our own studies, we did not find any anxiety-related behavioural differences in male mice from different inbred strains after being housed either singly or in social groups (unpublished observations). Other groups either found no (Rodgers and Cole, 1993), anxiolytic-like (Voikar et al., 2005) or anxiogenic-like (Ferrari et al., 1998) effects of individual housing in mice. For high-anxiety rats anxiogenic-like effects due to individual housing have been described as well (Ohl et al., 2002) One possible explanation for the inconsistency of these results might be the within-cage testing order of group-housed animals, as suggested by Lyte et al. (Lyte et al., 2005). They found behavioural differences in the EPM between first- and second-tested CF-1 male mice housed in pairs. First-tested animals made fewer closed arm entries, an indication of being less anxious. Chesler and colleagues (Chesler et al., 2002) were able to eliminate within-cage testing order effects on analgesic and nociceptive sensitivity by preventing the exposure of tested male Swiss Webster mice to naïve male conspecifics. Distress, due to the exposure to a novel environment, might thus be communicated, e.g. by ultrasonic calls (Liu et al., 2006) or odours and might even negatively affect welfare of the animals by modulating regulatory adaptive systems. Another factor potentially confounding results, e.g. of tests for anxiety, which has to be taken into consideration at least for male mice, is their territorial behaviour that can lead to aggressive encounters (Bisazza, 1981; Brain and Parmigiani, 2008; Van Loo et al., 2003; Van Oortmerssen, 1971). Importantly, it has also been shown that identical environmental experiences can lead to different responses due to differences in innate anxiety-levels (Ohl et al., 2001c).

Systematic research on the nature of environmental factors that are important for animal welfare is incomplete. However, this short selection of scientific results indicates that the effects of environmental conditions are related to the animal's adaptive abilities, and standardized conditions do not necessarily guarantee good animal welfare. Standardised housing conditions still remain a useful tool since i) it is unrealistic to create tailored conditions for every laboratory animal strain and ii) they allow for the systematic investigation of gene-environment interactions. In any case, detailed

knowledge about the emotional phenotype of the animals used therefore is essential for the reliable interpretation of experimental results.

Innate anxiety and adaptation

Selective breeding programmes in laboratory animals are generally focused on highly specific characteristics. However, selection may have an (unintended) impact on other characteristics, such as emotional traits, and may lead to reduced adaptive capacities, which can compromise biological functioning and thus may impair welfare and quality of life in animals (Ohl et al., 2008). Anxiety is not a unitary phenomenon: it can be divided in innate (trait) or situation evoked (state) anxiety (Belzung and Griebel, 2001; Ohl, 2005). However, it is extremely difficult to separate the two phenomena as animals with high trait anxiety often show high state anxiety as well. Habituation towards a novel stimulus, on the other hand, might show a discrepancy between state and trait anxiety. As trait anxiety is a durable characteristic of an animal, it depends less on environmental challenges and might show less adaptability over time.
High state anxiety evoked by environmental stimuli, will show adaptation over time, when cognitive components gain the upper hand. Strain comparisons reveal that the same environmental stimulation can elicit varying behavioural and physiological responses in rodents. Male rats selectively bred for high and low avoidance behaviour (high and low trait anxiety, respectively) in the elevated plus maze show pronounced differences in home cage behaviour when housed socially in groups of 3-5 in non-enriched cages (Henniger et al., 2000), with high anxiety rats being more inactive and affiliative than low anxiety rats. Notably, this contrasting behaviour is not paralleled by differences in stress hormone levels, indicating that both behavioural profiles lead to successful adaptation to the home cage environment. The same rat lines show extremely different behavioural responses to a novel environment, such as the modified hole board test (Ohl et al., 2001c). Although in contrast to their low anxiety counterparts high anxiety rats strongly avoid the unprotected area of the test environment, they still respond with higher stress-hormone responses than low anxiety rats. These results indicate that the more pronounced avoidance behaviour in high anxiety rats is not sufficient to cope with the novel environment. However, repeated exposure of high anxiety rats to the same test environment reveals fast habituation as reflected by a rapid decrease in avoidance behaviour over time (Ohl et al., 2002). Habituation reflects a waning of a behavioural response elicited over time or after repeated or prolonged exposure to the same environment/stimulus and is a form of non associative learning (Thompson and Spencer, 1966). It allows the animal to differentiate between biological meaningful stimuli to minimize reception and

processing of irrelevant sensory input (Eisenstein et al., 2001).
The cognitive nature of habituation to a novel environment has been demonstrated for example by effects of pharmacological compounds on learning and memory in rodents (Platel and Porsolt, 1982). Notably, gene-environment interactions might influence habituation processes and may cause a hypersensitive or hyposensitive i.e. non-adaptive response. Such habituation profiles can be seen in inbred mouse strains often used as contrasting experimental groups in anxiety research. Both low-anxiety rats and C57BL/6 mice initially show non-anxious behaviour in a novel environment, followed by no further habituation during repeated exposure (Ohl et al., 2002; Ohl et al., 2003). High anxiety rats as well as DBA/2 mice, initially being highly anxious, in contrast show rapid habituation during repeated exposition (Ohl et al., 2002; Ohl et al., 2003). Notably, initial high anxiety is paralleled by high cognitive performance in both species. In a different study, Thiel et al. (Thiel et al., 1999) found better habituation in rats with higher behavioural reactivity in a novel environment, indicating a close interaction between habituation and anxiety-related behaviour as well. If then anxiety behaviour is cognitively driven in an adaptive manner, it is most likely not to compromise welfare.
In contrast to the adaptive type of anxiety behaviour, the 129P3/J inbred mouse strain, frequently used as background strain for targeted mutagenesis (Cook et al., 2002; Simpson et al., 1997), shows a completely different profile (Salomons et al., 2010c): no habituation of anxiety-related behaviour can be seen over time. Cognitive testing shows that the 129P3/J mice are able to discriminate in a one trial object memory task, suggesting that impaired adaptation after repeated exposure is not caused by general cognitive deficits in this strain (Salomons et al., 2010c). Further research is certainly needed on these topics, but we hypothesize from our recent findings that high anxiety behaviour may not be a valid indicator for compromised welfare per se.
Besides genetically based innate (trait) anxiety, environmental conditions can have major influence on an animal's ability to adapt and can finally determine an individual's level of trait anxiety in later life. For example, chronic, inescapable or uncontrolled stress may lead to the impairment of normal regulatory adaptive systems and may result in high anxiety phenotypes. Early postnatally stressed mice are more susceptible to chronic stress during adulthood compared to non stressed control mice (Chung et al., 2005). Additionally, chronic stress during adolescence, created by forming an unstable social environment for a prolonged period of time (Schmidt et al., 2003) or exposing animals to variable stress (Marin et al., 2007), can result in a permanent alteration of centrally regulated stress systems and increased anxiety as well. It is of note that chronic stress can have different effects in individuals from the

same strain in that some individuals are more vulnerable to chronic stress exposure whilst others seem largely unaffected. This inter-strain has also been described for responses in other behavioural paradigms. For example Piazza et al. (Piazza et al., 1990) reported that outbred Sprague-Dawley rats can be separated in high and low responders in an amphetamine administration paradigm based on their locomotor response to novelty.
Similarly, Homberg (Homberg et al., 2004) reported individual differences in various aspects of cocaine self-administration in outbred Wistar rats following selection based on grooming behaviour or response to amphetamine. These results illustrate the importance of the individual plasticity within the brain's emotional circuits in actively maintaining an emotional homeostasis: While the individuals' innate level of anxiety determines its response to environmental stimulation, its emotional level will be modulated by the same stimulation as well. The interaction between anxiety and environment thus is highly dynamic, following the concept of allostasis.

Anxiety and welfare

Anxiety is an essential emotion which is highly conserved during evolution. In principle, anxiety is an adaptive reaction when an animal is confronted with danger or threat. Thus, anxiety enables an individual to escape from dangerous situations and to avoid them in the future, i.e. to adapt to environmental challenges. If, however, anxiety-responses are inappropriate, the individual's ability to adapt to external factors can be substantially compromised. In case of inappropriately low anxiety, the individual may be insufficiently prepared to avoid potentially dangerous situations. In case of inappropriately high anxiety, the individual may not be able to adapt to changing environmental stimuli, resulting in chronic stress and compromised welfare. As outlines in the above, negative emotions do, however, not necessarily compromise welfare, as long as they do not exceed adaptive capabilities *[Fig 1]*.
Non-adaptive anxiety responses not only compromise the animal's welfare, but may also fundamentally affect physiological functioning. In laboratory animals this may substantially affect experimental results even in non-behavioural studies. Such effects are likely to dramatically reduce the reliability of the study results obtained, ultimately leading to an undesirable increase in the number of experimental animals to be used.

Fig. 1

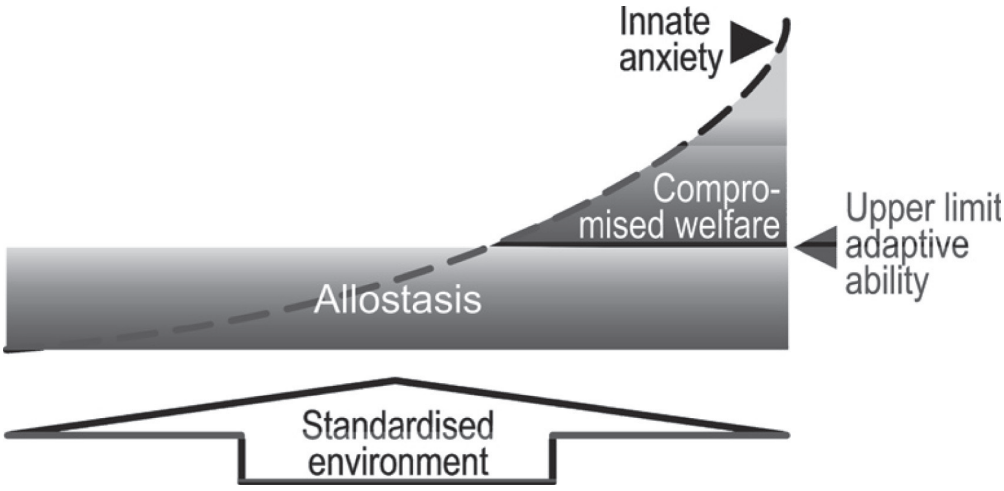

Figure 1: An increased level of innate anxiety does not necessarily result in compromised welfare. Based on the interaction between genetic susceptibility and environmental stimulation, each individual is characterized by its ability to adapt to challenges within a distinct range (allostasis). If the upper limit of this range is reached and adaptive abilities are exceeded, the individual's welfare will be compromised

Given the individual differences in anxiety discussed above, the questions arise whether 1) standardised housing conditions can guarantee good welfare for all strains of one species, and whether 2) specific demands in terms of housing and treatment can be hypothesized for animals differing in anxiety. Even if distinct housing conditions do not exceed the adaptive capabilities of anxious strains, adaptation will result in a specific behavioural profile, profoundly differing from less anxious strains. While the anxiety-phenotype may on the one hand be a confounding factor for experimental results, on the other hand it offers the opportunity to systematically investigate gene-environment interactions. However, researchers should be aware of the potential conflict between the need of standardized environmental and experimental conditions, the demanded reliability of experimental results and the welfare of experimental animals. Detailed knowledge about the emotional phenotype of the animals used is necessary to reach a balance between reliable experimental research and the welfare of laboratory animals.

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Chapter

3

Identifying emotional adaptation: behavioural habituation to novelty and immediate early gene expression in two inbred mouse strains

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Abstract

Normal anxiety is an adaptive emotional response. However, when anxiety appears to lack adaptive value, it might be defined as pathological. Adaptation in animals can be assessed for example by changes in behavioural responses over time, i.e. habituation. We hypothesize that non-adaptive anxiety might be reflected by impaired habituation. To test our hypothesis, we repeatedly exposed male mice from two inbred strains to a novel environment, the modified hole board. BALB/cJ mice were found to be initially highly anxious, but subsequently habituated to the test environment. In contrast, 129P3/J mice initially showed less anxiety-related behaviour compared to the BALB/cJ mice but no habituation in anxiety-related behaviour was observed. Notably, anxiety-related behaviour even increased during the experimental period. Complementary, 129P3/J mice did not show habituation in other parameters such as locomotor and exploratory activity whereas significant changes appeared in these behaviours in BALB/cJ mice.
Finally, the expression of the immediate early gene c-Fos differed between the two strains in distinct brain areas, known to regulate the integration of emotional and cognitive processes. These results suggest that 129P3/J mice might be a promising (neuro)-behavioural animal model for non-adaptive i.e. pathological anxiety.

Introduction

The assessment of anxiety-related behaviour in animals is based on the assumption that anxiety in animals is comparable to anxiety in humans (Hall, 1936) and physiological and behavioural responses to aversive stimuli are similar between humans and animals. Animal models of anxiety thus are hypothesised to be a valuable tool to investigate anxiety-related mechanisms per se.
However, the question remains what we can learn from animal models about pathological anxiety. The vast majority of anxiety research in animals is based on their exposure to novel stimuli, inducing biologically adaptive avoidance behaviour i.e. normal anxiety. An appropriate animal model for pathological anxiety in contrast should mirror a biological non-adaptive i.e. pathological response. Pathological anxiety in animals might be distinguished from normal anxiety as a persistent, uncontrollable emotion triggering physiological and behavioural responses lacking adaptive value (Ohl et al., 2008). Under standard laboratory conditions pathological anxiety in animals can only be identified by use of appropriate test systems forcing the animal to choose between adaptive-avoidance behaviour and non-adaptive avoidance behaviour. Adaptive avoidance behaviour could for example be expressed as habituation towards a novel stimulus over time. Correspondingly, pathological i.e. non-adaptive avoidance behaviour would be mirrored by a lack of habituation.
Habituation refers to the waning of a behavioural response elicited after repeated exposure to the same environment/stimulus and is a form of non-associative learning (Thompson and Spencer, 1966). It allows the animal to differentiate between biological meaningful and iterative stimuli to minimize reception and processing of irrelevant sensory input (Eisenstein and Eisenstein, 2006) and, thus, to adapt to the novel situation. Impaired habituation towards environmental stimuli can therefore be interpreted as being non-adaptive and consequently as a feature of pathological anxiety.
In the present study we investigated behavioural adaptation after repeated exposure to an initially aversive environment as possible indicator for (pathological) anxiety in two inbred mouse strains.
For our study we selected BALB/cJ (BALB/c) mice as potential model for pathological anxiety and 129P3/J (129P3) as non-pathologically anxious counterpart. The BALB/c strain has been reported to exhibit strong neophobic reactions when confronted with a novel compartment in the free exploration test (Griebel et al., 1993). In addition, BALB/c mice show strong and long lasting risk assessment (stretched attends) in an open field (Makino et al., 1991). These and other findings led to the suggestion that BALB/c mice may represent a model for pathological anxiety (Belzung and Griebel, 2001; Brooks et al., 2005; Makino et al., 1991; Sik et al., 2003).
Some substrains of the 129 inbred strain show specific characteristics

in several behavioural tasks (Montkowski et al., 1997; Simpson et al., 1997), such as less locomotor activity and more anxiety-related behaviour compared to for example C57BL/6 mice (Rodgers et al., 2002a; Tang and Sanford, 2005). However, the 129P3 strain has neither been reported to show high anxiety-related behaviour or other behavioural extremes, nor has it been hypothesised to model pathological anxiety. Since this substrain is widely used to obtain embryonic stem cells and is regarded as a commonly used mouse strain, we decided to use the 129P3 substrain as 'normal' counterpart to highly anxious BALB/c mice.
In order to investigate behavioural adaptation to novelty in the BALB/c and 129P3 strains, we repeatedly exposed them to a test environment under stressful (white light) and less stressful (red light) conditions. Subsequently, we performed a one-trial object recognition task (Sik et al., 2003) to test for cognitive performance independent of habituation. Numerous studies have shown that anxiety can affect cognition, raising the possibility that memory and anxiety interact (for review see Kalueff, 2007). In both rats and mice of high and low anxiety, the degree of anxiety was associated with cognitive performance (Ohl et al., 2002) with high anxiety being paralleled by increased cognitive performance. In addition to behavioural parameters, levels of plasma corticosterone (CORT) were determined and strain differences in the expression of c-Fos –belonging to the immediate early gene family of transcription factors and a marker for neural activity– in brain areas involved in emotional and cognitive processing were investigated.

Materials and methods

Ethical note

The experimental protocols were peer reviewed by the scientific committee of the Department of Animals, Science and Society (University of Utrecht, the Netherlands) and subsequently were reviewed and approved by the Animal Experiments Committee of the Academic Biomedical Centre Utrecht, The Netherlands. The Animal Experiments Committee based its decision on the Dutch implementation of the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the national 'Code on laboratory animal care and welfare' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003).

Animals and housing

Experiments were performed with naive, male BALB/cJ (BALB/c, stock number 000651, n=42) and male 129P3/J (129P3, stock number 000690, n=42; (Festing et al., 1999) mice obtained from the Jackson

Laboratory (Bar Harbor, Maine, USA). All animals were 7-8 weeks old at arrival and individually housed in Euro-standard Type II L cages (size: 365 x 207 x 140 mm, floor area 530 cm^2; Tecniplast, Buguggiate, Italy) provided with bedding material (autoclaved aspen chips; Abedd –Dominik Mayr KEG, Köflach, Austria), a tissue (KLEENEX® Facial Tissue, Kimberly-Clark Professional BV, Ede, The Netherlands), a handful of paper shreds (EnviroDry® nesting material, Tecnilab-BMI BV, Someren, The Netherlands) and a cardboard shelter for cage enrichment. Mice chow (CRM, Expanded, Special Diets Services Witham, England) and tap water were available ad libitum.
The mice were kept in the experimental room for 17 days under constant laboratory conditions for acclimatisation to the experimental room (reversed light/dark schedule, lights off between 6.00 and 18.00h and radio music played constantly as background noise). During this period the animals were handled three times a week by the person who also performed the behavioural tests. Relative humidity was kept at a constant level of approximately 50%, room temperature was sustained at 22 ± 2°C and ventilation rate was 15-20 air changes per hour. Testing equipment was already installed before arrival of the animals. The experimental period lasted 3, 5 or 14 days dependent on the type of experiment performed (first modified hole board (mHB) experiment: 14 days; second mHB experiment: 5 days; object recognition test (ORT): 3 days) in which the animals were continuously housed in the same experimental room.

The modified hole board (mHB)

The mHB represents a combination of an open field and a hole board (Ohl et al., 2001a). The experimental setup consisted of an opaque grey PVC box (100 x 50 x 50 cm) with a board (60 x 20 x 2 cm), which was made of the same material as the box, positioned in the middle of the box, thus representing the unprotected area comparable with the centre of an open field *[Fig S1a]*. On the board 20 cylinders (holes, 15 x 15 mm) were staggered in three lines. The area around the board was divided by black lines into 10 rectangles (20 x 15 cm) and 2 squares (20 x 20 cm). The board was additionally illuminated with a stage light (the illumination intensity could be regulated, see experiment 1 and 2) though the surrounding box was only illuminated with 1-5 lux. For testing, all animals were directly transferred from their home cage to the mHB and always placed in the same corner. Each trial lasted 5 minutes. After each trial the animal was transferred back to their home cage and the mHB was carefully cleaned with tap water and a damp towel. All tests were videotaped for raw data storage and behaviour was directly scored by a trained observer using the program Observer 4.0 (Noldus Information Technology, Wageningen, The Netherlands). The following behavioural parameters were measured and assigned

to different behavioural categories according to previous studies (Ohl et al., 2001b)*; a*) anxiety/avoidance behaviour of the unprotected area: the latency until the first board entry (latency board) the total number of board entries (board entries), the percentage of time spent on the board (time board) and risk assessment, measured as the number of stretched attend postures (stretched attends)*; b*) directed exploration: the latency until the first hole visit (latency hole), and the total number of holes visited (holes visited)*; c*) arousal or de-arousal: the percentage of time spent self-grooming (time grooming), the latency until the first self-grooming (latency grooming) and the total number of fecal boli (boli)*; d*) locomotor activity: the total number of line crossings (line crossings) and the total time spent immobile (time immobile)*; e*) general exploration: the total number of rearings in the box (rearings box) and on the board (rearings board) and the total number of hole explorations (hole explorations)*; f*) escape behaviour: the total number of jumps (jumps).

Object recognition test (ORT)

The test apparatus was a Euro-standard Type II L cage (size: 365 x 207 x 140 mm, floor area 530 cm^2; Tecniplast) without any bedding and equally divided in 6 rectangles by black lines *[Fig S1b]*. Light conditions in the test set-up were the same as in the experimental room, red light with an illumination intensity of approximately 1-5 lux. Two different objects were used that differed in height, colour, and shape (screw nut and die). Both objects were too heavy to be displaced by the animals and were always placed in the same corner of the apparatus, either one positioned at the same distance from the wall. For testing, the animals were directly transferred from their home cage and individually placed in the apparatus always in the same corner opposite of the objects. After each trial, the animals were transferred back to their home cage and the objects and the apparatus were carefully cleaned with tap water and a damp towel. The duration of the ORT was 10 minutes, during this period the behaviour was scored by a trained observer using the program Observer 4.0 (Noldus Information Technology). The following behaviours were scored in the ORT and assigned to different behavioural categories*; a*) object memory: the latency until the first exploration of the novel (latency novel object) and the familiar object (latency familiar object); the percentage of time spent exploring the novel (time novel object) and familiar object (time familiar object); The discrimination index (DI) was considered an index of discrimination between the novel and familiar object and calculated as follows; (total exploration time novel object –total exploration time familiar object)/ total exploration time novel object + total exploration time familiar object (based on (Sik et al., 2003)*; b*) risk assessment: the total number of

stretched attend postures (stretched attends)*; c*) general exploration: the total number of rearings (rearings)*; d*) locomotor activity: the total number of line crossings (line crossings) and the total time spent immobile (time immobile)*; e*) arousal or de-arousal: the percentage of time spent self-grooming (time grooming), the latency until the first self-groom (latency grooming) and the total number of fecal boli (boli)*; f*) escape behaviour: the total number of jumps (jumps).

Experiment 1; mHB red light

Male BALB/c (n=8) and 129P3 (n=8) were used. All testing was performed between 9.00 and 13.00h i.e. during the early activity phase of the mice. The board was lit by a red light spot to induce a light intensity difference of about 45 lux between the protected (1-5 lux, box) and the unprotected (50 lux, board) area.
Although mice are likely to be unable to detect the red light intensity difference, they can still be expected to avoid the central area of the testing environment due to thigmotaxis.
The animals were individually placed in the mHB and allowed to explore for 5 minutes per trial (4 trials per day, about one hour between trials) over 5 consecutive days. After a break of two days, a second series of mHB trials started with the same procedure as in the first week. Three hours following the last trial on day 10 (total of 40 trials) the animals were decapitated without anaesthesia, trunk blood was collected and the brains quickly removed.

Experiment 2; mHB white light

Male BALB/c (n=8) and 129P3 (n=8) were used. All testing was performed between 9.00 and 13.00h i.e. during the early activity phase of the mice. The board was lit by a white light spot to induce a light intensity difference of about 115 lux between the protected (1-5 lux, box) and the unprotected (120 lux, board) area. The animals were individually placed in the mHB and allowed to explore for 5 minutes per trial (4 trials per day, about one hour between trials) over 5 consecutive days. Three hours after the last trial on day 5 (total of 20 trials) the animals were decapitated without anaesthesia, trunk blood collected and the brains carefully removed. Since almost all behavioural parameters did not change during the last 20 trials in the first experiment, we decided to perform this experiment with only 20 trials.

Experimental 3; ORT

Male BALB/c (n=27) and 129P3 (n=27) mice were randomly assigned to three experimental groups (n=9 per experimental group).
All testing was performed between 9.00 and 13.00h i.e. during the early activity phase of the mice. All animals were allowed to familiarise with one of the two objects for 24 hours (randomised)

two days before the ORT in their home cage. Animals in the first experimental group were not allowed to familiarise with the apparatus (no exploration: group 1); animals in the second experimental group were allowed to explore the apparatus for 5 minutes one day before ORT without any objects being present (one exploration: group 2). Animals in the third experimental group were allowed to explore the apparatus 4 times for 5 minutes each (about one hour between explorations) one day before the ORT without any objects being present (4 times exploration: group 3). The ORT (10 min) took place two days after familiarisation with the familiar object and one day after free exploration of the test apparatus (except for the first group who performed the ORT 2 days after familiarisation with the object). Thirty minutes after the ORT, the animals were decapitated without anaesthesia and trunk blood was collected.

Corticosterone

In all experiments basal blood samples were collected four days before the start of the experimental period for plasma CORT determination. In the first mHB experiment, a second blood sample was collected three hours after the last trial on day 5. Three hours after the last trial on day 10, the animals were decapitated and trunk blood was collected to determine corticosterone values.
In the second mHB experiment, trunk blood was collected three hours after the last trial on day 5 when the animals were decapitated.
In the ORT experiment a blood sample was taken half an hour after the test to determine the CORT response after behavioural testing.
All blood samples were taken in a separate room in order to prevent that signals or odours from the sampled mice reached the remaining non-sampled animals (i.e. communication between animals in the absence of physical contact was prevented). The hallway and rooms were under red light conditions. Using tail vain incision (for basal blood samples and the second sample for experiment 1), a small blood sample was collected (± 50µl) and stored in prechilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Trunk blood after decapitation was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 12000 rpm, 4°C) and stored at -20°C until measurement.
Plasma CORT levels for the mHB experiments were measured by radioimmunoassay (RIA) with a Coat-A-Count Rat Corticosterone kit according to the protocol of the supplier (Diagnostic Products Corporation, Los Angeles, USA). Plasma CORT levels of the third experiment were measured by RIA with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MP Biochemicals, Amsterdam, The Netherlands).

c-Fos immunohistochemistry

Three hours after behavioural testing, the animals were decapitated. Only brains from the mHB experiments were used. Brains were removed and frozen in liquid (-80ºC) 2-methylbutane which was cooled with dry ice and stored at -80ºC. Coronal sections were cut (20 µm) and mounted on Menzel SuperFrost Plus slides (Menzel GmbH & Co, Braunschweig, Germany) and stored at -20 ºC. For the immunohistochemical detection of c-Fos, rabbit anti c-Fos (SC-52 Santa Cruz Biotechnology) was used. During the staining procedure the sections were rinsed several times after every step in 0.01 M PBS (pH 7.4). For increasing tissue permeability, the PBS contained 0.05% Tween 20 prior to rabbit anti c-Fos, Donkey-anti-rabbit IgG Biotin SP conjugated (Jackson ImmunoResearch Laboratories, Inc USA) incubation steps.
First, the sections were dehydrated. Endogenous peroxidise was blocked by treatment with H2O2 (0.1%) for 30 min. Sections were pre-incubated with 5% normal donkey serum (NDS) and 1% bovine serum albumine (BSA) in PBS (PBS-BSA 1% + NDS 5%) for 30 minutes prior to the rabbit anti c-Fos incubation (1:500 in PBS-BSA 1% + NDS 5%, 4oC 24h). Negative controls were incubated with the PBS-BSA 1% + NDS 5% solution. Next, the sections were incubated with donkey-anti-rabbit IgG Biotin SP conjungate (1:400 in PBS-BSA 1% + NDS 5%) for 45 minutes.
Subsequently the sections were incubated with avidin-horseradish peroxidise solution (1:400 in PBS-BSA 1% + NDS 5% VECTASTAIN® ELITE ABC Brunswich Chemie, Amsterdam) for 60 minutes and pre-incubated with diaminobenzidine tetrahydrochloride solution (DAB) containing nickel sulphate. For visualisation of bound peroxidise complexes, the substrate H2O2 (30%, 1:2000) was added to the DAB solution, and incubated for 5 minutes. Afterwards the sections were dehydrated in alcohol and coverslipped.

Image quantification

The images of brain sections were projected (10x magnification) and digitalized using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a high resolution digital camera interfaced with a computer. The following brain regions involved in anxiety have been investigated (Arzt and Holsboer, 2006; Muigg et al., 2007; Nguyen et al., 2006); medial prefrontal cortex (prelimbic), lateral septum (dorsal, intermediary and ventral), bed nucleus of the stria terminalis (medial anterior, lateral posterior and medial ventral), dentate gyrus (granular layer) paraventricular nucleus, dorsal medial hypothalamus and the amygdala (basolateral nucleus and central nucleus). The anatomical localisation was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas (Franklin and Paxinos, 1997). For each region at least two overt landmarks were used. For quantitative analysis of c-Fos positive cells,

the program Leica Qwin (image processing and analysis software, Cambridge, United Kingdom) was used. Left and right hemispheres were analysed in one section separately and averaged for each animal and calculated for stained neurons per mm^2.

Statistical analyses

Statistical analyses were performed using the software program SPSS for Windows (version 12.0.1; SPSS Inc., IL, USA).
Continuous data (plasma CORT, latency and relative duration of behavioural parameters) were summarized as means with standard error of the mean (SEM), whereas discrete data on the ordinal scale (total number of behavioural parameters) were represented as medians with the interquartile range (IQR). The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data. Group analyses using the Kolmogorov-Smirnov one sample test revealed a non-parametric distribution of several continuous parameters. These parameters, as well as the total number of behavioural parameters, were rank transformed (Conover and Iman, 1982). The (transformed) data from the mHB experiments were subsequently analysed using repeated measures ANOVA using Huyn-Feldt adjustment (trial number as within-subject factor and strain as between-subject factor). Survival analysis was not needed because no censored data was included.
Additionally, we wanted to examine the effects of different light conditions between the first and second mHB experiment.
A repeated measures ANOVA was carried out with light condition and strain as between-subject factors and trial as within-subject factor, Huyn-Feldt adjustment was applied. Though, these results were only considered to give an explorative overview since both experiments used two different animal batches and the mHB experiments were not carried out simultaneously. Results from the ORT were analysed by a two-way ANOVA using strain and experimental group as main factors. Additionally, a one sample t-test was performed in order to assess per strain and experimental group whether the discrimination index differed from zero.
The number of c-Fos positive cells was analysed using a two-way ANOVA for comparison of red and white light conditions (with strain and light conditions as main factors). Post hoc analyses for the mHB experiments, the ORT, CORT plasma levels and the number of c-Fos positive cells were done using an unpaired Student-t test for continues data and the Mann-Whitney U test for discrete data.
For ANOVA analyses, a probability value less than 0.05 was considered to be statistically significant. To minimize the risk of a Type 1 error due to multiple comparisons, the level of significance was corrected for the post hoc analyses using the Dunn-Šidák correction (Ludbrook, 1991). For experiment 1 (mHB red light) all

statistical analyses were performed over the 40 trials, but for sake of comparison, only the first 20 trials are shown in graphs.

Results

Experiment 1; mHB red light

A general overview of results can be found in *[Table S1]*.

Anxiety/avoidance behaviour: a significant strain, trial and trial x strain interaction was found for the latency until the first board entry ($F(1,14) = 6.252$, $P=0.025$; $F(39,546)= 4.012$, $P<0.001$ and $F(39,546)= 6.152$, $P<0.001$ resp.). BALB/c mice showed a higher latency in trial 1 (244.7 ± 27.1 s) compared to the last trial (33.9 ± 5.1s), whereas no significant difference was found between the first trial (48.6 ± 8.6 s) and the last trial (59.1 ± 10.1 s) of 129P3 mice *[Fig 1a]*.

For the total time spent on the board, significant strain ($F(1,14) = 7.322$ $P=0.017$) and trial ($F(39,546) = 2.299$ $P<0.001$) differences were found. BALB/c mice showed an increased time spent on the board during the experimental period (trial 1; 2.6 ± 1.3, trial 40; 14.2 ± 1.7 %), while 129P3 mice showed no difference between the first (14.5 ± 2.6 %) and the last trial (14.3 ± 2.1 %). For the number of board entries significant effects were found for trial ($F(39,546) = 3.244$, $P<0.001$) and trial x strain interactions ($F(39,546) = 4.261$, $P<0.001$). BALB/c mice showed an increasing number of board entries during the experimental period compared to the 129P3 strain (trial 1; 1 ± 5, trial 40; 16 ± 5; trial 1; 13.5 ± 7, trial 40; 13 ±7 nr).

Risk assessment: the number of stretched attends decreased over the trials in both strains (strain: $F(1, 14) = 37.227$ $P<0.001$; trial: $F(39,546) = 15.590$, $P<0.001$ and trial x strain interaction: $F(39,546) = 2.372$, $P<0.001$), although the BALB/c mice displayed more stretched attends than the 129P3 mice in the first trial (20 ± 23, 1 ± 9 resp.).

Locomotor activity: for the number of line crossings significant strain $F(1,14) = 19.253$, $P=0.001$), trial ($F(39,546)= 9.016$, $P<0.001$) and trial x strain interactions ($F(39,546)= 2.065$, $P<0.001$) were found *[Fig 2a]*. BALB/c mice showed a significant increase of line crossings during the experimental period ($P<0.0026$), whilst 129P3 did not show any change in the number of line crossings during this period.

General exploration: the number of rearings in the box also showed significant strain ($F(1, 14) = 27.571$, $P<0.001$), trial ($F(39,546)= 6.607$, $P<0.001$) and trial x strain interactions ($F(39,546)= 1.867$, $P<0.001$). Again, BALB/c mice showed a significant increase in the number of rearings ($P<0.0026$) during the experimental period whereas 129P3 did not.

Directed explorations: the latency until the first hole visit and the number of hole visits showed strain ($F(1,14) = 9.656$ $P=0.008$; $F(1,14) = 16.657$, $P=0.001$ resp.) and trial effects ($F(39, 546) = 4.012$; $P<0.001$; $F(39,546) = 3.463$, $P<0.001$ resp.). The latency until the first hole visit showed a decrease over the experimental period and the number of holes visited increased, though no significant interaction

between strain and trial were found.
Arousal/de-arousal: the total time spent grooming showed strain ($F(1,14) = 6.565$, $P=0.023$) and trial effects ($F(39,546)= 5.050$, $P<0.001$). In both strains an increase in the total time spent grooming was observed during the experimental period while the latency to groom decreased significantly over time.

Fig. 1 a+b

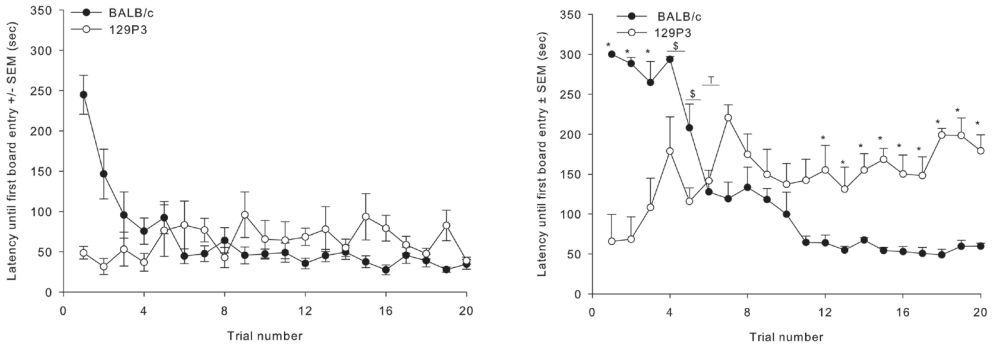

Figure 1: Mean (± SEM) latency until the first board entry under red light (left) and under white light conditions (right). Left; * = P<0.002561, $ = P<0.0026, T = P<0.0026 (BALB/c). Right; * = P<0.002561 and $ = P<0.0026 (BALB/c), T = P<0.0026 (129P3).

Fig. 2 a+b

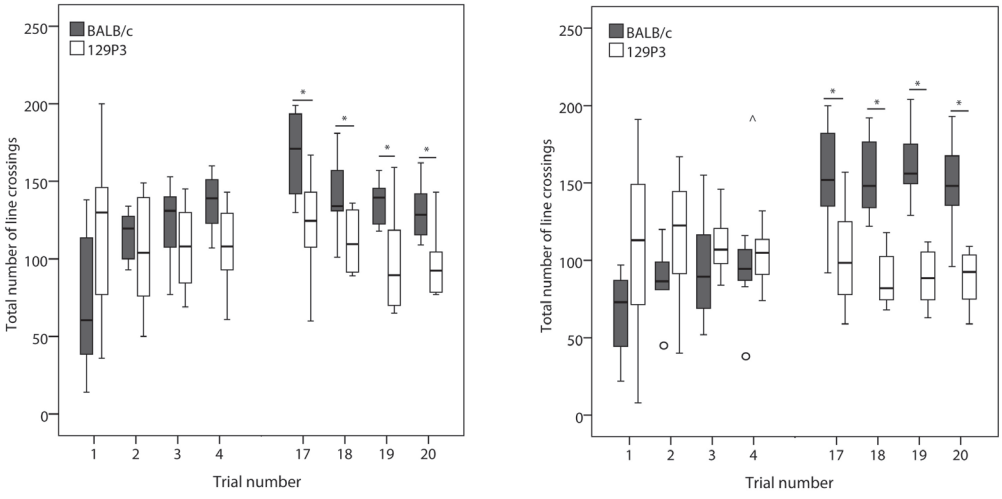

Figure 2: Median (± IQR) number of line crossings under red (left) and under white (right) light conditions.
Data are displayed for the first day (trial 1-4) and fifth day (trial 17-20). * = P<0.00256. Open circles represent outliers (1.5 x IQR) and ^ represent extreme cases (3 x IQR).

Experiment 2; mHB white light

A general overview is presented in *[Table S1]*.

Anxiety/avoidance behaviour: repeated measures ANOVA revealed a significant strain, trial and trial x strain interaction effect ($F(1,14) = 5.478$, $P=0.031$; $F(19,266) = 6.097$, $P<0.001$; $F(19,266) = 26.363$, $P<0.001$ resp.) for the latency until the first board entry *[Fig 1b]*.

A decrease was found in the latency until the first board entry in the BALB/c mice during the experimental period (trial 1, 300 ± 0.0 s, and trial 20, 59.8 ± 3.8 s). The 129P3 strain showed a shorter latency until the first board entry in the first trial (65.9 ± 3.8 s) compared to the BALB/c mice, but this latency clearly increased during the experiment (trial 20, 187.8 ± 16.1 s). The total time spent on the board revealed a significant trial ($F(19,266) = 5.334$, $P<0.001$) and trial x strain interaction effect ($F(19,266) = 9.334$, $P<0.001$). BALB/c mice showed an increase in total time spent on the board, while 129P3 mice showed a decrease over time. The number of board entries showed a significant effect for trial ($F(19,266) = 4.804$, $P<0.001$) and trial x strain interaction effect ($F(19,266) = 3.569$, $P<0.001$). BALB/c mice displayed an increase in the number of board entries during the experimental period though the 129P3 strain showed no difference during the same period.

Risk assessment: the number of stretched attends showed significant effects for strain ($F(1,14) = 5.898$ $P=0.029$), trial ($F(19,266) = 25.091$ $P<0.001$), and trial x strain ($F(19,266) = 23.450$, $P<0.001$). BALB/c mice showed a higher number of stretched attends compared to the 129P3 strain in the first trial (18±12, 3.5±5 resp.), although both strains showed a clear decrease during the experimental period.

Locomotor activity: the number of line crossings revealed a significant effect for strain (*[Fig 2b]*; $F(1, 14) = 7.190$ $P=0.018$) trial ($F(19, 266) = 3.569$ $P<0.001$) and a trial x strain interaction effect ($F(19, 266) = 10.242$ $P<0.00$, *[Fig 2b]*). While BALB/c mice showed an increase in locomotor activity over time, no change in locomotion was observed in 129P3 mice during the experimental period.

General exploration: The number of rearings in the box showed significantly strain ($F(1,14) = 5.598$ $P=0.029$) trial ($F(19,266) = 16.369$ $P<0.001$) and trial x strain ($F(19,266) = 3.151$ $P<0.001$) effects. Both strains showed an increase in general exploration during the experimental period.

Directed exploration: the latency until the first hole visit showed trial ($F(19,266) = 4.825$ $P<0.001$) and trial x strain interaction ($F(19, 266) = 2.249$, $P<0.001$) effects. A slight decrease was found in the latency until the first hole visit throughout the experimental period. The total number of hole visits revealed a significant strain ($F(1,14) = 2.187$, $P=0.018$) and trial effect ($F(19,266) = 5.077$, $P<0.001$), though only in BALB/c mice a slight increase in hole visits could be observed.

Arousal/de-arousal: the total time spent self-grooming and the

latency to the first self-groom showed only a trial effect ($F(19,266) = 4.301$, $P<0.001$ and $F(19,266) = 4.327$, $P<0.001$ resp.). In both strains an increase in the time spent grooming was observed. The latency to groom decreased over time in BALB/c mice while no obvious change was observed in 129P3 mice.

Experiment 1 and 2; red light versus white light

A repeated measures ANOVA was performed to explore the effect of light condition. These results can be found in the supplementary material *[Table S1]*.

Experiment 3; ORT

[Table S2] gives an overview of the results from the ORT.

Object memory: no significant differences were found for strain or experimental group in the discrimination index. Both strains showed a positive discrimination index. Additionally, we performed a one sample t-test to investigate whether the discrimination index differed significantly from zero (i.e. no discrimination between novel and familiar object). BALB/c mice showed a positive significant effect from zero ($P<0.037$) in all three experimental groups.

Although, 129P3 mice showed a positive significant effect in the first ($t = 2.887$, $P = 0.003$) and second experimental group ($t = 2.667$, $P=0.028$), no significant difference from zero was observed in the third experimental group ($t = 1.172$, $P=0.279$).

The latency to explore the novel object differed significantly between experimental groups ($F(1, 47) = 5.248$, $P=0.009$). Post hoc analyses showed that the latency until the first exploration of the novel object was significantly higher in the first experimental group compared to the third experimental group in BALB/c mice ($t = 2.901$, $P = 0.010$). Post hoc testing did not reveal any differences between the experimental groups in 129P3 mice. Additionally, in BALB/c mice a significant difference between the experimental groups was found for the latency until the first exploration of the familiar object ($F(1, 47) = 6.464$, $P=0.003$). Post hoc testing revealed a significantly higher latency to explore the familiar objects in the first experimental group compared to the third experimental group in BALB/c mice ($t = 3.586$, $P<0.0057$). No significant differences were found in 129P3 mice.

Risk assessment: slight, but significant strain differences were found for the number of stretched attends ($F(1,47) = 4.911$, $P=0.031$) and post hoc testing revealed that BALB/c mice showed more stretched attends than 129P3 mice in the first experimental group. Additionally, both strains showed less stretched attends in the third experimental group ($F(1,47) = 4.911$, $P=0.031$) compared to the first experimental group.

Locomotor activity and general exploration: strain effects were found for the number of line crossings ($F(1,47) = 9.352$, $P=0.004$)

and rearings (F (1,47) = 5.636, P=0.022). Post hoc analyses showed that 129P3 mice performed less rearings and line crossings compared to BALB/c mice in the second and third experimental group. Additionally, BALB/c mice showed a significant higher amount of line crossings in the third experimental group compared to the first experimental group. Immobility duration showed a strain effect (F (1,47) = 12.612, P=0.001) and an experimental group effect (F (2,47) = 3.345, P=0.044), though post hoc testing revealed no differences.

Corticosterone

A general overview of plasma CORT levels can be found in [Table S3].
mHB: A significantly higher CORT level after behavioural testing was observed in BALB/c mice compared to basal CORT levels under red light conditions (t = 2.850, P<0.0127). No significant differences were found between basal and after behavioural testing under this lighting condition in 129P3 mice. A significant strain difference was found in plasma CORT levels after behavioural testing under red light conditions (t = 4.315, P<0.0127). In experiment 1 BALB/c mice showed higher plasma CORT response compared to 129P3 mice (t= 4.568, P<0.0127).
ORT: 129P3 mice showed a significantly higher plasma CORT level after behavioural testing compared to basal levels in the first (t = 4.545, P<0.0127) and second (t = -3.187, P<0.0127) experimental group. No strain differences were observed in the first experimental group. Significant differences were found in the second experimental group between basal and after behavioural testing in BALB/c mice (t = 3.945, P<0.0127). Both strains showed a higher plasma CORT level after behavioural testing and 129P3 mice significantly higher than BALB/c mice (t = 3.589, P<0.0127) in the second experimental group.

c-Fos immunohistochemistry

Significant strain differences were found in the prelimbic cortex (*Fig 3a*, F (1,14) = 13.319, P=0.003) and the dorsal lateral septum (*Fig 3b*, F (1,15) = 5.882, P=0.029) only under white light conditions. BALB/c mice showed more c-Fos positive cells in both brain areas compared to 129P3 mice. No strain differences were found under red light conditions in all investigated brain areas *[Table S4]*.
Significant light condition effects were found in the amygdala (F (1, 20) = 8.404, P=0.009), bed nucleus of the stria terminalis (MA; F (1,20) = 18.875, P<0.001; LP; F(1,20) = 34.557, P<0.001; MV; F(1,20) = 20.203, P<0.001) paraventricular nucleus (F (1, 25) = 36.931, P<0.001) and the dorsal medial hypothalamus (F (1,20) = 16.628, P=0.001) *[Table S4]*. Under white light conditions, both strains showed an enhanced c-Fos expression in these areas compared to the red light condition. Post hoc testing showed a significant increase in c-Fos

positive cells under white light conditions in BALB/c mice in the amygdala (t = -4.073, P = 0.002), lateral and posterior part of the bed nucleus of the stria terminalis (t = -3.393, P = 0.005) and the paraventricular nucleus (t = -5.067, P<0.001) compared to red light conditions. In 129P3 mice, post hoc testing showed an increased number of c-Fos positive cells in all areas of the bed nucleus of the stria terminalis (LP; t = -5.321, P<0.001; MA; t = -3.523, P = 0.006; MV; t = -5.903), paraventricular nucleus (t = -3.500, P = 0.004) and the dorsal medial hypothalamus (t = -3.507, P = 0.006).

Fig. 3 a+b

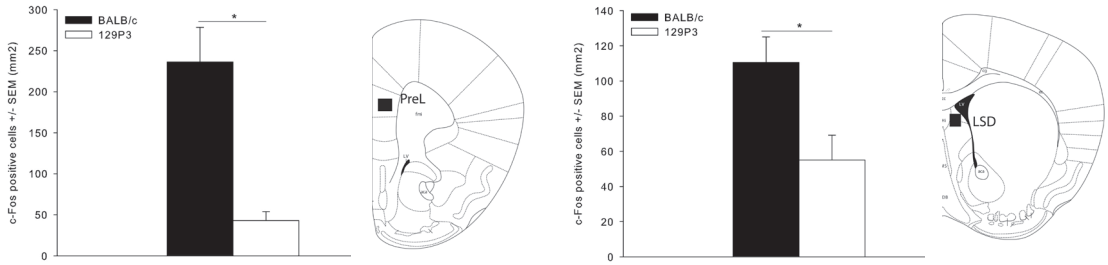

Figure 3: c-Fos expression (positive cells per mm^2 ± SEM) in the prelimbic cortex (PreL, left) and in the dorsal lateral septum (LSD, right) after mHB testing under white light conditions. Schematic diagrams, adapted from Franklin and Paxinos (1997) show the areas in which c-Fos expression was quantified, *P<0.01.

Discussion

In this study we investigated behavioural adaptation in two inbred mouse strains during repeated exposure to a test environment.
In mHB experiments, the BALB/c strain showed high initial avoidance behaviour as reflected by the long latency until the first board entry and the low total time spent on the board *[Fig 1 and Table S1]*. This finding is in agreement with previous studies, in which this strain showed high anxiety-related behaviour and neophobia (Belzung and Griebel 2001; Beuzen and Belzung 1995; Griebel et al., 1993; Ohl et al., 2001b). Following to their high initial anxiety, the BALB/c mice showed a rapid decrease in avoidance behaviour after the first trials, as well as an increase in locomotor activity and exploration *[Fig 1 and 2]*, demonstrating a rapid habituation to the test situation. The more averse white light condition slowed down habituation in BALB/c mice, which corresponds with earlier results in rats showing that higher illumination intensity in the open field increased avoidance behaviour compared to lower light intensity (Cunha and Masur, 1978; Hall et al., 2000; Valle, 1970).
However, in BALB/c mice habituation over time remained significant *[Fig 1]*, indicating an adaptive characteristic of avoidance behaviour, i.e. anxiety in these animals.
Surprisingly, and in contrast to BALB/c mice, the 129P3 strain

showed impaired habituation: the relatively low initial avoidance behaviour almost remained unchanged over time *[Fig 1]*, which at first sight may be explained by a floor effect. This explanation finally appeared to be unlikely, since under white light conditions even a trend towards sensitisation was observed, proving that 129P3 mice do not adapt to novelty. Compared to the BALB/c strain, the 129P3 mice were less active, which confirms previous studies (Bothe et al., 2004; Bothe et al., 2005). No change was observed in activity or exploration over time indicating that sensitisation was restricted to avoidance of novelty. This lack of habituation towards novelty in 129P3 mice may thus be hypothesized to mirror a non-adaptive anxiety profile, i.e. pathological anxiety.

However, the behavioural characteristic in 129P3 mice might as well reflect a primary cognitive deficit. We therefore investigated cognitive performance in the two strains using a one trial ORT, since this one trial learning paradigm allows to circumvent the confounding effect of habituation on cognitive performance. Further, we investigated object recognition during different stages of the habituation to the test environment to control for the influence of habituation on ORT performance.

Both strains were able to discriminate between the novel and the familiar object in the ORT *[Table S2]*. BALB/c mice showed a decrease in the latency to explore both objects when they were pre-exposed to the test environment, indicating that habituation again affected their novelty-avoidance behaviour.

In contrast, 129P3 mice showed no change in the latency to explore the objects after pre-exposure, confirming that 129P3 mice did not habituate to novelty. In the literature cognitive deficits in different 129 sub-strains have been reported (Kim et al., 2005; Sik et al., 2003; Montkowski et al., 1997) but to our knowledge, the 129P3/J sub-strain has never been tested in an object recognition task.

Our results suggest that 129P3 mice perform well in a one-trial object recognition paradigm and we conclude that their behavioural performance does not result from a general cognitive deficit.

Plasma CORT levels revealed no differences in 129P3 mice before and after mHB testing *[Table S3]*, suggesting that their avoidance behaviour towards the unprotected area during the experimental period prevents them of being stressed by novelty.

Interestingly, BALB/c mice showed an elevated level of plasma CORT after behavioural testing compared to basal levels only under red light conditions. This somehow counterintuitive effect might be explained by the fact that BALB/c mice spent more time in the unprotected area under red light conditions when compared to white light conditions. Since exploring the unprotected area might be translated as risk seeking behaviour, which is known to be paralleled by enhanced CORT levels (Crusio and Schwegler, 1987), this may account for the increased CORT-levels found after testing

the animals under less-stressful red-light conditions.
In the ORT we found higher plasma CORT levels 30 minutes after testing in 129P3 mice. Pre-exposure to the test environment failed to affect ORT results in 129P3 mice, confirming their lack of adaptation towards novelty.
Acute mild anxiogenic stimuli are known to increase neural activity in distinct brain areas, which can be measured by the expression of the immediate early gene c-Fos (Duncan et al., 1996; Hinks et al., 1996). Further, repeated exposure to a homotypic stressor produces habituation of c-Fos responses (Girotti et al., 2006; Melia et al., 1994). Interestingly, we found significant strain differences in c-Fos expression after habituation exclusively in the prelimbic cortex *[Fig 3a]* and dorsal lateral septum *[Fig 3b]* under white light conditions with BALB/c mice showing a higher amount of c-Fos positive cells compared to 129P3 mice. Although a direct correlation between the behavioural profile and the found differences in neural activity cannot be proven based on the present data, the parallel occurrence of these phenomena is of interest since a functional relationship between distinct brain areas and specific executive functions has been demonstrated earlier (Hebb et al., 2004; Morrow et al., 2000; Chen and Hertbert, 1996; Duncan et al., 1996; Mongeau et al., 2003; Singewald et al., 2003).
The prefrontal cortex mediates working memory processes, integrates information to guide subsequent behaviour (Goldman-Rakic, 1995) and processes emotional stimuli and behavioural flexibility (Davidson, 2002). Anxiogenic stimuli and drugs are known to stimulate c-Fos expression in this brain area (Hebb et al., 2004; Morrow et al., 2000; Singewald et al., 2003). Parts of the prefrontal cortex are considered to be fear/anxiety inhibiting (Morgan and LeDoux, 1995) and lesions of the dorsal prefrontal cortex including anterior cingulate and prelimbic cortex act anxiogenic (Sullivan and Gratton, 2002). The lateral septum plays a significant role in the processing of fear and anxiety-related processes and in the regulation of behavioural and physiological responses to an emotionally significant event (Sheehan et al., 2004). In the rat and mouse lateral septum, increased neural activity was found in response to various aversive and stressful stimuli (Chen and Herbert, 1995; Duncan et al., 1996; Mongeau et al., 2003). Findings indicate that elevated activity of the lateral septum is required for effective coping responses (Huang et al., 2004; Steciuk et al., 1999) and stress induced c-Fos expression was waned in chronically stressed rats (Auer et al., 2007; Chen and Herbert, 1995), while inhibition of the lateral septum increases anxiety in rodents (Ryabinin et al., 1999; Sheehan et al., 2004).
Neither in the amygdala, the central structure in regulating emotions (Lowry et al., 005; Singewald et al., 2003), nor in other brain areas known to show a stress induced c-Fos increase caused by a variety of anxiogenic stimuli and drugs (Nguyen et al., 2006; Singewald

et al., 2003), differences in c-Fos expression between strains were found in the present study. It might thus be hypothesised that the non-adaptive phenotype of 129P3 mice does not result from a primary dysregulation of emotional processing. We suggest that the lower c-Fos expression after the habituation procedure in both the prelimbic cortex and lateral septum of 129P3 mice compared to BALB/c mice may indicate that this strain is impaired in correctly integrating information about its environment and emotional processes, a prerequisite to adapt behavioural responses over time. Under white light conditions an increased c-Fos expression compared to red light conditions was found in both strains in almost every brain area investigated *[Table S4]*. This is in accordance with previous findings showing that higher illumination in the test environment leads to a higher c-Fos expression in various brain areas (Bouwknecht et al., 2007; Hale et al., 2006). Notably, under white light conditions BALB/c mice showed an increased c-Fos expression compared to red light conditions in the amygdala, while 129P3 mice did not show this increase. One possible interpretation might be that higher amygdala activity in BALB/c mice processes an adaptive response under the more stressful white light conditions, while 129P3 mice fails to discriminate between these two conditions.

In summary, the results reveal that initially highly anxious BALB/c mice rapidly habituated to the test environment. In contrast, 129P3 mice showed no habituation of anxiety-related behaviour. According to our hypothesis, the lack of behavioural habituation as observed in 129P3 mice might feature pathological anxiety. Lower neural activity of the prelimbic cortex and the lateral septum in 129P3 mice after the habituation procedure indicate that these animals may be unable to adequately integrate information about their environment and emotional processes. Further experiments are necessary to elucidate a functional relationship between the behavioural profile and the neural activity of specific brain areas and, furthermore, the central nervous mechanisms underlying the non-adaptive behavioural profile of 129P3 mice, to validate this mouse strain as a model for pathological anxiety.

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Supplementary material

Fig. S1 a+b

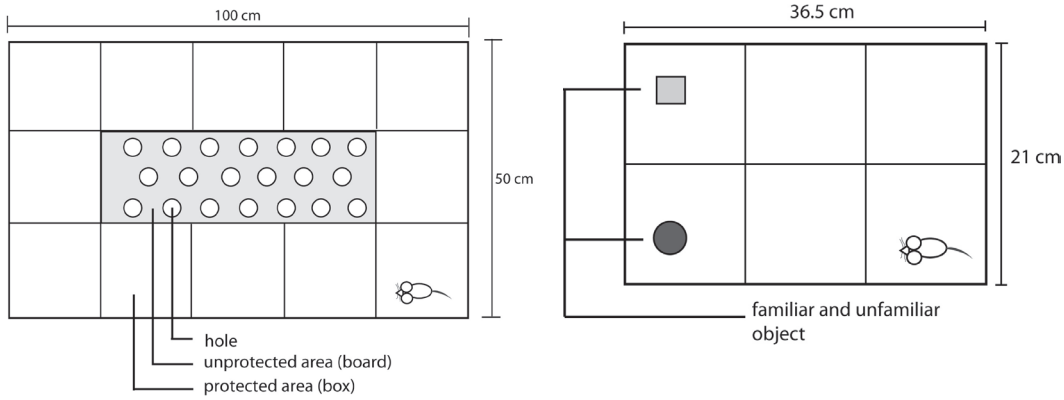

Figure S1: Schematic drawing of the modified hole board (mHB, a) and the object recognition test (ORT, b). *a*: The unprotected area (board) is illuminated with a red (50 lux, experiment 1) or white (120 lux, experiment 2) stage light hanging directly above the test set-up (surrounding box 1-5 lux red light). *b*: Free exploration of the test apparatus was done without any objects being present. One day after familiarisation with the test environment, the objects were placed in the test arena and the ORT was performed.

Table S1: Overview of the behavioural results under red light and white light conditions for the first (trial 1), twentieth (trial 20) and last trial (trial 40) in experiment 1 and the first (trial 1) and last trial of experiment 2 (trial 20).

	Significant effects			**Experiment 1 red light**						**Experiment 2 white light**			
	Red light	**White light**	**Red versus White light**	**BALB/c**			**129P3/J**			**BALB/c**		**129P3/J**	
Behavioural parameter				**Trial 1**	**Trial 20**	**Trial 40**	**Trial 1**	**Trial 20**	**Trial 40**	**Trial 1**	**Trial 20**	**Trial 1**	**Trial 20**
Latency board [sec]	T, S, T x S	T, S, T x S	L	244.7 ± 27.1	34.5 ± 6.8	33.9 ± 5.1	48.6 ± 8.6	55.1 ± 8.6	59.1 ± 10.1	300.0 ± 0.0	59.8 ± 3.8	65.9 ± 33.8	187.8 ± 16.1
Time board [%]	T, S	T, T x S	L	2.6 ± 1.3	15.1 ± 1.8	14.2 ± 1.7	14.5 ± 2.6	15.1 ± 2.4	14.3 ± 2.1	0 ± 0.0	6.7 ± 1.0	5.3 ± 1.1	1.2 ± 0.5
Board entries [nr.]	T, T x S	T, T x S	L	1.0 ± 5.0	16.0 ± 3	16 ± 5	13.5 ± 7	14 ± 9	13 ± 7	0 ± 0	5.5 ± 2	6.5 ± 5	2 ± 2
Stretched attends [nr.]	T, S, T x S	T, S, T x S	L	20.0 ± 23	0 ± 0	0 ± 0	1 ± 9	0 ± 0	0 ± 0	18 ± 12	0 ± 0	3.5 ± 5	0 ± 0
Line crossings [nr.]	T, S, T x S	T, S, T x S	ns	60.5 ± 83	34.5 ± 6.8	128.5 ± 30	118.5 ± 80	96.5 ± 22	92.5 ± 27	73 ± 50	148 ± 39	113 ± 92	92.5 ± 32
Time immobile [%]	ns	ns	ns	0.1 ± 0.3	0.2 ± 0.4	0.6 ± 1.6	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	1.2 ± 0.9	0 ± 0.0	0 ± 0.0	0.1 ± 0.1
Rearings box [nr.]	T, S, T x S	T, S, T x S	ns	9.5 ± 14	20.5 ± 10	21.5 ± 15	7 ± 12	12 ± 16	12.5 ± 16	4.5 ± 8	33.5 ± 26	10.0 ± 6	22 ± 23
Rearings board [nr]	ns	ns	ns	0 ± 0	1.5 ± 1.5	1.5 ± 3	0 ± 0	1 ± 1	1 ± 2	0 ± 0	0 ± 1	0 ± 0	0 ± 0
Hole explorations [nr.]	T	T, T x S	L	1 ± 10	4.0 ± 4	3.5 ± 7	8.5 ± 5	6 ± 4	4.5 ± 7	0.5 ± 1	5.5 ± 3	9 ± 6	3 ± 3
Latency hole [sec]	T, S	T, T x S	L	294.3 ± 5.1	128.2 ± 15.9	115.3 ± 29.3	269.3 ± 18.6	143.1 ± 123.3	153.7 ± 28.7	300.0 ± 0.0	201.1 ± 25.6	300.0 ± 0.0	277.1 ± 14.6
Holes visited [nr.]	T, S	T,S	L	0 ± 0	7 ± 2	7 ± 4	0 ± 1	4 ± 3	4 ± 4	0 ± 0	1 ± 5	0 ± 0	0 ± 1
Time grooming [%]	T, S	T	ns	0.2 ± 0.1	1.2 ± 1.7	1.4 ± 0.3	0.1 ± 0.1	0.3 ± 0.2	0.9 ± 0.2	0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.6 ± 0.2
Latency grooming [sec]	T, S	T	ns	292.6 ± 5.3	174.6 ± 22.2	185.6 ± 40.8	296.1 ± 3.9	159.7 ± 28.2	129.0 ± 26.4	300.0 ± 0.0	168.0 ± 41.5	291.5 ± 8.9	272.2 ± 22.9
Boli [nr.]	T, S, T x S	T, S	ns	5.5 ± 4	7 ± 2	8 ± 3	3.5 ± 4	2 ± 2	1 ± 4	7 ± 3	7.5 ± 5	4 ± 2	3 ± 2
Jumps [nr.]	T	ns	L	0 ± 0	4 ± 6	4 ± 3	0 ± 0	1 ± 2	1 ± 2	0 ± 0	0 ± 2	0 ± 0	0 ± 0

Results are presented as mean (± SEM) for continues data, for discrete data on the ordinal scale the results are presented as median (± IQR).
A repeated measure ANOVA was performed using trial as within factor and strain (or light) as between factor. A P value less than 0.05 was considered significant. nr =number, sec=seconds, T=trial effect, S=strain effect, T x S = trial x strain interaction, L = light condition effects, ns=non-significant.

Table S2: Overview of behavioural results from the object recognition test.

	BALB/c			**129P3/J**			
Behavioural parameter	**Exp group 1**	Exp group 2	Exp group 3	Exp group 1	Exp group 2	Exp group 3	Sig
DI	0.2 ± 0.1	0 .2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	ns
Latency novel object [sec]	45.5 ± 13.4^{a}	10.5 ± 2.4	6.7 ± 1.4^{a}	16.9 ± 3.2	6.2 ± 1.3	6.9 ± 1.0	E, S x E
Latency familiar object [sec]	57.7 ± 17.8^{a}	16.1 ± 5.3	10.1± 3.3^{a}	16.5 ± 4.2	7.2 ± 1.6	10.0 ± 3.3	E
Time novel object [sec]	145.4 ± 22.3	107.0 ± 11.4	110.7 ± 12.1	117.1 ± 16.5	96.7 ± 22.5	78.2 ± 15.9	ns
Time familiar object [sec]	83.2 ± 8.7	66.4 ± 8.0	70.6 ± 7.9	76.5 ± 16.8	57.5 ± 11.0	102.1 ± 33.3	ns
Stretched attends [nr.]	3 ± 7 ^{ac}	1 ± 4	0 ± 0^{a}	1 ± 2^{bc}	0 ± 2	0 ± 0^{b}	S, E
Line crossings [nr.]	139 ± 98^{b}	197 ± 114^{a}	215 ± 72^{bc}	138 ± 50	110 ± 89^{a}	164 ± 62^{c}	S, S x E
Time immobile [%]	1.4 ± 1.0	6.6 ± 4.0	4.3 ± 1.6	9.6 ± 5.0	43.8 ± 21.1	12.2 ± 4.6	S, E
Rearings [nr.]	61 ± 71	95 ± 45^{a}	86 ± 61	67 ± 29	54 ± 54^{a}	81 ± 25	S
Time grooming [%.]	3.3 ± 1.0	3.6 ± 1.7	3.2 ± 0.5	5.4 ± 1.5	3.0 ± 1.0	3.8 ± 0.8	ns
Latency grooming [sec]	271.5 ± 49.5	258.1 ± 62.3	207.3 ± 41.6	218.4 ± 30.8	188.4 ± 40.2	224.7 ± 54.3	ns
Boli [nr.]	10 ± 4a	9 ± 4b	11 ± 5c	6 ± 3a	6 ± 2b	6 ± 3c	S
Jumps [nr.]	0 ± 1	1 ± 2	1 ± 2	0 ± 2a	0 ± 3	1 ± 6a	E

Results are presented as mean (± SEM) for continuous data, for discrete data on the ordinal scale the results are presented as median (± IQR). A two-way ANOVA was performed using experimental group and strain as main factors. A P value less than 0.05 was considered significant. E = experimental group effect, S = strain effect, S x E = strain x experimental group interaction effect, ns = non-significant and Sig = Significance (P<0.05). The same superscript letters in one row represent significant differences revealed by post hoc testing (P<0.0057).

Table S3: Blood plasma CORT levels before and after behavioural testing.

		Before behavioural testing (nmol/l)		After behavioural testing (nmol/l)	
		BALB/c	**129P3/J**	**BALB/c**	**129P3/J**
Exp 1; mHB red light		109.5 ± 47.2 [a]	99.9 ± 20.7	284.1 ± 38.3 [ab]	103.0 ± 17.2 [b]
Exp 2; mHB white light		106.6 ± 23.9	111.1 ± 30.0	91.6 ± 22.3	126.4 ± 28.7
Exp 3: ORT	**Exp group 1**	219.7 ± 27.0	194.6 ± 20.2 [a]	357.0 ± 60.9	399.5 ± 76.1 [a]
	Exp group 2	176.8 ± 11.7 [a]	205.1 ± 27.4 [b]	284.7 ± 20.5 [ac]	449.3 ± 62.8 [bc]
	Exp group 3	210.3 ± 37.2	202.6 ± 42.7	216.2 ± 24.2	395.3 ± 74

Results are represented as mean (± SEM).
Repeated measures ANOVA was performed with basal and after behavioural testing as within factor and strain as between factor. Post hoc analyses revealed significant strain differences in experiment 1 after behavioural testing ($aP<0.0127$). In experiment 3, significant strain differences were found in experimental group 2 after behavioural testing ($bP<0.0127$).

Table S4: Overview of the number of c-Fos positive cells in different brain areas under red and white light conditions in the mHB. PreL (prelimbic cortex), LSD (dorsal lateral septum), LSI (intermediary lateral septum), V (ventral lateral septum), BSTMA (bed nucleus of the stria terminalis, medial anterior part), BSTLP (bed nucleus of the stria terminalis, lateral posterior part), BSTMV (bed nucleus of the stria terminalis, medial ventral part), DG (dentate gyrus), PVN (paraventricular nucleus), DMH (dorsal medial hypothalamus), BLA (basolateral amygdala), CeA (central nucleus of the amygdala), dlPAG (dorsolateral part of periaqueductal grey), dmPAG (dorsomedial part of the periaqueductal grey), lPAG (lateral part of the periaqueductal grey) and the vlPAG (ventrolateral part of the periaqueductal grey).

	BALB/c		**129P3/J**		
	Red light	**White light**	**Red light**	**White light**	**Sig**
PreL	119.9 ± 11.0[a]	236.3 ± 42.1[a,b]	50.0 ± 15.3	43.0 ± 10.9[b]	S, L
LSD	58.8 ± 12.8	110.5 ± 14.5[a]	65.6 ± 18.3	55.0 ± 14.1[a]	S
LSI	87.6 ± 21.6	132.0 ± 27.3	70.1 ± 14.3	96.9 ± 17.8	ns
LSV	111.4 ± 24.9	177.1 ± 45.3	73.5 ± 8.5	143.4 ± 41.5	ns
BSTMA	88.8 ± 15.8	236.2 ± 36.2	119.4 ± 16.6[a]	285.3 ± 27.7[a]	L
BSTLP	55.9 ± 8.1[a]	131.9 ± 15.0[a]	42.5 ± 7.3[b]	144.7 ± 17[b]	L
BSTMV	47.0 ± 10.1	91.3 ± 14.6	41.0 ± 10.5a	157.9 ± 14.8a	L
DG	71.5 ± 9.6	189.3 ± 52.0	71.7 ± 7.2	119.8 ± 13.6	L
PVN	148.3 ± 43.0[a]	1140.1 ± 108.6[a]	295.7 ± 73.8[b]	1040.5 ± 105.4[b]	L
DMH	66.7 ± 5.6	177.1 ± 24.9	83.9 ± 9.9[a]	178.8 ± 16.0[a]	L
BLA	71.4 ± 11.0[a]	324.4 ± 29.1[a]	177.0 ± 30.4	263.4 ± 24.4	L
CeA	76.9 ± 8.7[a]	198.3 ± 21.5[a]	165.1 ± 35.4	90.1 ± 13.7	L

Results are represented as mean number of c-Fos positive cells per mm2 (± SEM). Sig = significance, S = significant for strain, L = significant for light condition, ns = non-significant. The same superscript letters in one row represent significant differences revealed by post hoc testing (P<0.0127).

Chapter

4

Susceptibility of a potential animal model for pathological anxiety to chronic mild stress

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Abstract

When anxiety-related behaviour in animals appears to lack adaptive value, it might be defined as pathological. Adaptive behaviour can be assessed for example by changes in behavioural responses over time i.e. habituation. Thus, non-adaptive anxiety would be reflected by a lack of habituation. Recently, we found that 129P3/J mice are characterized by non-adaptive avoidance behaviour after repeated test exposure. The present study was aimed at investigating the sensitivity of the behavioural profile of these animals to exposure to a chronic mild stress (CMS) paradigm followed by repeated exposure to the modified hole board test. If the behavioural profile of 129P3/J mice mirrors pathological anxiety, their behavioural habituation under repeated test exposure conditions should be affected by CMS treatment. The results confirm the profound lack of habituation with respect to anxiety-related behaviour in both control and CMS treated mice. Additionally, CMS treated animals revealed a lower exploratory behaviour, reduced locomotor activity and increased arousal-related behaviour over time when compared to control individuals, proving an extension of their impaired habituation behaviour. Although no effects of CMS treatment on plasma corticosterone levels were found, higher immediate early gene expression in the bed nucleus of the stria terminalis and the ventrolateral periaqueductal grey in CMS treated mice indicated that 129P3/J mice are susceptible to the negative effects of CMS treatment at both the behavioural and the functional level. These results support the hypothesis that 129P3/J mice might be an interesting model for pathological anxiety.

Introduction

Anxiety in general is an adaptive emotional response which allows the individual to adapt to environmental challenges. However, anxiety responses as well can become dysfunctional, probably resulting in a chronic state of high anxiety and even in anxiety disorders. In humans, anxiety disorders are characterized by excessive anxiousness and worry occurring for at least 6 months (DSM IV). Although 'worry' can hardly be proven to exist in animals, distinct physiological and behavioural responses to aversive stimuli are similar between humans and animals (Hall,1936; Livesey, 1986). Animal models of anxiety can thus be a valuable tool to investigate anxiety-related mechanisms per se. However, reliable identification of pathological anxiety in animals requires more consideration. In animals, pathological anxiety might be distinguished from normal anxiety as a persistent, uncontrollable emotion triggering physiological and behavioural responses lacking adaptive value (Ohl et al., 2008). Thus, the absence of adaptive value of anxiety-related behaviour may be defined as a core symptom of pathological anxiety in animals. At the behavioural level adaptation can be assessed by changes in behavioural responses over time, that is, habituation to a given stimulus. Therefore, non-adaptive anxiety in rodents would be mirrored by a lack of habituation towards a novel, unprotected area, i.e. non-decreasing avoidance behaviour over time. Interestingly, a lack of habituation is a characteristic found in anxiety disorders patients. For example, it was found that the physiological response in highly anxious persons increased with repeated exposure to a certain sequence of sounds, while this response in non-anxious persons diminished (Lader et al.,1967). Other studies have reported rigid responsiveness, reduced initial autonomic responses to stress with slower recovery and diminished habituation in anxious individuals (Hoehn-Saric et al., 1989; Hoehn-Saric and McLeod, 1988). Clinical observations made by Beck, support the idea that high anxious persons tend to show less adaptive capacity (Beck et al., 1985). With repeated exposure to a threatening situation, the average individual showed more confidence and less anxiety, while the highly anxious individuals became more anxious during re-exposures. Recently, we found that individuals from the 129P3/J mouse strain are characterized by a such a lack of habituation during repeated exposure to a test environment and, under more aversive conditions, even a slight sensitisation to this test environment was observed (Salomons et al., 2010c). Notably, BALB/cJ as well as DBA/2 mice, inbred mouse strains which have previously been reported to be highly anxious (Belzung and Berton, 1997), show rapid habituation to the same test environment (Ohl et al., 2003; Salomons et al., 2010c). Moreover, in 129P3/J mice compared to rapidly habituating BALB/c mice, a lower c-Fos expression after the habituation procedure in distinct brain areas (e.g. prelimbic cortex and lateral septum) was

found (Salomons et al., 2010c) indicating that this strain may be impaired in correctly integrating information about its environment and emotional processes, a prerequisite to adapt behavioural responses over time. Additionally, it is known that 129 substrains show general deficits in fear extinction which is also associated with impaired or altered neural processing (Camp et al., 2009; Hefner et al., 2008; Herry and Mons, 2004).

From these results we concluded that BALB/c mice represent a high but non-pathological anxiety-profile, while 129P3/J mice might be an interesting animal model for pathological anxiety. The present study aims at further validating the non-adaptive nature of the behavioural profile in 129P3/J mice. More specifically, we investigated the sensitivity of the behavioural profile in 129P3/J mice to pre-exposure to a chronic mild stress (CMS) paradigm (for review see Willner, 1997). By means of the CMS paradigm, rodents were exposed to a variety of mild stressors over a period of weeks. Application of CMS procedures in rats and mice has been found to induce behavioural, physiological and neurological alterations, some of which have been observed in human depression (de Kloet et al., 2005; Nemeroff, 1988; Sapolsky, 2003; Willner, 1997). The CMS paradigm is thought to be well-suited to investigate causal factors in susceptibility to stress.

The effects of CMS treatment on anxiety-related behaviour are contradictory. While some authors have reported increased anxiety-related behaviour after CMS treatment (Griebel et al., 2002), others have found no effects (Mineur et al., 2006) or even decreased anxiety-related behaviour (D'Aquila et al., 1994; Rossler et al., 2000; Schweizer et al., 2009). The anxiolytic effects of CMS have been described as 'anomalous' and are generally interpreted as either being due to blunted emotionality or to methodological differences (Willner, 2005). It is of note that most studies on the effects of CMS treatment on anxiety-related behaviour have been performed using an acute, single test exposure. Moreover, variability in response to CMS treatment has been hypothesised to be due to differences in susceptibility to stressful events (Ducottet et al., 2004; 2005).

If pathological anxiety is characterized by non-adaptive changes over time in 129P3/J mice, the behavioural profile of these animals should be susceptible to CMS treatment and treatment effects should become visible under repeated test exposure conditions.

Material and methods

Ethical note

The experimental protocols were peer reviewed by the scientific committee of the Department of Animals in Science and Society, Utrecht University, The Netherlands and were subsequently approved by the Animal Experiments Committee of the Academic Biomedical Centre Utrecht, The Netherlands.
The Animal Experiments Committee based its decision on the Dutch implementation of the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the 'Principles of Laboratory Animal Care' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003 *www.springerlink.com/content/86881171278wt787/fulltext.html* - CR20#CR20).

Animals and housing

Experiments were performed with 28 naïve male 129P3/J mice (129P3, stock number 000690, (Festing et al., 1999) obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). At arrival they were 7-8 weeks of age and randomly assigned to two groups of 14 animals each (control group and CMS group) and housed in separate rooms to insure that the CMS did not affect the control group. The animals were housed individually in Eurostandard Type II cages (size: 36.5 x 21 x 14 cm; Tecniplast, Buguggiate, Italy) with bedding material (autoclaved aspen chips; Abedd –Dominik Mayr KEG, Köflach, Austria), a tissue (KLEENEX® Facial Tissue, Kimberly-Clark), a cardboard shelter and a handful of paper shreds (EnviroDry®, Technilab-BMI BV, Someren, The Netherlands).
The mice were kept in the test room for 17 days under constant conditions for acclimatisation to the experimental room under a reversed 12/12 hour light/dark cycle (lights off between 6.00 and 18.00h) and a radio played constantly as background noise. During this period the animals were handled three times a week by the experimenter who also performed behavioural testing. Food (Expanded, Special Diets Services Witham, England) and tap water were available ad libitum. All testing took place in the animals housing room and equipment was installed before the animals arrived. Relative humidity was kept at a constant level of approximately 50%, room temperature was sustained at 22°C ± 2 and ventilation rate was 15-20 air changes per hour.

CMS procedure

The CMS protocol was adapted from Ducottet (2004; 2005) and slightly modified *[see Table 1]*. Various environmental and social stressors were applied once per day in a random order so that they were unpredictable for the animals. All stressors, except for the 24 hours light, were applied to the animals randomly between 6.00 and 18.00h *[see Table 1]*. One person applied the stressors while another person performed the mHB tests. The animals were exposed to the CMS protocol for a period of 14 days.

Table 1: The chronic mild stress procedure.

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Week 1	Empty cage without sawdust (1 1/2 h)	Cage tilt at 45 ° (3 h)	Social stress (3 h)	Empty cage with 1 cm layer of 35°C water (10 min)	Lights on (1 h)	Reversal dark-light cycle(24 h)	Reversal dark-light cycle (24 h)
Week 2	Cage tilt at 45 ° (3 h)	Damp sawdust (4 h)	Social stress (2 h)	Lights on (3 x 30 min, >2h between)	Empty cage with 1 cm layer of 35°C water (10 min)	Cage tilt at 45 ° (3 h)	Empty cage without sawdust (1 h)

The modified hole board (mHB)

The mHB consisted of an opaque grey PVC box (100 x 50 x 50 cm) with a board, made of the same material as the box, positioned in the middle of the box (60 x 20 x 2 cm), thus representing the unprotected area comparable with the centre of an open field (Ohl et al., 2001a). On the board 20 cylinders (diameter 15 mm) were staggered in three lines. The area around the board was divided by black lines into 10 rectangles (20 x 15 cm) and 2 squares (20 x 20 cm). The board was illuminated with an additional stage light (white light 120 lux), though the surrounding box was only illuminated with red light (1-5 lux). For the investigation of food intake inhibition, animals were habituated to a familiar food object, which they received for three days (at 9.00h) before the experimental period in their home cage. The familiar (almonds, piece of 45 mg) and unfamiliar food (Dustless Precision Pellets, 45 mg, Bio-Serv, Frenchtown, USA) objects were always placed in the same corner of the mHB, either one was positioned at the same distance from the wall. For testing, all animals were directly transferred from their home cage to the mHB and always placed in the same corner diametrically opposed to the corner in which the food objects were placed and allowed to explore the mHB for 5 minutes per trial. After each trial the mHB was carefully cleaned with tap water and a damp towel.

All tests were videotaped for raw data storage and behaviour was directly scored by a trained observer using the program Observer 4.0 (Noldus Information Technology, Wageningen, The Netherlands). The following behavioural parameters were measured and assigned to different behavioural categories according to previous studies (Ohl et al., 2001b); *a*) avoidance behaviour towards the unprotected area: the latency until the first board entry (latency board), the total number of board entries (board entries), the percentage of time spent on the board (time board); *b*) risk assessment, measured as the number of stretched attend postures (stretched attends) and the latency until the first stretched attend (latency stretched attend); *c*) directed exploration: the latency until the first hole visit (latency hole), and the total number of holes visited (holes visited) (a hole was counted as visited when the mouse dipped the nose below the rim of the hole); *d*) arousal or de-arousal: the percentage of time spent self-grooming (time grooming), the latency until the first self-grooming event (latency grooming), the total number of self-grooming events (self-groomings) and the total number of fecal boli (boli); *e*) locomotor activity: the total number of line crossings (line crossings), the latency until the first line crossing (latency line crossing), the total time spent immobile (time immobile) and the latency until the first immobility event (latency immobility); *f*) general exploration: the total number of rearings in the box (rearings box) and the board (rearings board), the latency until the first rearing in the box (latency rear box) and on the board (latency rearings board), the total number of hole explorations (hole exploration) and the latency until the first hole exploration (latency hole exploration) (a hole was counted as explored when the animal's nose was directed to a hole; direct contact with the hole was not necessary); *g*) food intake inhibition: the latency until the first exploration of the familiar (latency familiar food) and unfamiliar food (latency unfamiliar food); *h*) escape behaviour: the total number of jumps (jumps).

Corticosterone

Basal blood samples were collected from both groups 4 days before the start of the CMS procedure (7 days after arrival) to determine basal levels of plasma corticosterone (CORT). Trunk blood was collected 3 hours after the last mHB trial to determine CORT levels after behavioural testing. An extra blood sample was taken from the CMS group one day before the start of the behavioural experiments in order to determine CORT levels after two weeks of CMS treatment. All blood sampling and decapitation took place in a separate room adjacent to the experimental room (in order not to disturb circadian rhythm of the mice, the hallway and rooms were under red light conditions). A small blood sample was collected (± 50µl) using tail vain incision and stored in pre-chilled Microvette tubes

(CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Trunk blood after decapitation was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 20.000 g, 4°C) and stored at -20°C until measurement. Plasma CORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

Experimental set-up

All behavioural experiments were performed between 9.00 and 13.00h during the early activity phase of the animals. After 17 days of habituation the control animals were placed in the mHB 4 times per day over 5 consecutive days (5 minutes per trial, one hour between trials). Three hours after the last trial the animals were decapitated, trunk blood was collected and brains were removed. After arrival, the animals of the CMS group were left undisturbed for 7 days after which CMS treatment started and continued for 14 days. Subsequently, the animals were placed in the mHB using the same procedure as the animals from the control group. Three hours after the last trial animals were decapitated, trunk blood was collected and brains were removed.

c-Fos immunohistochemistry and image quantification

After decapitation, the brains were removed and frozen in -80°C 2-methyl-butane which was cooled with dry ice and stored at -20°C. Coronal sections of 20 µm were cut and mounted on Menzel SuperFrost Plus slides (Menzel GmbH&Co, Braunschweig, Germany) and stored at -20°C. The sections were processed for c-Fos immunohistochemistry as described previously (Salomons et al., 2010c) First, dilution with a polyclonal primary antibody (1:1000, SC-52 Santa Cruz Biotechnology, Santa Cruz, USA), and a donkey-anti-rabbit IgG Biotin SP conjugated secondary antibody (1:400, Jackson ImmunoResearch Laboratories, Inc USA).
Cells containing a nuclear brown-black reaction product were considered as c-Fos positive cells and counted in the following brain areas which are known to be involved in anxiety (Arzt and Holsboer, 2006; Muigg et al., 2007; Nguyen et al., 2006) (numbers correspond with the Bregma level where c-Fos analyses were performed); medial prefrontal cortex (prelimbic, + 1.78), lateral septum (dorsal, intermediary and ventral, + 0.86), bed nucleus of the stria terminalis (medial ventral + 0.38; medial anterior and lateral posterior + 0.14), paraventricular nucleus (-0.82), dentate gyrus (granular layer, -1.34), dorsal medial hypothalamus (-1.58), the amygdala (basolateral nucleus and central nucleus, -1.58) and the periaqueductal gray (dorsolateral, dorsomedial, lateral and ventrolateral, -4.72).

The anatomical localisation of c-Fos positive cells was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas (Franklin and Paxinos, 1997). For each region at least two overt landmarks were used. For quantitative analysis of c-Fos positive cells, the program Leica Qwin (image processing and analysis software, Cambridge, United Kingdom) was used. Left and right hemispheres were analysed in one section separately and averaged for each animal and calculated for stained neurons per mm^2.

Statistical analyses

Statistical analyses were performed using the software program SPSS for Windows (version 15.0.1; SPSS Inc., IL, USA).
Continuous data (CORT, latency and relative duration of behavioural parameters (represented as mean ± standard error of the mean, SEM)), were first tested for Gaussanity using the Kolmogorov-Smirnov test. Homoscedasticity was tested by Levene's test.
Group analyses using the Kolmogorov-Smirnov one sample test revealed a non-parametric distribution of several continuous parameters. These parameters, as well as the total number of behavioural parameters, were rank transformed (Conover and Iman, 1982). The (transformed) data from the mHB experiments were subsequently analysed using repeated measures ANOVA using Huyn-Feldt adjustment (trial number as within-subject factor and treatment as between-subject factor).Survival analysis was not needed because no censored data was included. The number of c-Fos positive cells was analysed by using a one-way ANOVA for comparison of the treatment conditions. Post hoc analyses for the mHB and CORT results were done using an unpaired Student-t test for continuous data and the Mann-Whitney U test for discrete data. For ANOVA analyses, a probability value less than 0.05 was considered to be statistically significant. To minimize the risk of a Type 1 error due to multiple comparisons, the level of significance was corrected for the post hoc analyses using Dunn Sidak correction (Ludbrook, 1991).

Results

mHB A summary of mHB results for trial 1 and trial 20 is listed in *[Table S1]*.

Avoidance behaviour: repeated measures ANOVA revealed a significant trial difference for the latency until the first board entry *[Fig 1a]*, the total time spent on the board *[Fig 1b]* and the number of board entries ($F(19,475) = 20.57$, $P<0.001$; $F(19,475) = 17.36$, $P<0.001$; $F(19,475) = 21.56$, $P<0.001$ resp.). No significant effects were found for treatment and trial x treatment interactions ($P>0.05$). The latency until the first board entry significantly increased during the experimental period whereas the total time spent on the board and the number of board entries significantly decreased during the

experimental period in both groups *[see Table 2]*. Risk assessment: the total number of stretched attends showed a significant trial ($F_{(19,475)} = 22.23$, $P<0.001$), treatment ($F_{(1,25)} = 10.01$, $P=0.004$) and trial x treatment effect ($F_{(19,475)} = 2.14$, $P=0.004$). Both groups showed a decrease in the number of stretched attends during the experimental period, while CMS treated animals showed more stretched attends during the experimental period. The latency until the first stretched attend increased during the experimental period in both groups (trial: $F_{(19,475)} = 28.78$, $P<0.001$), though CMS treated animals showed a lower latency until the first stretched attend compared to the control group ($F_{(1,25)} = 16.52$, $P<0.001$).
Locomotor activity: the total number of line crossings *[Fig 3a]* showed a significant trial ($F_{(19,475)} = 4.61$, $P<0.001$), treatment ($F_{(1,25)} = 37.18$, $P<0.001$) and trial x treatment ($F_{(19,475)} = 9.73$, $P<0.001$) effect. The CMS treated group displayed a decrease in the number of line crossings during the experimental period, though the control group showed no change during the experimental period. The latency until the first line crossing decreased during the experimental period in both groups ($F_{(19,475)} = 2.62$, $P<0.001$), but no treatment effects were found. The CMS animals showed an increase in the time spent immobile during the experimental period *[Fig 2a]*, whereas in the control group no significant difference over time was found (trial: $F_{(19,475)} = 11.17$, $P<0.001$; treatment: $F_{(1,25)} = 36.34$, $P<0.001$; trial x treatment: $F_{(19,475)} = 7.44$, $P<0.001$). Additionally, the latency until the first immobility decreased significantly over time while CMS treated animals compared to the control animals showed a lower latency until the first immobility (trial: $F_{(19,475)} = 10.53$, $P<0.001$; treatment: $F_{(1,25)} = 22.75$, $P<0.001$; trial x treatment: $F_{(19,475)} = 6.59$, $P<0.001$)
General exploration: significant differences were found between the groups in general exploratory behaviour. The total number of rearings in the box *[Fig 3b]* showed a trial ($F_{(19,475)} = 17.43$, $P<0.001$), treatment ($F_{(1,25)} = 21.79$, $P<0.001$) and trial x treatment ($F_{(19,475)} = 14.90$, $P<0.001$) effect. The control group displayed an increase in the amount of rearings in the box during the experimental period whereas the CMS treated animals showed no difference over time. A decrease in latency until the first rearing in the box was observed in control animals, while no change was seen in CMS treated animals (trial: $F_{(19,475)} = 2.60$, $P<0.001$; treatment: $F_{(1,25)} = 5.28$, $P=0.030$; trial x treatment: $F_{(19,475)} = 4.04$, $P<0.001$). No significant effects were found on the number of rearings on the board or the latency until the first rearing on the board. Both groups rarely displayed this behaviour in the unprotected area.
The number of hole explorations significantly decreased over time in both groups (trial: $F_{(19,475)} = 9.58$, $P<0.001$; treatment: $F_{(1,25)} = 13.27$, $P<0.001$ and trial x treatment: $F_{(19,475)} = 3.04$, $P<0.001$), while the latency until the first hole exploration increased in both groups

(F (19,475) = 9.50, P<0.001).
Directed exploration: No significant differences were found for the number of holes visited or the latency until the first hole visit.
Food intake inhibition: the latency to explore the familiar food decreased over time in both groups (trial: F (19,475) = 17.93, P<0.001), although no significant treatment effects were observed. No significant effects were found in the latency to explore the unfamiliar food objects.
Arousal/de-arousal: CMS treated animals significantly spent more time grooming during the experimental period than the control group (trial: F (19,475) = 10.10, P<0.001; treatment: F (1,25) = 102.48, P<0.001; trial x treatment: F (19,475) = 5.61, P<0.001, [*Fig 2b]).* Additionally, CMS treated animals showed a decrease in the latency until the first self-grooming compared to the control group (treatment F (1,25) = 100.8, P<0.001). CMS treated animals showed an increase in the number of grooming events during the experimental period and more grooming events compared to control animals (trial: F (19,475) = 8.52, P<0.00; treatment: F (1,25) = 108.46, P<0.001; trial x treatment: F (19,475) = 4.96, P<0.001). CMS treated animals showed a significantly higher number of boli compared to the control group (F (1,25) = 10.67, P<0.001), though in both groups no change during the experimental period was observed.
Escape behaviour: No significant effects were found on the number of jumps.

Fig. 1 a+b

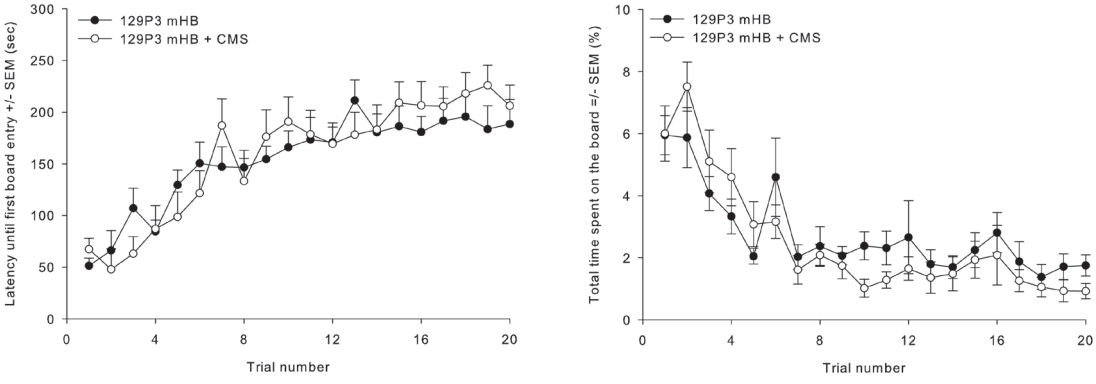

Figure 1: Latency until the first board entry (left) and the total time spent on the board (right) for control animals and CMS treated animals across the experimental period. Data are represented as mean ± SEM. Significant trial effects were observed in both groups for both parameters (P<0.05). No significant effects of CMS treatment were found. Both groups showed an increase in avoidance behaviour during the experimental period.

Fig. 2 a+b

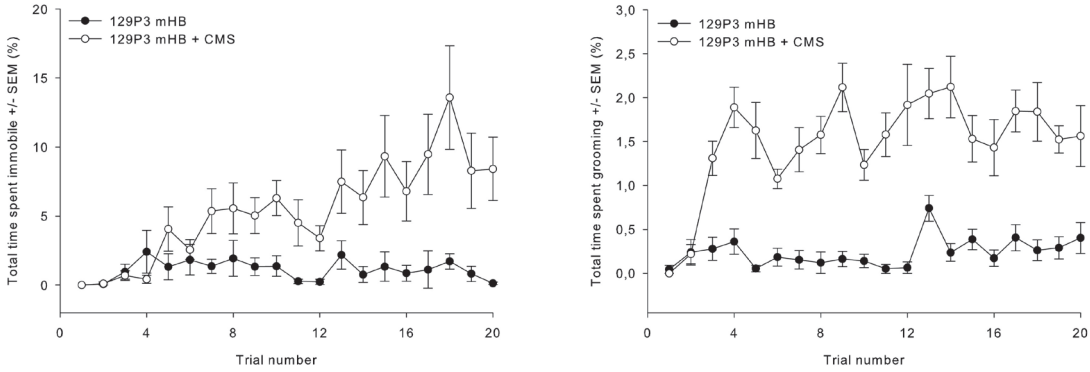

Figure 2: The total time spent immobile (left) and the total time spent grooming (right) for control animals and CMS treated animals across the experimental period. Data are represented as mean ± SEM. Significant trial, treatment and trial *treatment interaction was found for both parameters (P<0.05). CMS treated animals spent more time immobile and more time grooming compared to control animals during the experimental period.

Fig. 3 a+b

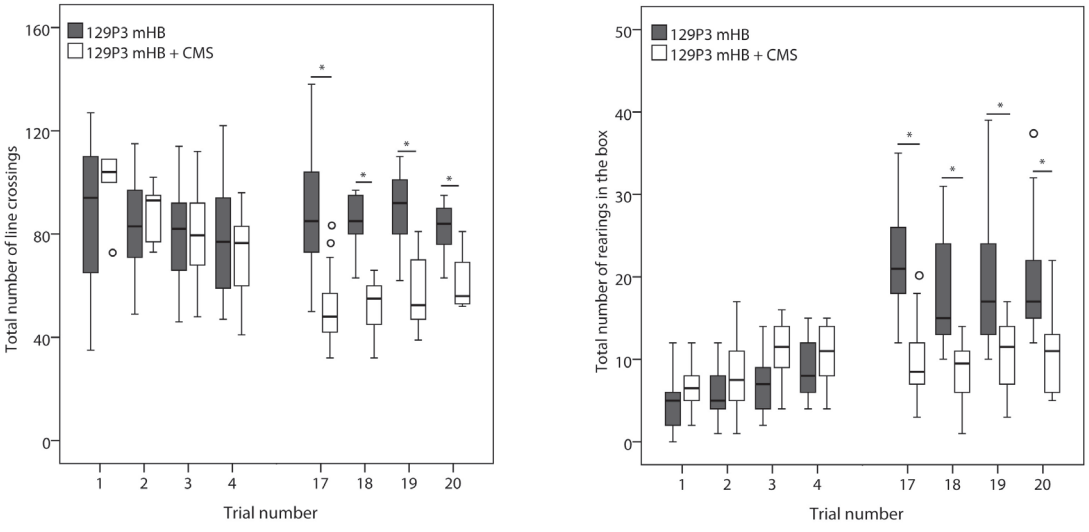

Figure 3: The total number of line crossings (left) and the total number of rearings (right) for trials 1-4 (day 1) and trials 17-20 (day 5) expressed as the median ± IQR. *P<0.00271. Open circles represent outliers (1.5.x IQR).

Corticosterone

Repeated measures ANOVA between basal CORT levels and CORT levels after mHB testing showed a significant difference ($F(1,23) = 5.54$, $P=0.029$), although no treatment effects were found ($F(1,23) = 0.15$, $P=0.747$). Post hoc testing did not reveal a significant difference. Neither did the basal CORT levels differ from those after the CMS procedure, nor did plasma levels after the CMS procedure and after mHB testing differ *[see Table 2]*.

Table 2: CORT levels before CMS and mHB and after CMS and mHB testing.

	Basal	After CMS	After mHB
129P3/ mHB	107.3 ± 15.6	-	172.5 ± 26.4
129P3/J mHB +CMS	98.6 ± 20.5	110.7 ± 12.3	153.0 ± 17.4

c-Fos immunohistochemistry

Significant treatment effects were found for the lateral posterior part of the bed nucleus stria terminalis ($t = -2.38$, $P=0.002$), and the ventrolateral part of the periaquaductal gray ($t = -2.14$, $P=0.045$). CMS treated animals showed more c-Fos activity compared to the control group in these brain areas. No significant treatment effects were found in all other brain areas investigated *[see Table S2]*.

Discussion

The non-adaptive anxiety phenotype of 129P3 mice was further validated by the results of the present study: firstly, previous findings were replicated in that impaired behavioural habituation, as reflected by an increase in anxiety-related behaviour over time (Salomons et al., 2010c, again were found in control (i.e. non-stressed) 129P3 mice. Secondly, CMS treatment affected the behavioural profile in stressed 129P3 mice by inhibiting exploratory and locomotor behaviour over time. Thus, chronically stressed 129P3 mice show an impaired habituation profile across all motivational systems observed, while this characteristic is restricted to avoidance behaviour in control animals.

It is important to note that, in contrast to the present study, most studies on the effects of CMS treatment on anxiety-related behaviour have been done under single acute test exposure. In general, the findings of CMS treatment on anxiety-related behaviour are controversial. For example, CMS- treated rats (D'Aquila et al., 1994) and -female mice (Rossler et al., 2000) displayed an anxiolytic-like profile in the elevated plus maze in which they showed no clear preference for the closed arms compared to the open arms. Similar anxiolytic effects were observed after CMS treatment in the mHB in which C57Bl/6 mice displayed increased locomotor activity and increased time spent on the aversive area

(Schweizer et al., 2009). In contrast, also no effects (Mineur et al., 2006) as well as an increase in anxiety-related behaviour in the elevated plus maze (Griebel et al., 2002) after CMS treatment have been described. The fact that we were not able to detect any effects of CMS treatment on anxiety-related avoidance behaviour might be due to a floor effect. Even untreated 129P3 mice almost completely avoided the unprotected area of the test arena and further avoidance could thus not have been achieved by CMS treated animals.
In contrast, exploratory and locomotor behaviour in CMS treated animals markedly decreased during the experimental period while immobility duration steadily increased in contrast to control animals. These results contradict findings of other studies, which have found hyperlocomotion in several mouse strains after CMS treatment (Mineur et al., 2006; Schweizer et al., 2009; Strekalova et al., 2005). Again, these studies investigated locomotor and exploratory behaviour during a single acute exposure. In one other study however, the effect of CMS on habituation in the open field was investigated (Dubovicky and Jezova, 2004). Rats exposed to 6-min open field sessions on four consecutive days displayed slower habituation, although it did not reach significance. Nevertheless, it can be concluded that the 129P3 mice were susceptible to the CMS treatment, which was sufficient to compromise habituation in locomotor and exploratory activity and caused general inactivity *[Fig 2a]* in CMS treated mice.
Markedly, food intake inhibition did not differ between control and CMS-treated mice treatment suggesting that food intake motivation was not altered in stressed 129P3 mice. Since changes have been observed in consummatory behaviour after chronic stress mainly in sucrose intake (Ducottet et al., 2004) it can be suggested that our CMS protocol was not sufficient to induce this characteristic.
Arousal–related behaviour was affected by CMS treatment as reflected by a higher percentage of time spent grooming *[Fig 2b]*, more grooming events and a lower latency to start grooming in comparison with the control group. Additionally, CMS treated animals showed a higher defecation frequency during the experimental period. Ducottet (Ducottet et al., 2004) found that mice subjected to CMS displayed less grooming activity and consequently had a dirtier fur state. Generally, grooming can be dissected in comfort and stress grooming, the latter being characterized by short frequent bursts of grooming (Kalueff and Tuohimaa, 2004). Additionally, in rodents, grooming can play an important role in behavioural adaptation to stress (Kalueff and Tuohimaa, 2004). Increased arousal-related behaviour in CMS treated mice in the present study suggests that these mice perceived repeated test exposure as more stressful than control animals.
However, the increased arousal-related behaviour was not accompanied by changes in CORT levels, suggesting that

HPA-axis activity was not chronically affected by CMS treatment. Other studies have found increased CORT levels in male Swiss mice (Li et al., 2008) and ICR mice (Mao et al., 2009) after CMS treatment, though these treatments were of a longer duration (Ducottet and Belzung, 2005; Mao et al., 2009) and applied more stressors per day in comparison to our CMS protocol. In the study of Li (2008), increased CORT levels were accompanied by no change in locomotor activity in the open field. The lack of stress hormone response in CMS treated animals in our study suggests that sustained behavioural inhibition is necessary to compensate the exposure to a testing environment, which the animals are not able to habituate to.
The immediate early gene c-Fos is generally used as a functional anatomical mapping tool for neural circuits underlying neuroendrocine-, autonomical- and behavioural responses induced by stress (Kovacs, 1998). Several anxiogenic stimuli are known to induce c-Fos expression in a wide range of anxiety-related brain areas (Duncan et al., 1996; Gray and McNaughton, 1983; Hinks et al., 1996). Additionally, c-Fos responses have been found to wane after a number of homotypic stressors such as repeated restraint (Girotti et al., 2006; Melia et al., 1994). In a previous experiment we found strain differences in c-Fos expression after repeated behavioural testing between BALB/c and 129P3 mice exclusively in the prelimbic cortex and lateral septum (Salomons et al., 2010c). Interestingly, in the present study a higher c-Fos expression was observed in the lateral posterior part of the bed nucleus of the stria terminalis (BSTLP) and the ventrolateral part of the periaqueductal gray (vlPAG) in CMS treated animals compared to control animals *[see Table S2]*.
The BST is thought to integrate information from several stress-related brain areas, to be a key relay in stress circuitry (Herman et al., 2005; Makino et al., 2002) and shows an increase in c-Fos expression after treatment with anxiogenic drugs (Singewald et al., 2003).
The PAG is well known to integrate limbic input and plays a role in behavioural and autonomic responses (Behbehani, 1995; Bernard and Bandler, 1998). Upon stimulation, the ventrolateral PAG has been found to produce passive reactions of quiescence and immobility, and decreased vigilance (Bernard and Bandler, 1998). Excitation of the ventrolateral PAG elicits anxiety-like reactions that are characterized by freezing behaviour.
Additionally, the ventrolateral PAG has been proposed to be exclusively involved in the passive emotional coping reaction (Lim et al., 2008; McNaughton and Corr, 2004). We therefore hypothesise that the higher c-Fos activity as found in the present study in CMS treated animals might indicate that CMS treatment intensified the sustained perception of the repeated test exposure as being aversive in 129P3 animals. It might further be suggested that, as a consequence, CMS treated animals were less able to cope with testing conditions compared to control mice.

It should be noted that absence of data on basal c-Fos expression in naïve animals results in a limitation to the interpretation of these results, since a direct correlation between the behavioural profile and neural activity cannot be proven. Nevertheless, the parallel occurrence of increased c-Fos activity in the BSTLP and vlPAG and sustained behavioural inhibition in CMS treated animals does strongly suggest that 129P3 mice are susceptible to the effects of CMS treatment.

A final point of consideration is that most behavioural differences between control and CMS treated animals were observed after several trials. 'Anomalous' findings after CMS treatment such as anxiolysis or hyperlocomotion as reported in the literature have mostly been found after a single acute test exposure. We propose that a single test exposure might be insufficient to identify distinct behavioural characteristics and that non-adaptive anxiety can only be identified by behavioural changes over time and, thus, after repeated test exposure.

In summary, the present results emphasise that 129P3 mice represent a mouse model for pathological anxiety with a high translational value. In parallel to findings in humans (Hoehn-Saric et al., 1989; Hoehn-Saric and McLeod, 1988), 129P3 mice are susceptible to repeated exposure to stress, which is indicated by an intensified lack of habituation after chronic mild stress. It is of note that our previous hypothesis that the behavioural characteristic in 129P3 mice may be caused by an impaired integration of the gathered information about the environment and the animals' emotional perception is paralleled by the suggestion from human studies that in contrast to less anxious persons, the anxious person is unable to make a distinction between what is safe and what is not (Beck et al., 1985). We conclude that the further investigation of non-adaptive anxiety behaviour over time can significantly contribute to our understanding of pathological anxiety in both animals and humans.

Supplementary material

Table S1: Overview of significant main effects found by Repeated Measures ANOVA and average per behavioural parameter for trial 1 and 20 (± SEM) in the mHB. Behavioural parameters are assigned to different behavioural categories.

			129P3/J mHB		129P3/J mHB + CMS	
Behavioural category	**Behavioural parameter**	**Sig**	**Trial 1**	**Trial 20**	**Trial 1**	**Trial 20**
Avoidance	Latency until first board entry [sec]	T	51.3 ± 7.5	188.5 ± 23.4	67.3 ± 10.4	206.2 ± 19.9
	Total time spent on board [%]	T	6.0 ± 0.6	1.8 ± 0.3	6.0 ± 0.9	0.9 ± 0.3
	Total number of board entries [nr.]	T	7.0 ± 4.0	2.0 ± 2.0	4.0 ± 5.0	1.0 ± 2.0
Risk assessment	Total number of stretched attends [nr.]	T, Tr, T*Tr	7.0 ± 1.0	0.0 ± 0.0	5.0 ± 1.0	0.0 ± 0.0
	Latency until first stretched attend [sec]	T, Tr, T*Tr	6.9 ± 1.0	300.0 ± 0.0	5.1 ± 1.0	265.3 ± 19.6
Locomotor activity	Total number of line crossings [nr.]	T, Tr, T*Tr	94.0 ± 23.0	88.0 ± 15.0	101.0 ± 12.0	56.0 ± 16.0
	Latency until first line crossing [sec]	T	6.4 ± 2.2	1.8 ± 0.3	3.5 ± 0.6	1.7 ± 0.2
	Total time spent immobile [%]	T, Tr, T*Tr	0.0 ± 0.0	0.1 ± 0.3	0.0 ± 0.0	8.4 ± 2.3
	Latency until first immobility [sec]	T, Tr, T*Tr	300.0 ± 0.0	266.1 ± 23.4	300.0 ± 0.0	84.0 ± 25.0
General exploration	Total number of rearings in the box [nr.]	T, Tr, T*Tr	16.0 ± 13.0	17.0 ± 7.0	9.0 ± 7.0	11 ± 8.0
	Latency until first rearing in the box [sec]	T, Tr, T*Tr	129.4 ± 25.4	5.4 ± 5.0	61.3 ± 13.4	68.4 ± 11.3
	Total number of rearings on the board	ns	0.0 ± 0.0	0.0 ± 1.0	0.0 ± 0.0	0.0 ± 1.0
	Latency until first rearing board [sec]	ns	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	291.3 ± 8.4
	Total number of hole explorations [nr.]	T, Tr, T*Tr	8.0 ± 5.0	5.0 ± 1.0	5.0 ± 5.0	0.0 ± 2.0
	Latency until first hole exploration [sec]	T	31.0 ± 5.8	202.4 ± 20.3	41.9 ± 10.6	192.6 ± 16.7
Directed exploration	Total number of hole visits [nr.]	ns	0.0 ± 0.0	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0
	Latency until first hole visit [sec]	ns	300.0 ± 0.0	281.7 ± 12.9	300 ± 0.0	300.0 ± 0.0
Food intake inhibition	Latency first exploration unfamiliar food	ns	252.3 ± 23.6	237.9 ± 29.1	173.4 ± 28.1	145.1 ± 36.2
	Latency first exploration familiar food [sec]	T	90.6 ± 19.0	20.3 ± 6.9	82.3 ± 21.5	9.8 ± 2.6
Arousal	Total time spent grooming [%]	T, Tr, T*Tr	0.0 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	1.6 ± 0.4
	Latency until first self groom [sec]	T, Tr, T*Tr	89.7 ± 10.3	239.5 ± 25.5	300.0 ± 0.0	142.8 ± 24.5
	Total number of self-groomings [nr.]	T, Tr, T*Tr	0.0 ± 0.5	0.5 ± 0.5	0.0 ± 0.0	5.0 ± 1.0
	Total number of boli [nr.]	Tr	2.0 ± 2.0	1.0 ± 1.0	2.0 ± 3.0	2.0 ± 3.0
Escape	Jumps [nr]	ns	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR.
Repeated measures ANOVA was performed using trial as within factor and treatment as between factor. A P value less than 0.05 was considered significant. Sig = significance nr = number, sec=seconds, T = trial effect, Tr = treatment effect, T x Tr = trial x treatment interaction, ns = non-significant.

Table S2: Overview of the number of c-Fos positive cells after behavioural testing. PreL (prelimbic cortex), LSD (dorsal lateral septum), LSI (intermediary lateral septum), V (ventral lateral septum), BSTMA (bed nucleus of the stria terminalis, medial anterior part), BSTLP (bed nucleus of the stria terminalis, lateral posterior part), BSTMV (bed nucleus of the stria terminalis, medial ventral part), DG (dentate gyrus), PVN (paraventricular nucleus), DMH (dorsal medial hypothalamus), BLA (basolateral amygdala), CeA (central nucleus of the amygdala), dlPAG (dorsolateral part of periaqueductal grey), dmPAG (dorsomedial part of the periaqueductal grey), lPAG (lateral part of the periaqueductal grey) and the vlPAG (ventrolateral part of the periaqueductal grey).

	Treatment		
	mHB	**mHB + CMS**	**Significance**
PreL	1.4 ± 1.1	8.1 ± 1.5	ns
LSD	11.4 ± 2.9	17.9 ± 5.1	ns
LSI	5.5 ± 1.0	7.0 ± 1.2	ns
LSV	4.9 ± 2.5	8.4 ± 2.6	ns
BSTMA	16.1 ± 4.9	25.1 ± 6.6	ns
BSTLP	3.1 ± 1.5	15.8 ± 3.5	P = 0.002
BSTMV	9.3 ± 2.3	12.5 ± 3.1	ns
DG	5.0 ± 1.0	6.3 ± 1.1	ns
PVN	15.0 ± 6.6	10.0 ± 3.4	ns
DMH	22.9 ± 7.9	7.1 ± 1.0	ns
BLA	1.9 ± 0.7	2.7 ± 0.8	ns
CeA	0.0 ± 0.0	1.7 ± 1.3	ns
dlPAG	5.8 ± 1.7	4.9 ± 1.09	ns
dmPAG	0.7 ± 0.7	5.2 ± 2.8	ns
lPAG	2.0 ± 1.0	10.4 ± 5.2	ns
vlPAG	0.3 ± 1.0	12.7 ± 3.8	P = 0.045

Results are represented as mean number of c-Fos positive cells per mm^2 (± SEM). ns = non-significant.

Chapter

5

Behavioural habituation to novelty and brain area specific immediate early gene expression in female mice of two inbred strains

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Abstract

In mice, emotional adaptation might be assessed by changes in behavioural responses towards novelty over time (i.e. habituation), with non-adaptive anxiety being expressed by a lack of habituation. Recently we found that male 129P3/J mice showed such a profound lack of habituation in comparison to male BALB/cJ mice. From these results we concluded that male 129P3/J mice might model non-adaptive, i.e. pathological anxiety. As a first step in the process of assessing the generalizability of our results, we investigated whether these results were robust across gender. Therefore we replicated our previous study in female individuals. Results from the present study reveal behavioural habituation towards novelty, i.e. an adaptive phenotype in female BALB/cJ mice. In contrast, females of the 129P3/J strain were characterized by a lack of habituation, similar as their male counterparts. Compared to female BALB/cJ, female 129P3/J mice showed lower neural activity in brain areas known to regulate the integration of emotional and cognitive processes. Extending the results found in males, female 129P3/J mice revealed increased post-testing plasma corticosterone levels and higher neural activity in brain areas related to emotional processing than females of the BALB/cJ strain. Taken together our results demonstrate that both genders of the 129P3/J mouse strain are characterized by a non-adaptive anxiety-phenotype, strengthening the hypothesis that the 129P3/J strain may be a promising (neuro)-behavioural model for pathological anxiety.

Introduction

Anxiety disorders are amongst the most prevalent types of psychiatric disorders and affect about 10-17% percent of the world population (Kessler et al., 2005; Somers et al., 2006). Commonly used animal models of anxiety are based on the exposure of animals to novel stimuli, inducing biologically adaptive avoidance behaviour i.e. normal anxiety. An appropriate animal model for pathological anxiety in contrast should mirror a biological non-adaptive i.e. pathological response (Ohl et al., 2008). Furthermore, characteristics of pathological anxiety should be detectable in both genders of a potential animal model (van der Staay et al., 2009).

One procedure to differentiate between normal and pathological anxiety may be the evaluation of behavioural habituation to a given stimulus over time. Non-adaptive anxiety would then be mirrored by a lack of habituation (Salomons et al., 2010c).

Recently we found that male 129P3/J mice are characterized by such a lack of habituation and under more aversive experimental conditions even reveal sensitisation (Salomons et al., 2010c).

In contrast, for example male BALB/cJ mice which have been reported to be highly anxious (Belzung and Berton, 1997; Belzung and Griebel, 2001; Griebel et al., 1993; Makino et al., 1991; Salomons et al., 2010c) show rapid habituation to the same test environment (Salomons et al., 2010c), a phenomenon which has been reported for other inbred rodent strains used as models for anxiety as well (Ohl et al., 2002; Ohl et al., 2003). In addition to their behavioural characteristics, distinct brain areas (e.g. prelimbic cortex and lateral septum) in 129P3/J male mice compared to rapidly habituating BALB/cJ male mice revealed a lower c-Fos expression after the habituation procedure (Salomons et al., 2010c). This finding suggests that individuals from the 129P3/J strain may be impaired in correctly integrating information about their environment and emotional processes, a prerequisite to adapt behavioural responses over time. From these results we concluded that male 129P3/J mice might be an interesting animal model for pathological anxiety.

As a first step in the process of assessing the generalizability (external validity) of our results, we investigated whether these results were robust across gender. Notably, in humans, woman are more likely to develop anxiety disorders and are twice as often diagnosed with anxiety disorders than men (Zender and Olshansky, 2009).

Despite this well known fact, the vast majority of preclinical research on pathological anxiety in animal models is done in male individuals (Blanchard et al., 1991; Blanchard et al., 1995). We therefore replicated our previous study with female individuals.

Materials and methods

Ethical note

The experimental protocols were peer reviewed by the scientific committee of the Department of Animals in Science and Society (Utrecht University, The Netherlands) and subsequently reviewed and approved by the Animal Experiments Committee of the Academic Biomedical Centre Utrecht -The Netherlands.
The Animal Experiments Committee based its decision on the Dutch implementation of the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the national 'Code on laboratory animal care and welfare' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003).

Animals and housing

Naive female BALB/cJ (BALB/c, stock nr. 000651, n=10) and 129P3/J mice (129P3, stock nr. 000690, n=10) were obtained from the Jackson Laboratory (Bar Harbour, Maine, USA) and arrived at the age of 6-7 weeks . Upon arrival they were housed individually in Eurostandard Type II cages (size: 26.7cm x 20.7cm x 14 cm, Tecniplast, Buguggiate, Italy) provided with bedding material (Lignocel®, J. Rettenmaier & Söhne GmbH, Germany), a tissue (KLEENEX® Facial Tissue, Kimberly-Clark Professional BV, Ede, The Netherlands) and a shelter for cage enrichment. Mice chow (CRM, Expanded, Special Diets Services, Witham, England) and tap water were available ad libitum. The mice were kept in the experimental room for 17 days under constant laboratory conditions at the animal facilities of the Netherlands Vaccine Institute (Bilthoven, the Netherlands) for acclimatisation to the experimental room under a 12h reversed dark/light cycle (lights on between 18.00h and 6.00h) and a radio played constantly as background noise. During this period all animals were handled three times a week between 9.00 and 11.00h by the person who also performed the behavioural tests. At the beginning of the experimental period, the animals were 8-9 weeks old. All behavioural testing took place in the animal's housing room and equipment was installed before the animals arrived.
Relative humidity was kept at a constant level of approximately 50%, room temperature was sustained at 22°C ± 2°C and ventilation rate was 15-20 air changes per hour.

The modified hole board (mHB)

The mHB consisted of an opaque grey PVC box (100 x 50 x 50 cm) with a board, made of the same material as the box, positioned in the middle of the box (60 x 20 x 2 cm), thus representing the unprotected

area comparable with the centre of an open field (Ohl et al., 2001a). On the board 20 cylinders (diameter 15 mm) were staggered in three lines. The area around the board was divided by black lines into 10 rectangles (20 x 15 cm) and 2 squares (20 x 20 cm). The board was illuminated with an additional stage light (white light 120 lx), though the surrounding box was only illuminated with red light (1-5 lx). For the investigation of food intake inhibition, the animals were familiarized with a small piece of almond (45 mg, given with a forceps) in their home cage on three consecutive days (at 9.00h) prior to behavioural testing. During testing, the familiar (almonds) and unfamiliar food (Dustless Precision Pellets, 45 mg, Bio-Serv, Frenchtown, USA) objects were always placed in the same corner of the mHB, either one was positioned at the same distance from the wall. For testing, one animal at the time was directly transferred from its home cage to the mHB and always placed in the same corner diametrically opposed to the corner in which the food objects were placed and allowed to explore the mHB for 5 minutes per trial (4 trials per day, on 5 consecutive days, total of 20 trials). After each trial, the animal was placed back in its home cage and the mHB was carefully cleaned with tap water and a damp towel. All tests were videotaped for raw data storage and behaviour was directly scored by a trained observer using the program 'The Observer 5.0' (Noldus Information Technology, Wageningen, The Netherlands). The following behavioural parameters were measured and assigned to different behavioural categories according to previous studies (Ohl et al., 2001b); *(a)* avoidance behaviour directed towards the unprotected area: the latency until the first board entry (latency board), the percentage of time spent on the board (time board) and the total number of board entries (board entries); *(b)* risk assessment, measured as the number of stretched attend postures (stretched attends) and the latency until the first stretched attend (latency stretched attend); *(c)* locomotor activity: the total number of line crossings (line crossings), the latency until the first line crossing (latency line crossing), the total time spent immobile (time immobile) and the latency until the first immobility event (latency immobility); *(d)* general exploration: the total number of rearings in the box (rearings box) and on the board (rearings board), the latency until the first rearing in the box (latency rear box) and on the board (latency rearings board), the total number of hole explorations (hole explorations) and the latency until the first hole exploration (latency hole exploration) (a hole was counted as explored when the animal's nose was directed to a hole; direct contact with the hole was not necessary); *(e)* directed exploration: the total number of holes visited (holes visited, a hole was counted as visited when the mouse dipped the nose below the rim of the hole) and the latency until the first hole visit (latency hole); *(f)* food intake inhibition: the latency until the first exploration of the unfamiliar

(latency unfamiliar food) and familiar food object (latency familiar food); *(g)* arousal or de-arousal: the percentage of time spent self-grooming (time grooming), the latency until the first self-grooming event (latency grooming), the total number of self-grooming events (self-groomings) and the total number of fecal boli (boli); *(h)* escape behaviour: the total number of jumps (jumps).

Corticosterone

Basal blood samples were collected 4 days before the start of mHB testing to determine basal levels of plasma corticosterone (CORT) (basal). Basal sampling took place at least 3 hours after the onset of the dark period and 5 hours before the light period to minimize variations in CORT levels due to circadian rhythmicity (Oshima et al., 2003). Trunk blood was collected 2h after the last mHB trial to determine CORT levels after behavioural testing (non-basal).
All blood sampling and decapitation took place in a separate room adjacent to the experimental room (in order not to disturb circadian rhythm of the mice, the hallway and rooms were kept under red light conditions). Basal blood samples were collected (± 50µl) using tail vain incision and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin.
Trunk blood after decapitation was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 12.000 rpm, 4°C) and stored at -20°C until analysis. CORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

c-Fos immunohistochemistry and image quantification

After decapitation, the brains were removed and frozen in -80°C 2-methyl-butane which was cooled with dry ice and stored at -20°C. Coronal sections of 20 µm were cut and mounted on Menzel SuperFrost Plus slides (Menzel GmbH&Co, Braunsweg, Germany) and stored at -20°C. The sections were processed for c-Fos immunohistochemistry as described previously (Salomons et al., 2010c). First, dilution with a polyclonal primary antibody (1:1000, SC-52 Santa Cruz Biotechnology, Santa Cruz, USA), and a donkey-anti-rabbit IgG Biotin SP conjugated secondary antibody (1:400, Jackson ImmunoResearch Laboratories, Inc, USA) was done.
Cells containing a nuclear brown-black reaction product were considered as c-Fos positive cells and counted in the following brain areas which are known to be involved in anxiety (Arzt and Holsboer, 2006; Muigg et al., 2007; Nguyen et al., 2006) (numbers correspond with the Bregma level in which c-Fos analyses was done); medial prefrontal cortex (prelimbic, +1.78), lateral septum (dorsal, intermediary and ventral, +0.86), bed nucleus of the stria terminalis

(medial ventral, +0.38, medial anterior and lateral posterior, +0.14), dentate gyrus (granular layer, -1.35), paraventricular nucleus (-0.82), dorsal medial hypothalamus (-1.58), amygdala (basolateral nucleus and central nucleus, -1.58) and periaqueductal gray (dorsolateral, dorsomedial, lateral and ventrolateral, -4.72). The anatomical localisation of c-Fos positive cells was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas (Franklin and Paxinos, 1997). For each region at least two overt landmarks were used. For quantitative analysis of c-Fos positive cells, the program Leica QWin (image processing and analysis software, Cambridge, United Kingdom) was used. Left and right hemispheres were analysed in one section separately and averaged for each animal and calculated for stained neurons per mm^2.

Statistical analyses

Statistical analyses were performed using the software program SPSS for Windows (version 15.0.1; SPSS Inc., IL, USA). Continuous data (CORT, latency and relative duration of behavioural parameters (represented as mean ± standard error of the mean, SEM)), were first tested for Gaussanity using the Kolmogorov-Smirnov test. Homoscedasticity was tested by Levene's test. Group analyses using the Kolmogorov-Smirnov one sample test revealed a non-parametric distribution of several continuous parameters. These parameters, as well as the total number of behavioural parameters (represented as median ± interquartile range, IQR), were rank transformed (Conover and Iman, 1982). The (transformed) data from the mHB experiment were subsequently analysed using repeated measures ANOVA with Huyn-Feldt adjustment (trial number as within-subject factor and treatment as between-subject factor). All data including outliers were used in the analyses.
Post hoc analyses were done using an unpaired Student-t test for continuous data and the Mann-Whitney U test for discrete data. For ANOVA analyses, a probability value less than 0.05 was considered to be statistically significant. To minimize the risk of a Type 1 error due to multiple comparisons, the level of significance was corrected for the post hoc analyses using Dunn Sidak correction (Ludbrook, 1991).

Results

mHB

A general overview of mHB results for all behavioural parameters for the first (trial 1) and the last trial (trial 20) can be found in [Table S1].
Avoidance behaviour: a significant strain ($F(1,18) = 4.794$, $P<0.04$), trial ($F(19,342) = 1.634$, $P<0.05$) and trial x strain interaction ($F(19,342) = 14.496$, $P<0.001$) was found for the total time spent on the board. BALB/c mice spent initially less time on the board compared to 129P3 mice (trial 1: 0.0 ± 0.0% vs. 30.5 ± 7.7%).

Across the trials, the total time spent on the board increased in BALB/c mice (trial 20: 3.9 ±1.1), while it decreased in 129P3 mice (trial 20: 3.5± 1.2%). The number of board entries and the latency until the first board entry showed only a significant trial x strain interaction ($F(19,342) = 14.948$, $P<0.001$, $F(19,342) = 15.846$, $P<0.001$ resp.). The latency until the first board entry decreased in BALB/c mice across trials while the number of board entries and the latency until the first board entry increased in 129P3 mice *[Fig 1a]*.

Risk assessment: both strains showed a decrease in the number of stretched attends across trials (trial: $F(19,342) = 13.163$, $P<0.001$, trial x strain interaction $F(19,342) = 7.807$, $P<0.001$). No strain effects were found.

Locomotor activity: a significant strain ($F(1,18) = 65.582$, $P<0.001$), trial ($F(19,342) = 4.560$, $P<0.001$) and trial x strain interaction ($F(19,342) = 16.735$, $P<0.001$) was observed for the number of line crossings *[Fig 2a]*. BALB/c mice showed a general increase in the number of line crossings across trials whereas no change was observed in 129P3 mice. No significant differences were found for the total time spent immobile and the latency until the first immobility event within and between strains.

General exploration: for the number of rearings in the box a significant strain ($F(1,18) = 24.363$, $P<0.001$), trial ($F(19,342) = 3.143$, $P<0.001$) and trial x strain interaction ($F(19,342) = 6.343$, $P<0.001$) was found *[Fig 2b]*. BALB/c mice displayed a general increase across trials in the number of rearings in the box while the latency until the first rearing in the box decreased ($F(19,342) = 4.466$, $P<0.001$). Compared to BALB/c mice, the 129P3 mice showed a general decrease in the number of rearings in the box and an increase in the latency until the first rearing in the box ($F(1,18) = 11.906$, $P<0.001$). No significant effects were found for the number of rearings on the board or the latency until the first rearing on the board, since this behaviour was hardly displayed by both strains.

Significant trial and trial x strain interactions were found for the number of hole explorations ($F(19,342) = 2.269$, $P<0.002$, $F(19,342) = 13.727$, $P<0.001$) and the latency until the first hole exploration ($F(19,342) = 2.451$, $P<0.002$, $F(19,342) = 14.465$, $P<0.001$). No significant strain differences were found. Compared to BALB/c mice, 129P3 mice showed an increase in the latency until the first hole exploration.

Directed exploration: a significant trial and trial x strain interaction was found for the number of hole visits ($F(19,342) = 3.145$, $P<0.002$, $F(19,342) = 5.086$, $P<0.001$) and the latency until the first hole visit ($F(19,342) = 3.102$, $P<0.002$, $F(19,342) = 5.274$, $P<0.001$). BALB/c mice initially performed less hole visits compared to 129P3 mice, whereas this behaviour increased across trials. 129P3 mice showed initially more hole visits, but those decreased across trials.

Food intake inhibition: the latency time until the first exploration

of the familiar food object decreased over time in BALB/c mice, whereas 129P3 mice showed no general change across trials (trial: $F(19,342) = 7.275$, $P<0.001$; strain: $F(1,18) = 11.472$, $P<0.003$; trial x strain: $F(19,342) = 7.274$, $P<0.00$, *[Fig 1b]*). For the latency until the first exploration of the unfamiliar food a trial ($F(19,342) = 4.189$, $P<0.001$) and a trial x strain interaction ($F(19,342) = 1.183$, $P<0.017$) was found. Both strains showed a decrease in the latency until the first exploration of the unfamiliar food across trials, while this decrease was more apparent in BALB/c mice.

Fig. 1 a+b

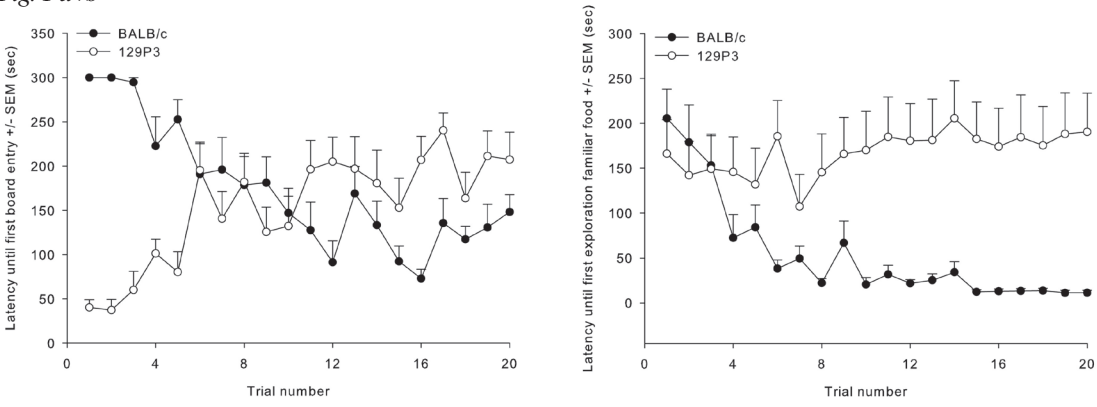

Figure 1: Mean (± SEM) latency until the first board entry (left) and latency until the first exploration of the familiar food (right).

Fig. 2 a+b

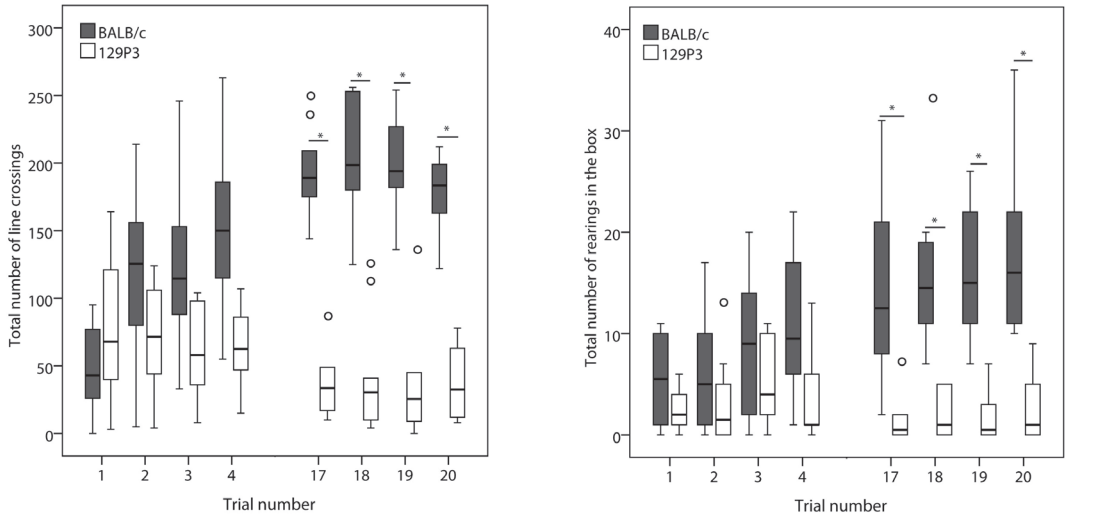

Figure 2: Median (± IQR) number of line crossings (left) and number of rearings in the box (right). Data are displayed for the first day (trial 1-4) and the last day (trials 17-20). Open circles represent outliers (1.5 x IQR).

Arousal/de-arousal: the total time spent grooming increased while the latency to the first self-grooming event decreased across trials in both strains, but this did not reach significance. Only for the total number of grooming events a significant trial x strain interaction was found ($F(19,342) = 1.671$, $P<0.039$). A significant trial ($F(19,342) = 2.354$, $P<0.001$), strain ($F(1,18) = 12.328$, $P<0.002$) and trial x strain interaction ($F(19,342) = 2.797$, $P<0.001$) was found for the total number of boli. BALB/c mice displayed a decrease in the number of boli, while 129P3 mice showed no change in the number of boli across trials.
Escape behaviour: no significant effects for the number of jumps were found.

Corticosterone

Basal CORT levels *[Fig 3]* did not significantly differ between the strains. BALB/c mice did not show a significant difference between basal and non-basal (after behavioural testing) CORT levels, while 129P3 mice showed significantly higher CORT levels after behavioural testing ($t = -6.897$, $P<0.001$). Compared to BALB/c mice, non-basal CORT levels were significantly higher in 129P3 mice ($t = 5.120$, $P<0.001$).

Fig. 3

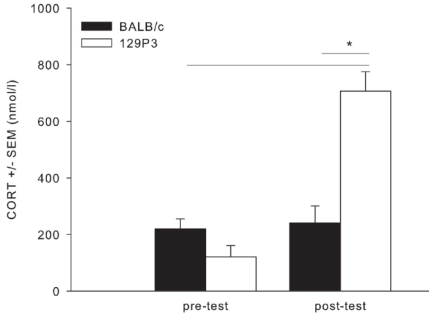

Figure 3: Mean (± SEM) CORT levels before (pre-test) and after (post-test) behavioural testing. *P<0.001.

Fig. 4

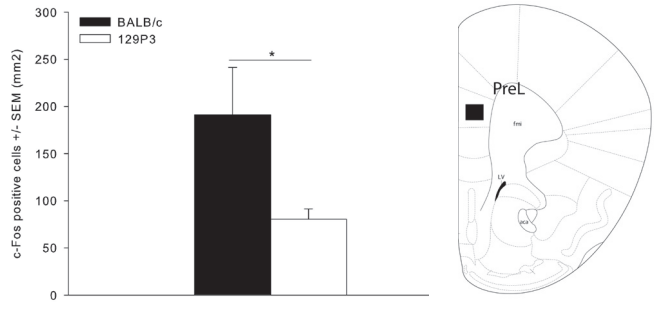

Figure 4: Mean (± SEM) c-Fos positive cells in the prelimbic (PreL) cortex after mHB testing. Schematic diagram adapted from Franklin and Paxinos shows the brain area in which c-Fos expression was quantified.* P<0.005

c-Fos immunohistochemistry

Significant higher c-Fos expression was found in the prelimbic cortex ($t = 2.253$, $P = 0.029$, *[Fig 4]*) and the dentate gyrus ($t = 2.014$, $P = 0.049$) in BALB/c mice compared to 129P3 mice. While lower c-Fos expression was found in BALB/c mice in the medial anterior part of the bed nucleus stria terminalis ($t = -3.567$, $P = 0.002$) and in the central nucleus of the amygdala ($t = -2.198$, $P = 0.041$) compared to 129P3 mice. In all other brain areas investigated, no significant differences between strains were found *[see Table S2]*.

Discussion

The present study was aimed at evaluating the habituation profile of female 129P3 mice compared to BALB/c females after repeated exposure to a test environment. Overall, female BALB/c mice were found to be characterized by high initial avoidance behaviour, followed by fast habituation, while 129P3 mice revealed relatively low initial avoidance behaviour, which increased over time, indicating a profound lack of habituation towards the test environment. These results are in accordance with previously reported strain characteristics of male 129P3 and BALB/c individuals (Salomons et al., 2010c), thus emphasizing the potential value of the 129P3 inbred strain as an animal model for pathological anxiety.
In more detail, female BALB/c individuals showed high initial avoidance behaviour compared to female 129P3 mice as reflected by a higher latency to enter the unprotected area and the low time spent on the board of the mHB *[Fig 1a]*. Following their high initial anxiety, female BALB/c mice showed a rapid decrease in the latency to enter the unprotected area and spent more time on the board during the experimental period. Differently from their male counterparts, female BALB/c mice showed increased locomotor and exploratory activity over time, being indicated by an increasing number of line crossings and rearings across the experimental trials. These findings are in agreement with studies investigating BALB/c mice and other mouse strains in which females tend to be more active than their male counterparts (Bothe et al., 2005). Overall, female BALB/c mice showed behavioural habituation to the test environment and, thus, adaptive anxiety-related behaviour comparable to their male counterparts. In contrast to female individuals of the BALB/c strain, female 129P3 mice revealed impaired behavioural habituation.
In fact, their avoidance behaviour even increased over time *[Fig 1a]*, while locomotor and exploratory activity remained unchanged during the experimental period. Compared to female BALB/c mice, female 129P3 mice showed less locomotor and exploratory activity *[Fig 2a,b]*. The habituation profile as observed in female 129P3 mice towards novelty thus mirrors a non-adaptive anxiety profile, which is in accordance to earlier results in males of the same strain (Salomons et al., 2010c).

Strengthening the conclusions from behavioural observations, strain differences of central-nervous c-Fos activity patterns previously reported for male BALB/c and 129P3 mice could be reproduced in females as well. Compared to female BALB/c mice, 129P3 mice showed less c-Fos activity in the prelimbic cortex *[Fig 4]* and dentate gyrus. The prefrontal cortex and hippocampus are known to integrate information to guide subsequent behaviour and activation of these areas can inhibit stress responses (Davidson, 2002; Goldman-Rakic, 1995; McEwen, 1999). In contrast to female BALB/c mice, higher c-Fos expression was found in the central nucleus of the amygdala and bed nucleus of the stria terminalis in female 129P3 mice. The latter finding even extends the strain differences observed in male BALB/c and 129P3 mice, though we cannot exclude that other factors may have influenced this result. As the central nucleus of the amygdala is a brain area known to predominantly process conditioned emotional responses (Davis, 2006; Wilensky et al., 2006), the bed nucleus is thought to be a key relay in the stress circuitry (Herman et al., 2005; Makino et al., 2002), it can be hypothesized that female 129P3 mice perceive the repeated testing conditions as more stressful than male 129P3 mice. This hypothesis is confirmed by the notion that, in contrast to male 129P3 mice, females firstly showed no habituation to familiar food, indicating a sustained inhibition of the motivation to intake a palatable food object, which is known to be caused by stress (Ducottet and Belzung, 2005; Schweizer et al., 2009; Willner, 2005). Secondly, CORT levels after behavioural testing were increased in female 129P3 mice compared to basal levels, a stress response which was not found in male 129P3 mice. Although CORT levels after the first mHB trial were not investigated in the present experiment, it can be concluded that after 20 mHB trials, exposure to the testing conditions was still perceived as stressful by the animals. It is of note that the findings in female 129P3 mice summarize to a behavioural profile, which is comparable to that of 129P3 males exposed to chronic mild stress followed by repeated testing (Salomons et al., 2010b). Chronic mild stress in male 129P3 mice caused sustained inhibition in locomotor and exploratory activity in addition to sustained avoidance behaviour, approving an intensification of their impaired habituation profile. Female 129P3 mice in the present study obviously experienced a comparable stress level due to the repeated testing. One possible explanation for this gender difference might be that the individual housing conditions applied to experimental animals in both studies to avoid testing order effects (Arndt et al., 2009) were more stressful for female 129P3 mice than for their male counterparts. It has been reported earlier that for example female CD-1 mice, housed individually for one week, showed increased anxiety-related behaviour, reduced exploration and locomotion compared to group-housed females (Palanza, 2001), and individually housed female C57Bl/6 mice eventually revealed

increased anxiety as well (Martin and Brown, 2010). Although the results on the effects of single housing are not consistent (Arndt et al., 2009) it cannot be excluded that these housing conditions are perceived as stressful by female 129P3 mice, resulting in comparable behavioural and physiological effects as has been reported for a CMS procedure in male individuals of this strain (Salomons et al., 2010b). However, it cannot be ruled out that environmental factors other than single housing might have influenced the behavioural phenotype of female 129P3 mice, such as the handling procedure or the transport from the breeder. The comparison between the two mouse strains used in this experiment and the fact that female BALB/c mice were treated identically as female 129P3 mice, still allow for the interesting notion that female 129P3 mice are more susceptible to the negative effects of such influences, and that this susceptibility results in an impaired habituation to novelty.

Another point of consideration is the potential influence of the female's estrous cycle on behaviour and hormones, which has not been controlled for in our experiment. It is well known that rapidly changing and fluctuating ovarian hormones can affect emotionality, such as anxiety (Bartolomucci et al., 2009; Carey et al., 1995; Halbreich and Kahn, 2001; Ter Horst et al., 2009). As a matter of fact, we cannot exclude possible effects of estrous cycle on the adaptive capacity of female BALB/c and 129P3 mice. However, as animals from both strains were housed in the same room, we can exclude that a synchronization per strain occurred and that a possible estrous cycle effect was responsible for the reported between-strain effects. In contrast, an estrous cycle effect could have led to an increase of variation within the experimental groups and, thus, would rather have produced false negative results then false positive results.

In summary, it appears that the strain characteristics with respect to behavioural habituation previously shown in male individuals are also present in female 129P3 and BALB/c mice. The latter strain is characterized by high initial avoidance behaviour, followed by fast habituation, thus a non-pathological anxiety phenotype.

In contrast, 129P3 mice reveal a profound lack of habituation with females being even more sensitive to the repeated testing than their male counterparts. In addition to the behavioural observations, these conclusions are grounded on increased post-testing CORT levels, sustained locomotor and exploratory inhibition and increased c-Fos expression in the amygdala and bed nucleus stria terminalis in female 129P3 mice. Lower c-Fos activity in the prelimbic cortex and dentate gyrus in 129P3 mice compared to BALB/c mice suggest an impaired integration of information by higher cortical areas guiding subsequent adaptive behaviour in the 129P3 strain.

Further experiments will have to elucidate the central nervous mechanisms underlying the non-adaptive profile in 129P3 mice to validate this mouse strain as a model for pathological anxiety.

Supplementary material

Table S1: Overview of significant main effects found by repeated measures ANOVA and average per behavioural parameter for trial 1 and 20 (± SEM) in the mHB. Behavioural parameters are assigned to different behavioural categories.

			BALB/c		**129P3/J**	
Behavioural category	**Behavioural parameter**	**Sig**	**Trial 1**	**Trial 20**	**Trial 1**	**Trial 20**
Avoidance	Latency until first board entry [sec]	T*S	300.0 ± 0.0	148.1 ± 19.6	40.2 ± 8.7	207.3 ± 31.1
	Total time spent on board [%]	T, T*S ,S	0.0 ± 0.0	3.9 ± 1.1	30.5 ± 7.7	3.5 ± 1.2
	Total number of board entries [nr.]	T*S	0.0 ± 0.0	3.0 ± 2.0	11.0 ± 8.0	1.5 ± 4.0
Risk assessment	Total number of stretched attends [nr.]	T, T*S	29.5 ± 15.0	0.0 ± 0.0	0.5 ± 5.0	0.0 ± 1.0
	Latency until first stretched attend [sec]	T, T*S	12.8 ± 3.0	300.0 ± 0.0	177.7 ± 44.7	235.0 ± 31.3
Locomotion	Total number of line crossings [nr.]	T, T*S ,S	43.0 ± 56.0	183.5 ± 40.0	68.0 ± 88.0	32.5 ± 55.0
	Latency until first line crossing [sec]	T, T*S ,S	68.1 ± 27.3	2.6 ± 0.5	8.2 ± 3.0	60.0 ± 18.5
	Total time spent immobile [%]	ns	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.7
	Latency until first immobility [sec]	ns	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	292.4 ± 7.6
General exploration	Total number of rearings in the box [nr.]	T, T*S, S	5.5 ± 10.0	16.0 ± 13.0	2.0 ± 4.0	1.0 ± 5.0
	Latency until first rearing in the box [sec]	T, T*S, S	110.6 ± 35.4	53.6 ± 9.1	136.2 ± 36.9	183.2 ± 38.3
	Total number of rearings on the board [nr.]	ns	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Latency until first rearing board [sec]	ns	300.0 ± 0.0	294.2 ± 5.8	300.0 ± 0.0	289.9 ± 7.9
	Total number of hole explorations [nr.]	T, T*S	0.0 ± 0.0	4.0 ± 4.0	13.5 ± 9.0	1.0 ± 4.0
	Latency until first hole exploration [sec]	T, T*S	287.0 ± 10.8	105.1 ± 18.3	43.2 ± 17.3	207.6 ± 29.0
Directed exploration	Total number of hole visits [nr.]	T, T*S	0.0 ± 0.0	1.5 ± 2.0	2.0 ± 7.0	0.0 ± 2.0
	Latency until first hole visit [sec]	T,T*S	300.0 ± 0.0	209.6 ± 21.2	142.4 ± 39.3	243.5 ± 27.7
Food intake inhibition	Latency first exploration unfamiliar food [sec]	T, T*S	300.0 ± 0.0	32.4 ± 35.5	251.1 ± 32.8	214.8 ± 43.4
	Latency first exploration familiar food [sec]	T, T*S ,S	205.6 ± 32.5	11.5 ± 2.5	166.2 ± 37.6	190.5 ± 43.1
Arousal	Total time spent grooming [%]	ns	0.2 ± 0.2	0.8 ± 0.5	0.8 ± 0.6	2.45 ± 1.3
	Latency until first self groom [sec]	ns	298.3 ± 1.7	178.0 ± 40.5	235.6 ± 28.5	231.2 ± 32.4
	Total number of self-groomings [nr.]	T*S	0.0 ± 0.0	0.5 ± 1.0	0.0 ± 1.0	0.0 ± 1.0
	Total number of boli [nr.]	T, T*S ,S	4.0 ± 4.0	1.5 ± 4.0	3.0 ± 2.0	3.5 ± 2.0
Escape	Total number of jumps [nr.]	ns	0.0 ± 0.0	1.0 ± 4.0	0.0 ± 0.0	0.0 ± 0.0

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR. Repeated measures ANOVA was performed using trial as within factor and strain as between factor. A P value less than 0.05 was considered significant. Sig: significance; nr, number; sec, seconds; T, trial effect; S, strain effect; T *S, trial x strain interaction; ns, non-significant.

Table S2: Overview of the number of c-Fos positive cells after behavioural testing. PreL (prelimbic cortex), LSD (dorsal lateral septum), LSI (intermediary lateral septum), V (ventral lateral septum), BSTMA (bed nucleus of the stria terminalis, medial anterior part), BSTLP (bed nucleus of the stria terminalis, lateral posterior part), BSTMV (bed nucleus of the stria terminalis, medial ventral part), DG (dentate gyrus), PVN (paraventricular nucleus), DMH (dorsal medial hypothalamus), BLA (basolateral amygdala), CeA (central nucleus of the amygdala), dlPAG (dorsolateral part of periaqueductal grey), dmPAG (dorsomedial part of the periaqueductal grey), lPAG (lateral part of the periaqueductal grey) and the vlPAG (ventrolateral part of the periaqueductal grey).

	BALB/c	**129P3**	**Significance**
PreL	20.2 ± 5.4	8.4 ± 1.9	P = 0.029
LSD	11.0 ± 3.8	15.4 ± 3.9	ns
LSI	10.9 ± 2.4	8.9 ± 2.3	ns
LSV	11.2 ± 2.7	18.8 ± 7.2	ns
BSTMV	10.8 ± 3.1	13.7 ± 3.4	ns
BSTMA	11.6 ± 4.3	42.8 ± 10.9	P = 0.002
BSTLP	6.1 ± 2.3	7.4 ± 2.3	ns
PVN	26.6 ± 5.2	38.8 ± 5.7	ns
DG	57.9 ± 8.6	41.4 ± 7.3	P = 0.049
DMH	45.0 ± 8.7	59.9 ± 8.3	ns
BLA	31.4 ± 5.2	19.9 ± 3.5	ns
CeA	5.6 ± 2.3	14.1 ± 4.7	P = 0.04
dlPAG	25.8 ± 8.4	29.5 ± 5.4	ns
dmPAG	35.6 ± 10.9	46.6 ± 10.0	ns
lPAG	27.3 ± 6.5	27.7 ± 47.1	ns
vlPAG	30.6 ± 4.9	42.5 ± 11.6	ns

Results are represented as mean number of c-Fos positive cells per mm^2 (± SEM). ns, non-significant.

BOX 1: Habituation profiles in 129S2/Sv mice

The 129P3 mouse strain is a member of a large family of 129 substrains originating from one common ancestor (Festing et al., 1999; Simpson et al., 1997). Since they share a common genetic background, we hypothesized that the non-adaptive phenotype of the 129P3 mice as shown in the previous chapters could be assumed for other 129 substrains as well. For this purpose, we tested two other 129S2 mouse strains, the 129S2/SvPasCrl (129SCrl, Charles River, the Netherlands) and the 129S2/SvHsd (129SHsd, Harlan, the Netherlands) by repeatedly exposing them to the mHB test, a similar experiment as described in Chapter 3 (experiment 2), with similar acclimatization period and housing conditions.

Results confirm a non-adaptive phenotype of both mouse strains as reflected by significantly increased avoidance behaviour of the unprotected area during the experimental period (*[Fig 1a]*; trial: $F(19.266) = 5.851$, $P < 0.01$; trial x strain $(19.266) = 3.788$, $P<0.01$) (*[Fig 1b]*; trial: $F(19.266) = 6.801$, $P<0.01$; trial x strain $(19.266) = 2.765$, $P<0.01$). Both strains showed no change in locomotor *[Fig 2a]* and exploratory activity *[Fig 2b]* during the experimental period, although 129SHsd mice showed significantly more general activity than 129SCrl mice. This was also indicated by the time spend immobile, which increased during the testing period in both strains whereas 129SCrl mice spend more time immobile than 129SHsd mice ($F(1.14) = 10.654$, $P < 0.01$). No significant differences were found in CORT levels before and after behavioural testing.
For a complete overview of behavioural results, see *[Table S1]*.

Fig. 1 a+b

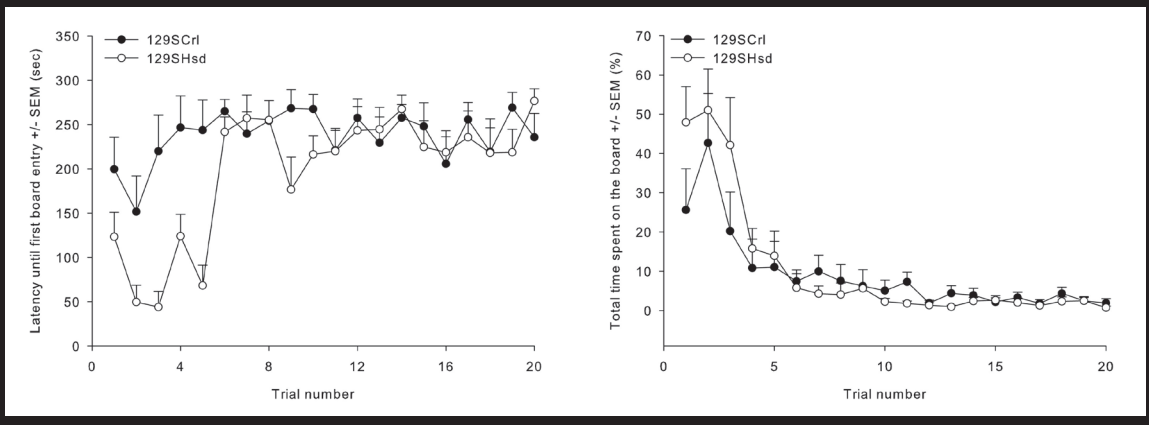

Figure 1: Mean (± SEM) latency until the first board entry (left) and the total time spend on the board (right).

Fig. 2 a+b

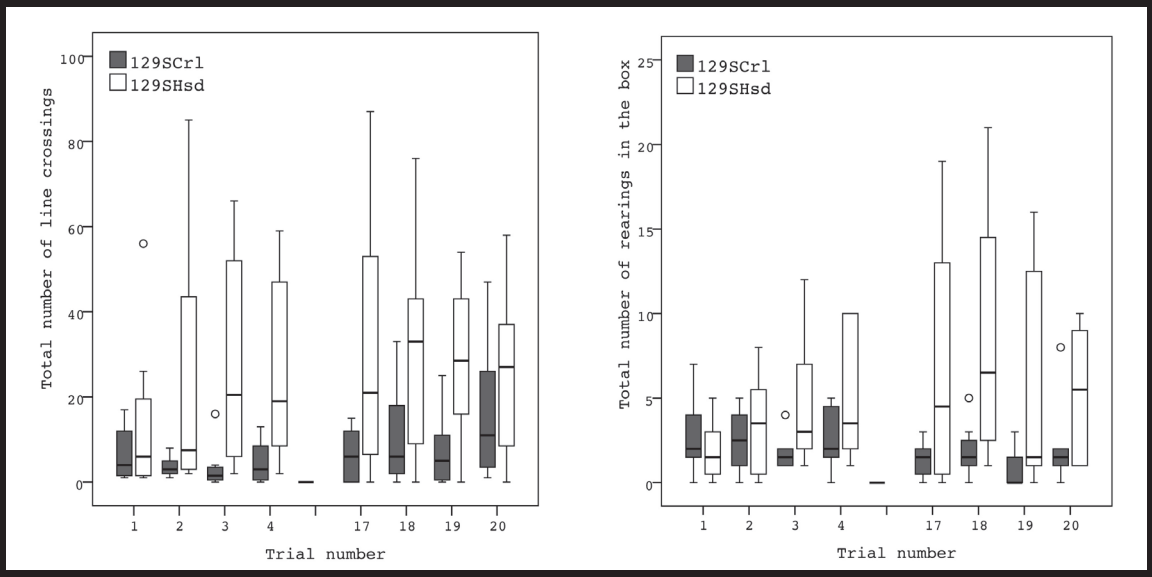

Figure 2: Median (± IQR) number of line crossings (left) and rearings in the box (right). Data are displayed for the first day (trials 1-4) and the last day (trials 17-20).

Both 129S2/Sv strains were characterized by impaired habituation towards novelty similar as their related 129P3 counterparts. In contrast to 129P3 mice, 129S2/Sv mouse strains showed very little general activity, which was even more pronounced in the 129SCrl mice. Though 129 substrains share a common ancestor, they show quite some genetic variability (Simpson et al., 1997). Several studies have found differences in anxiety-related behaviour, activity levels and cognition among 129 substrains (Bothe et al., 2004; Bothe et al., 2005; Montkowski et al., 1997). The 129S2/Sv mouse line in particular has been found to show brain anomalies (Balogh et al., 1999). It is therefore not surprising that habituation profiles differ across the 129 substrains. Nevertheless, the lack of habituation as observed in both 129S2/Sv strains in the present study is probably confounded by generally low activity levels. Dissociation between anxiety behaviour and locomotor activity cannot be made based on the present results and hence 129S2/Sv mice are not suitable for further investigation of a potential pathological anxiety profile.

Chapter

6

Impaired integration of emotional and cognitive processes results in a lack of habituation in 129P3 mice: involvement of glutamatergic neurotransmission

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Abstract

Previous studies have demonstrated a profound lack of habituation in 129P3/J mice compared to the habituating, but initially more anxious, BALB/cJ mice. The present study investigated whether this non-adaptive phenotype of 129P3/J mice is primarily based on anxiety-related characteristics. To test this hypothesis and extend our knowledge on the behavioural profile of 129P3/J mice, the effects of the (putative) anxiolytics diazepam and the metabotropic glutamate receptor 5 (mGlu5) antagonist 2-methyl-6-(phenylethynyl) pyridine (MPEP) treatment on intrasession habituation, between-trial sensitization and cognitive performance were investigated.

Behavioural findings validated the initially high, but habituating phenotype of BALB/cJ mice, while 129P3 mice were characterized by impaired intrasession habituation.

While the lowest dose of diazepam and all doses of MPEP tested, improved the habituation profile, higher diazepam doses caused marked sedation in 129P3/J mice suggesting a deficit in GABAergic functioning. MPEP on the other hand had almost no anxiolytic effects in BALB/cJ mice, while it reduced stress-induced plasma corticosterone responses only in 129P3 mice. These results were complemented by a reduced expression of the immediate early gene c-Fos after MPEP treatment in brain areas related to emotional processes and increased c-Fos expression in higher integrating brain areas compared to vehicle-treated 129P3/J mice.

Cognitive performance was improved in 129P3/J mice after MPEP treatment, but no treatment effects were found on between-trial sensitization. These results suggest that the behavioural profile of 129P3/J mice may be the result of a dysfunctional integration of cognitive and emotional processes.

Introduction

When anxiety in animals appears to lack adaptive value which severely interferes with the normal interaction of the sufferer with its physical and social environment has been be defined as pathological anxiety (Ohl et al., 2008). At the behavioural level, adaptation in animals can be assessed by changes in behavioural responses over time, i.e. habituation to a given stimulus. Therefore, non-adaptive anxiety may be mirrored by a lack of habituation, being expressed for example by non-decreasing avoidance behaviour directed towards an unprotected area. Recently, we found that 129P3/J mice are characterized by a profound lack of habituation to the modified hole board test while BALB/cJ mice, which have been reported to be highly anxious (Belzung and Berton, 1997; Belzung and Griebel, 2001), show rapid habituation to the same test environment (Salomons et al., 2010c). In addition, in 129P3/J mice c-Fos expression was found to be lower after the habituation procedure in distinct brain areas (e.g. prelimbic cortex and lateral septum) in comparison to BALB/cJ mice (Salomons et al., 2010c). This suggests that individuals of the 129P3/J strain may be impaired in correctly integrating information about the environment and emotional processes, a prerequisite to adapt behavioural responses over time. In a subsequent study we further demonstrated that exposure to chronic mild stress prior to repeated behavioural testing intensified the habituation profile in 129P3/J mice (Salomons et al., 2010b). From our results we concluded that 129P3/J mice may represent an interesting animal model for pathological anxiety.

Still, the question remains whether the profound lack of habituation in 129P3 mice is primarily based by anxiety-related characteristics, since avoidance behaviour may be confounded by other motivational systems, such as locomotor activity (Belzung and LePape, 1994; Ohl, 2005; Rodgers et al., 2002a) as well as by cognitive processes (Dere et al., 2002; Leussis and Bolivar, 2006; McNaughton, 1997). If the habituation profile in 129P3/J mice was primarily based on anxiety-related characteristics, anxiolytic treatment should affect the animals' behaviour by reducing their lack of ability to habituate. To test this hypothesis, two anxiolytic compounds were used in the present study: the benzodiazepine diazepam as the classical gold standard anxiolytic, and the metabotropic glutamate receptor 5 antagonist (mGlu5) 2-methyl-6-(phenylethynyl)pyridine (MPEP). Although diazepam has proved its anxiolytic efficacy over decades (Faravelli et al., 2003; Shader and Greenblatt, 1993) and exerts anxiolytic effects in both rats and mice (Griebel et al., 1998; Pellow et al., 1985), this compound is known to induce severe side effects at the cognitive level (amnesia) as well as in activity (sedation) (Faravelli et al., 2003). Therefore, the putative anxiolytic MPEP was included in the present study. MPEP has been demonstrated to induce anxiolytic effects in several animal models of anxiety with low risks of side

effects (Gasparini et al., 1999; Nordquist et al., 2007; Spooren et al., 2000; Spooren et al., 2001).
Since our previous studies evaluated inter-session habituation (between trials) in two different tests, we were now interested in extending our knowledge on the behavioural profile in 129P3/J mice by investigating intra-session habituation as well. Therefore, the two mouse strains BALB/cJ and 129P3/J, were tested after acute treatment with either diazepam or MPEP for 30 minutes in the open field (intra-session habituation), repeatedly in the elevated plus maze (inter-session habituation/sensitization), and an object recognition test (cognitive performance), respectively. In addition to behavioural parameters, levels of plasma corticosterone (CORT) were determined. Finally, the expression of c-Fos, a member of the immediate early gene family of transcription factors and a marker for neural activity, in brain areas involved in emotional and cognitive processing was investigated.

Material and methods

Ethical note

The experimental protocols were peer reviewed by the scientific committee of the Department of Animals in Science and Society, University of Utrecht, The Netherlands and subsequently were approved by the Animal Experiments Committee of the Academic Biomedical Centre Utrecht, The Netherlands. The Animal Experiments Committee based its decision on the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the 'Principles of Laboratory Animal Care' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003).

Animals and housing

Naive male BALB/cJ (BALB/c, stock nr. 000651, n=113) and 129P3/J (129P3, stock nr. 000690 mice, n=113, Festing et al., 1999) were obtained from the Jackson Laboratory (Bar Harbour, Maine, USA) and housed individually in Eurostandard Type II cages (size: 26.7 x 20.7 x 14 cm, Tecniplast, Buguggiate, Italy) provided with bedding material (Lignocel®, J. Rettenmaier & Söhne GmbH, Germany), a tissue (KLEENEX® Facial Tissue, Kimberly-Clark Professional BV, Ede, The Netherlands) and a shelter for cage enrichment. Mice chow (CRM, Expanded, Special Diets Services Witham, England) and tap water were available ad libitum.
For all behavioural experiments, the mice were acclimated to the experimental room for 17 days at the animal facilities of the Netherlands Vaccine Institute under a reversed dark/light cycle (lights on between 18.00h and 6.00h) and radio played constantly

as background noise. During this period the animals were handled three times a week by the person who also performed the behavioural tests. All behavioural testing took place in the animal's housing room and equipment was installed before the animals arrived. Relative humidity was kept at a constant level of approximately 50%, room temperature was sustained at 22°C ± 2 and ventilation rate was 15-20 air changes per hour.

Drugs

Diazepam (BUFA, the Netherlands) and 2-methyl-6-(phenylethynyl)-pyridine (MPEP, Hoffmann-La Roche, Basel, Switzerland) were prepared in 0.1% Tween 80 and saline in a volume of 10 ml/kg and injected intraperitoneally (i.p.). in the experimental room 30 minutes prior to behavioural testing. Vehicle treatment consisted of 0.1% Tween 80 and saline and was injected i.p. 30 minutes prior to behavioural testing.

Open field (OF)

Behavioural testing took place between 9.00h and 13.00h, during the early activity phase of the animals. The OF apparatus consisted of a circular grey PVC arena, 80 cm in diameter and 33 cm high grey PVC walls *[Fig S1a]*. The arena was divided by red concentric circles in an outer zone, inner zone and centre area. Extra lines radiating out from the centre were placed on the floor as indicator for locomotion. One extra light bulb (red light) was fastened above the arena making the light intensity in the OF about 5-10 lux. Each animal was individually placed in the OF, always starting from the same position. After each trial the OF was carefully cleaned with tap water and a towel. Behaviour was directly scored by a trained observer using the program Observer 5.0 (Noldus Technology, The Netherlands) for 30 minutes after which the animals were placed back in their home cage. The following behaviours assigned to different behavioural categories (Ohl et al., 2001a) were scored in the OF: *a*) avoidance behaviour: the latency until the first centre entry (latency centre), the total time spent in the centre (centre duration) and the number of centre entries (centre entries); *b*) risk assessment: the number of stretched attends (stretched attends) *c*) locomotion: the number of line crossings (line crossings), the total time spent immobile (immobility duration) and the latency until the first immobility event (latency immobility); *d*) general exploration: the number of rearings (rearings); *e*) arousal/de-arousal: the total time spent grooming (grooming duration), the latency until the first self-grooming event (latency grooming) and the total number of defecations (defecations). All these parameters (except the latencies and the total number of defecations) were evaluated in time intervals of 5 minutes each in order to test for intrasession habituation.

Elevated plus maze (EPM)

Behavioural testing in the EPM took place between 9.00h and 13.00h during the early activity phase of the animals. The EPM comprised two open arms (28 x 6 x 16 cm) and two closed arms(28 x 6 x 16 cm) that extended from a central platform (6 x 6 cm). The apparatus was constructed from grey PVC (grey floor, grey arms) and elevated to a height of 84 cm above floor level. The apparatus was indirectly lit by a small light bulb hanging above the maze (red light, 5-10 lux). Each animal was individually placed on the central platform facing a closed arm and behaviour was directly scored by a trained observer for 5 minutes using the program Observer 5.0. After each trial the animal was transferred back to its home cage and the maze was carefully cleaned with tap water and a towel. The following behaviours were scored and assigned to different behavioural categories; *a*) avoidance behaviour: the total time spent on the open arms (time open arms), the number of open arm entries (open arm entry); *b*) risk assessment: the number of stretched attends (stretched attends); *c*) locomotion: the total number of arm entries (total arm entries), the total time spent immobile (immobility duration) and the latency until the first immobility event (latency immobility); *d*) general exploration: the total number of rearings (rearings), the total number of head dippings (head dips) and the total number of end explorations (end explorations); *e*) arousal/de-arousal: the total time spent grooming (grooming duration) and the latency to the first self-grooming event (latency grooming). These parameters were measured on two consecutive days to test for intersession sensitisation.

Object recognition test (ORT)

Behavioural testing in the ORT took place between 9.00h and 13.00h during the early activity phase of the animals. The test apparatus was a Eurostandard Type II L cage (size: 36.5 x 21 x 14 cm; Techniplast, Buguggiate, Italy) without any bedding and equally divided in 6 squares by black lines on the floor *[Fig S1b]*. Light conditions were the same as in the experimental room, red light with an illumination intensity of approximately 5 lux. Two different objects were used that differed in colour, material and shape (nut and die). Both objects were considered too heavy to be displaced by the animals.
All animals were allowed to familiarize with one of the two objects for 24h in their home cage (randomized) one day before the ORT. Both objects were always placed in the same corner of the apparatus, either one was positioned at the same distance from the wall.
For testing, the animals were individually placed in the apparatus always in the same corner opposite of the objects. After each trial, the animals were transferred back to their home cage and the objects and the apparatus were carefully cleaned with tap water

and a damp towel. The duration of the ORT was 10 minutes, during this period the behaviour was scored by a trained observer using the program Observer 5.0. The following behaviours assigned to different behavioural categories were scored in the ORT; *a*) object memory: the latency until the first exploration of the novel and the familiar object (latency novel/familiar object); the percentage of time spent exploring the familiar and novel objects (time novel/familiar object); the discrimination index (DI) was considered an index of discrimination between the novel and familiar objects and calculated as followed; (total exploration time novel object –total exploration time familiar object)/ total exploration time novel object + total exploration time familiar object (based on (Sik et al., 2003); *b*) risk assessment: the number of stretched attends (stretched attends); *c*) locomotion: the number of line crossings (line crossings), the total time spent immobile (immobility duration) and the latency until the first immobility (latency immobility); *d*) general exploration: the number of rearings (rearings); *e*) arousal or de-arousal: the total time spent self-grooming (grooming duration) and the latency until the first self-grooming event (latency grooming).

Experimental procedures

Open field: in the OF test a dose response curve was made. A total of 56 mice per strain was used and randomly assigned to 3 different dose groups per compound and one vehicle group (n=8 per group). Diazepam (1, 3 or 5 mg/kg), MPEP (3, 10 or 30 mg/kg) or vehicle were i.p. injected 30 minutes before behavioural testing.
Thirty minutes after behavioural testing a blood sample was taken and 120 minutes after testing the animals were decapitated and brains were removed.
Elevated plus maze: based upon the results found in the OF test, we chose one dose of each compound to induce a minimum behavioural effect without causing sedation. A total of 30 mice per strain was used (n=10 per group). On day 1, the animals were placed in the EPM without any pharmacological treatment and were allowed to explore the apparatus for 5 minutes (Trial 1). On day 2, the animals were treated with diazepam (1 mg/kg), MPEP (10 mg/kg) or vehicle 30 minutes before behavioural testing and allowed to explore the EPM for another 5 minutes (Trial 2). Thirty minutes after behavioural testing a blood sample was taken and 120 minutes after testing the animals were decapitated and brains were removed.
Object recognition test: a total of 27 mice per strain was used (n=9 per group). On day 1, the familiar object was placed in the home cage. On day 2, the animals were treated with Diazepam (1 mg/kg), MPEP (10 mg/kg) or vehicle 30 minutes before behavioural testing. Thirty minutes after the ORT, a blood sample was taken and 120 minutes after testing the animals were decapitated and brains were removed.

Corticosterone

In all experiments basal blood samples (basal) were collected 13 days after arrival at our facilities and four days before the start of the experimental period for plasma corticosterone (CORT) determination. Thirty minutes after behavioural testing a second blood sample was taken (non-basal). For blood sampling, mice were transported individually in their home cage to an adjacent laboratory (in order not to disturb circadian rhythm of the mice, the hallway and rooms were under red light conditions). By using tail vain incision a small blood sample was collected (±50µl) and stored in prechilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Blood samples were centrifuged (10 min at 12000rpm, 4°C) and stored at -20°C until measurement. CORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

c-Fos immunohistochemistry and image quantification

Two hours after behavioural testing, the animals were decapitated. Brains from the OF and EPM (vehicle, 1 mg/kg diazepam and 10 mg/kg MPEP, n=8 per group) were processed for c-Fos immunohistochemistry. Brains were removed and frozen in liquid (-80°C) 2-methylbutane which was cooled with dry ice and stored at -80°C. Coronal sections were cut (20 µm) and mounted on Menzel SuperFrost Plus slides (Menzel GmbH & Co, Braunschweig, Germany) and stored at -20 °C.

The sections were processed for c-Fos immunohistochemistry as described previously (Salomons et al., 2010c), dilution with a polyclonal primary antibody (1:1000, SC-52 Santa Cruz Biotechnology, Santa Cruz, USA), and a donkey-anti-rabbit IgG Biotin SP conjugated secondary antibody (1:400, Jackson ImmunoResearch Laboratories, Inc USA). Cells containing a nuclear brown-black reaction product were considered as c-Fos positive cells and counted in several brain areas which are known to be involved in anxiety (Arzt and Holsboer, 2006; Muigg et al., 2007; Nguyen et al., 2006): medial prefrontal cortex (prelimbic, PreL), lateral septum (dorsal, LSD; intermediary, LSI; ventral, LSV), bed nucleus of the stria terminalis (medial anterior, BSTMA; lateral posterior, BSTLP; medial ventral, BSTMV), dentate gyrus (granular layer, DG), paraventricular nucleus (PVN), dorsal medial hypothalamus (DMH), amygdala (basolateral nucleus, BLA; central nucleus CeA) and the periaqueductal gray (dorsolateral, dlPAG; dorsomedial, dmPAG; lateral, lPAG; ventrolateral,vl PAG). The anatomical localisation was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas (Franklin and Paxinos, 1997).

For each region at least two overt landmarks were used.
For quantitative analysis of c-Fos positive cells, the program Leica Qwin (image processing and analysis software, Cambridge, United Kingdom) was used. Left and right hemispheres were analysed for stained neurons per mm2 and calculated for one section separately and averaged for each animal.

Statistics

Statistical analyses were performed using the software program SPSS for Windows (version 15.0.1; SPSS Inc., IL, USA).
Continuous data (CORT, latency and relative duration of behavioural parameters were summarized as means with standard error of the mean (SEM), whereas discrete data on the ordinal scale (total number of behavioural parameters) were represented as medians with the interquartile range (IQR). The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data. Group analyses using the Kolmogorov-Smirnov one sample test revealed a non-parametric distribution of several continuous parameters. These parameters, as well as the total number of behavioural parameters, were rank transformed (Conover and Iman, 1982).
Results from the OF and the EPM were analysed using a repeated measures ANOVA with time interval (or trial for the EPM experiment) as within factor and treatment and strain as between factor, Huyn-Feldt adjustment was applied. Results from the ORT and c-Fos positive cells were analysed by a two-way ANOVA using strain and treatment as main factors. Survival analysis was not needed because no censored data were included. Post hoc analyses for all experiments were done using an unpaired Student-t test for continuous data and the Mann-Whitney U test for discrete data.
For ANOVA analyses, a probability value less than 0.05 was considered to be statistically significant. For post hoc analyses the Dunn-Sidak correction ($\alpha = 1 - 0.951/q$, q = number of comparisons) was used to correct for the number of comparisons.

Results

Open field

In [Tables S1] (diazepam) and [S2] (MPEP) a general overview of behavioural results can be found.

Avoidance behaviour: in general, vehicle-treated BALB/c mice initially showed more avoidance behaviour towards the unprotected centre of the OF compared to vehicle-treated 129P3 mice.
A significant time interval effect was found ($F(3,28) = 8.834$, $P<0.001$). Post hoc testing revealed only a significant increase in centre entries in vehicle-treated BALB/c mice ($P<0.001$), while no significant difference in centre entries occurred in the course of the testing trial in 129P3 mice. Diazepam dose dependently decreased

the latency to enter the centre (F (3,64) = 3.678, P<0.001, *[Fig 1a]*) and increased the number of centre entries (F (5,1354) = 16.027, P<0.001) in BALB/c mice compared to vehicle-treated BALB/c mice. 10 mg/kg MPEP increased the latency to enter the centre area in BALB/c mice [*Fig 1b]*, though the total time spent in the centre increased. 129P3 mice showed a lower latency to enter the centre *[Fig 1a]* and more time spent in the centre after 1 mg/kg of diazepam treatment (F (3,28) = 4.924, P<0.05) compared to vehicle-treated 129P3 mice. The higher doses diazepam (3 and 5 mg/kg) increased the latency in 129P3 mice and decreased the time spent in the centre area (F (3,28) = 8.834, P<0.05). All doses of MPEP treatment in 129P3 mice increased the number of centre entries (F (3,28) = 3.941, P<0.05) compared to vehicle treatment, though no effect of MPEP was found on the total time spent in the centre. The latency to enter the centre area decreased after application of 10 and 30 mg/kg MPEP in 129P3 mice *[Fig 1b]*. The total number of centre entries was higher after treatment with 3 and 30 mg/kg MPEP compared to vehicle treatment in 129P3 mice. A significant time interval effect (F (5,65) = 6.826, P<0.001) was found after all dosages of MPEP treatment and both strains showed an increase in the time spent in the centre during the experimental period.

Fig. 1 a+b

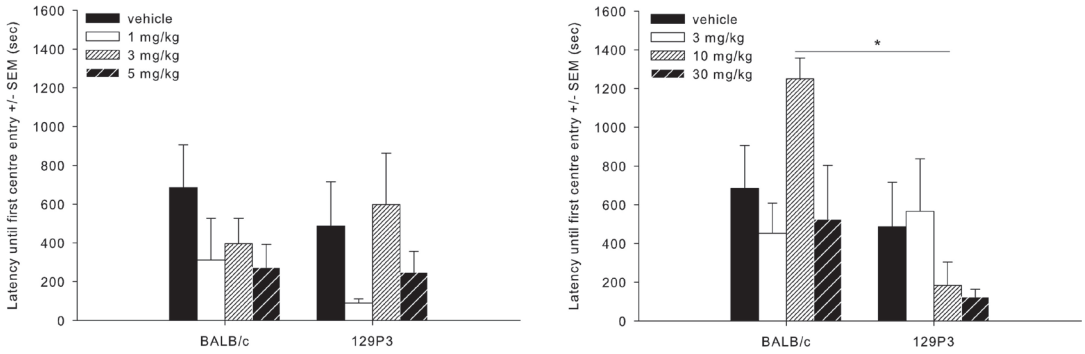


Figure 1: Mean latency until first centre entry (± SEM) in the OF after diazepam (left) or MPEP (right) treatment. * = P<0.0057.

Risk assessment: vehicle-treated BALB/c and 129P3 mice showed a significant decrease in the number of stretched attends during the experimental period (F (5,70) = 17.431, P<0.001). All dosages of diazepam treatment decreased the number of stretched attends in the first time interval compared to vehicle treatment in both strains (F (5,135) = 48.021, P<0.001) and were found to decrease the number of stretched attends during the experimental period. MPEP treatment also decreased the number of stretched attends during the experimental period in both strains (F (5,70) = 37.120, P<0.001). Compared to vehicle treatment, 10 and 30 mg/kg MPEP decreased the number of stretched attends in the first time interval

only in 129P3 mice, no effects of all dosages of MPEP were found in BALB/c mice.

Locomotion: after vehicle treatment, an increase in the number of line crossings was observed during the experimental period in BALB/c mice ($F(5,70) = 2.394$, $P<0.05$), though this was not observed in 129P3 mice. In general after all treatments, BALB/c mice showed more line crossings than 129P3 mice. All dosages of diazepam significantly increased the number of line crossings in the first time interval compared to vehicle treatment in BALB/c mice ($F(5,70) = 8.135$, $P<0.05$). Only the lowest dose of diazepam increased the number of line crossings in 129P3 mice, the higher doses (3 and 5 mg/kg) significantly decreased the number of line crossings in the first time interval ($P<0.001$). All dosages of MPEP treatment increased the number of line crossings during the experimental period in BALB/c mice ($F(5,70) = 6.234$, $P<0.05$), though in the first time interval there is no significant difference from vehicle treatment. The highest dose of MPEP (30 mg/kg) increased the number of line crossings in 129P3 mice compared to vehicle- treated animals in the first time interval, while the other dosages (10 and 30 mg/kg) did not affect the number of line crossings during the experimental period.

No effects were found on immobility duration or latency until the first immobility event in vehicle-treated animals of either strain during the experimental period. None of the doses of diazepam had effects on immobility duration in BALB/c mice, though the latency until the first immobility event significantly increased after 1 mg/kg diazepam application and decreased after 3 and 5 mg/kg ($F(5,70) = 4.294$, $P<0.05$). During the whole experimental period, 3 and 5 mg/kg diazepam treatment significantly increased the time spent immobile in 129P3 mice compared to vehicle-treated animals and BALB/c mice ($F(1,14) = 11.633$, $P<0.001$). Additionally, 129P3 mice seemed to show a dose dependent decrease in the latency until the first immobility event after diazepam treatment. None of the MPEP doses tested had effects on immobility parameters in BALB/c mice. In 129P3 mice, 10 mg/kg MPEP significantly increased the latency until the first immobility ($F(5,70) = 5.314$ $P<0.001$) and decreased the time spent immobile ($F(5,70) = 3.373$, $P<0.05$).

General exploration: vehicle-treated animals of both strains showed an increase in the number of rearings during the experimental period ($F(5,70) = 21.226$, $P<0.001$). 1 mg/kg diazepam increased the number of rearings in the first time interval in both strains compared to vehicle treatment. In contrast, 3 and 5 mg/kg decreased the number of rearings in the first time interval in both strains ($F(5,70) = 8.326$, $P<0.05$). All three dosages of MPEP increased the number of rearings during the experimental period in both strains ($F(5,70) = 16.363$, $P<0.001$), though no significant effects were found on the total number of rearings in the first time interval compared to vehicle-treated animals.

Arousal/de-arousal: no significant effects were found on arousal-related parameters in either strain after vehicle treatment.
Grooming duration in BALB/c mice increased during the experimental period under treatment with 5 mg/kg diazepam compared to vehicle-treated treatment ($F(5,70) = 12.086$, $P<0.05$). In 129P3 mice, all dosages of diazepam significantly increased the time spent grooming ($F(5,70) = 6.033$, $P<0.001$). In BALB/c mice, 3 and 10 mg/kg MPEP increased the time spent grooming in the first time interval compared to vehicle-treatment. In contrast, 30 mg/kg seemed to decrease the time spent grooming and significantly increased the latency until the first grooming event in BALB/c mice ($P=0.002$). No significant effects on grooming behaviours were observed in 129P3 mice after MPEP treatment.
No significant strain differences were observed in the number of defecations after vehicle treatment. Every dosage of diazepam decreased the number of defecations in BALB/c mice ($F(5,70) = 6.472$, $P<0.05$). In 129P3 mice only 3 and 5 mg/kg decreased the number of defecations ($P<0.05$). After 30 mg/kg MPEP treatment, 129P3 mice showed a lower number of defecations than BALB/c mice ($P<0.05$), though no significant difference with vehicle-treated animals were observed.

Elevated plus maze

In *[Table S3]* a general overview of behavioural results from the EPM can be found.
Avoidance behaviour: vehicle-treated animals in Trial 2 showed a decrease in the percentage of time spent on the open arm ($F(1,18) = 6.215$, $P<0.05$, *[Fig 2]*) and less open arm entries were observed ($F(1,18) = 11.551$, $P<0.05$) in both strains compared to the first trial. Diazepam further decreased the percentage of time on the open arm in both strains in the second trial ($F(1,18) = 12.254$, $P<0.001$) and a decrease in the number of open arm entries was observed ($F(1,18) = 6.215$, $P<0.05$) compared to Trial 1. Additionally, BALB/c mice spent significantly less time on the open arm compared to 129P3 mice after diazepam treatment ($F(1,19) = 7.508$, $P<0.001$). After MPEP treatment both strains showed significantly less time on the open arm in the second trial compared to the first trial ($F(1,19) = 6.209$, $P<0.001$). Strain effects were observed in the number of open arm entries after MPEP treatment ($F(1,19) = 32.279$, $P<0.001$). Post hoc analyses revealed that untreated 129P3 mice made more open arm entries in trial 1 compared to untreated BALB/c mice.
Risk assessment: vehicle-treated animals showed a significant decrease in the number of stretched attends in the second trial compared to the first trial in both strains ($F(1,19) = 19.202$, $P<0.001$). After diazepam and MPEP treatment a significant reduction in the number of stretched attends was also observed ($F(1,19) = 13.110$, $P<0.001$, $F(1,19) = 10.349$, $P<0.001$) in both strains, though no

significant difference with vehicle-treated animals was found. Locomotion: significant trial ($F(1,19) = 10.427$, $P<0.001$) and strain ($F(1,19) = 4.716$, $P<0.001$) effects were found for the total number of arm entries after vehicle treatment. Both strains showed less total arm entries on the second trial compared to the first trial. Moreover, BALB/c mice appeared to show less total arm entries than 129P3 mice. Diazepam treatment in the second trial did not have an effect in BALB/c mice. 129P3 mice showed a decrease in the total number of arm entries after diazepam treatment ($F(1,19) = 8.450$, $P<0.001$) compared to the first trial, though this was not significantly different from vehicle-treated 129P3 mice. MPEP treatment significantly decreased the number of total arm entries in the second trial 129P3 mice ($F(1,19) = 26.784$ $P<0.001$), though post hoc analyses revealed no effect of MPEP in BALB/c mice.

Fig. 2

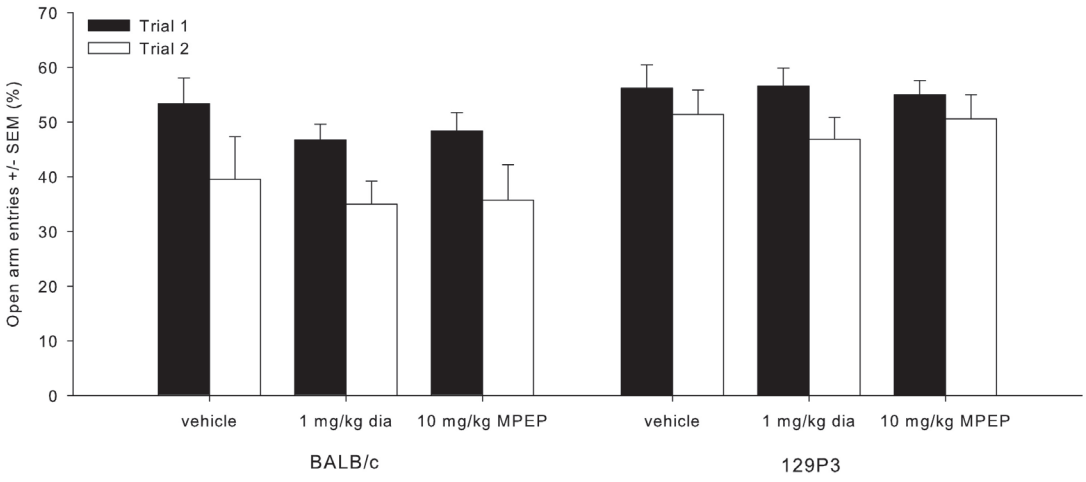


Figure 2: Mean percentage open arm entries (± SEM) in the EPM after vehicle, diazepam or MPEP treatment. Data are displayed for trial 1 and trial 2 in BALB/c (left) and 129P3 (right) mice.

Vehicle-treated BALB/c mice spent more time being immobile in the second trial compared to the first trial. No significant effects were found in 129P3 mice. Diazepam treatment increased the time spent immobile in the second trial compared to the first trial in both strains ($F(1,19) = 8.373$, $P<0.001$). A significant trial effect on immobility duration was found after MPEP treatment ($F(1,19) = 6.672$, $P<0.001$). Although, in 129P3 mice, MPEP treatment increased immobility duration while in BALB/c mice immobility duration decreased. General exploration: vehicle-treated animals showed a decrease in the total number of rearings ($F(1,19) = 5.935$, $P<0.001$) and head dips ($F(1,19) = 43.558$, $P<0.001$) in the second trial compared to the first untreated trial in both strains. In trial 1, vehicle treated BALB/c mice significantly displayed more rearings than vehicle-treated

129P3 mice (P=0.003). Diazepam treatment increased the number of rearings between the trials in BALB/c mice, though diazepam had no effect on rearings in 129P3 mice ($F(1,19) = 10.565$, $P<0.001$). Diazepam had no effect on the number of head dips in BALB/c mice, though in 129P3 mice, head dips significantly decreased in the second trial ($F(1,19) = 19.967$, $P<0.001$). MPEP treatment had no significant effect on the number of rearings and head dips in both strains. No significant effects of treatment were found on the number of end explorations in both strains, since they were hardly displayed.
Arousal/de-arousal: no differences were observed in grooming duration in vehicle-treated animals of both strains.
Diazepam ($F(1,19) = 4.631$, $P<0.001$) and MPEP ($F(1,19) = 17.033$, $P<0.001$) treated animals showed increased grooming duration between trials in both strains. Post hoc analyses revealed a higher grooming duration in trial 2 compared to trial 1 in MPEP-treated BALB/c mice (P=0.008), though no significant differences were found with vehicle treated animals.
No significant differences were found in the number of defecations between trials in vehicle-treated animals of both strains.
After diazepam treatment, significant strain differences were found ($F(1,19) = 34.546$, $P<0.001$). Post hoc analyses showed that diazepam-treated BALB/c mice defecated more than diazepam-treated 129P3 mice (P=0.001, P=0.005 resp). Significant trial ($F(1,19) = 18.833$, $P<0.001$) and strain ($F(1,19) = 12.149$, $P<0.001$) effects were found after MPEP treatment. Post hoc analyses revealed that MPEP treated BALB/c mice defecated more than MPEP-treated 129P3 mice (P=0.000).

Object recognition test

[Table S4] summarizes behavioural results from the ORT.
Object memory: the discrimination index showed a significant strain effect ($F(1,44) = 6.718$, $P<0.013$). Post hoc testing revealed a higher discrimination index in BALB/c mice compared to 129P3 mice after vehicle treatment *[Fig 3]*. No treatment effects on the discrimination index were found. Additionally, we performed a one-sample t-test to investigate whether the discrimination index differed significantly from zero (i.e. no discrimination between novel and familiar object). This revealed that vehicle- and MPEP-treated BALB/c mice showed a positive discrimination index ($t = 4.057$, $P=0.005$, $t=4.436$, $P=0.003$ resp., *[Fig 3]*). No discrimination difference was observed after diazepam treatment. In vehicle-, diazepam- and MPEP-treated 129P3 mice, no significant positive discrimination index was found. MPEP treatment seemed to slightly attenuate this, although it did not reach significance. No significant strain or treatment effects were found for the latency to explore the novel or the familiar object.
Risk assessment: significant treatment effects were found for the number of stretched attends ($F(2,44) = 4.316$ $P<0.013$).

After post hoc testing, a significantly higher number of stretched attends in MPEP-treated BALB/c mice compared to vehicle-treated (P=0.002) and diazepam-treated (P=0.001) BALB/c mice was found. No effects of treatment on the number of stretched attends in 129P3 mice were found.

Locomotion: strain differences were observed in the total number of line crossings ($F(1,44) = 57.097$, $P<0.001$). Vehicle-treated BALB/c mice showed more line crossings than vehicle-treated 129P3 mice (P=0.016). Additionally, diazepam-treated (P=0.005) and MPEP-treated (P=0.010) BALB/c mice showed more line crossings than 129P3 mice. The latency to the first immobility event ($F(1,44) = 6.125$, $P<0.004$) was higher and immobility duration ($F(1,44) = 30.683$, $P<0.001$) was lower in vehicle-, diazepam- and MPEP- treated BALB/c mice compared to 129P3 mice.

General exploration: significant strain differences were found in the number of rearings ($F(1,44) = 69.815$, $P<0.001$). Vehicle-treated (P=0.023), diazepam-treated (P=0.000) and MPEP-treated (P=0.004) BALB/c mice, showed a higher number of rearings compared to 129P3 mice.

Arousal/de-arousal: treatment effects were found for the latency until the first self-grooming event ($F(1,44) = 6.834$, $P<0.013$). Vehicle–treated BALB/c mice showed a higher latency until the first self-grooming event compared to diazepam-treated BALB/c mice. Diazepam-treated 129P3 mice showed a higher latency until the first self-grooming event compared to MPEP-treated 129P3 mice, though no difference with vehicle-treated animals was observed. No significant treatment or strain effects were found on grooming duration.

Fig. 3

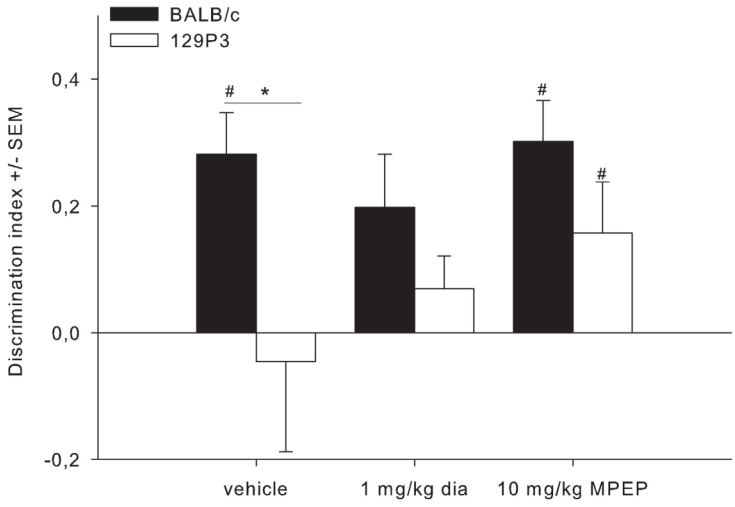


Figure 3: Discrimination index (± SEM) after vehicle, diazepam or MPEP treatment in the ORT. * = P<0.0057, # = significantly different from zero (P<0.0057).

CORT

In *[Table S5]* a general overview of CORT results from the OF, EPM and ORT can be found.

OF: Significant differences between basal and non-basal CORT levels were observed in both strains after vehicle treatment ($F(1,14) = 25.780$, $P<0.001$, [*Fig 4/5]*). Post hoc analysis revealed a significantly higher non-basal value for both BALB/c and 129P3 mice ($P<0.00512$) in vehicle-treated animals. Diazepam treatment in BALB/c mice significantly decreased non-basal CORT levels compared to vehicle treatment *[Fig 4]*. In 129P3 mice, only 1 mg/kg diazepam decreased CORT levels as compared to vehicle-treated animals and no difference between basal and non-basal CORT levels were observed. In contrast, 3 and 5 mg/kg diazepam significantly increased non-basal CORT levels compared to basal levels ($P<0.00512$, *[Fig 4]*).

No significant difference between MPEP and vehicle treatment on non-basal CORT levels were observed in either strain *[Fig 5]*.

Basal CORT levels compared to non-basal levels after every dosage of MPEP was found to show a significant difference (3 mg/kg: $F(1,11) = 6.198$, $P<0.05$; 10 mg/kg: $F(1,11) = 7.840$, $P<0.05$; 30 mg/kg: $F(1,11) = 18.019$, $P<0.05$). Post hoc testing revealed a higher non-basal CORT level compared to basal levels in 10 mg/kg and 30 mg/kg MPEP treated BALB/c mice *[Fig 5]*.

EPM: Non-basal CORT levels were significantly increased compared to basal levels in vehicle treated animals of both strains ($F(1,15) = 45.457$, $P<0.001$). Diazepam significantly reduced the non-basal CORT level in BALB/c mice compared to non-basal values of vehicle-treated animals ($P=0.000$). No significant effects were found in 129P3 mice after diazepam treatment. MPEP treatment decreased non-basal CORT levels in BALB/c mice compared to vehicle treatment ($P=0.004$). In MPEP-treated 129P3 mice, non-basal CORT levels were significantly increased compared to basal levels ($P=0.003$) and compared to MPEP-treated BALB/c mice ($P=0.004$).

ORT: Significant strain ($F(1,23) = 23.444$, $P<0.001$) and treatment effects $F(1,33) = 35.552$, $P<0.001$) were observed in CORT levels before and after ORT testing. 129P3 mice showed a significantly higher basal CORT level than BALB/c mice in all treatment groups. Post hoc testing revealed a significantly higher CORT level in BALB/c mice after ORT testing after vehicle ($t = 4.236$, $P<0.001$) and diazepam ($t = 4.328$, $P=0.005$. In vehicle-treated 129P3 mice, a significantly higher CORT level was observed after ORT testing ($t = 4.667$, $P=0.002$). After diazepam and MPEP treatment no differences were observed between basal and non-basal CORT levels in 129P3 mice.

Fig. 4

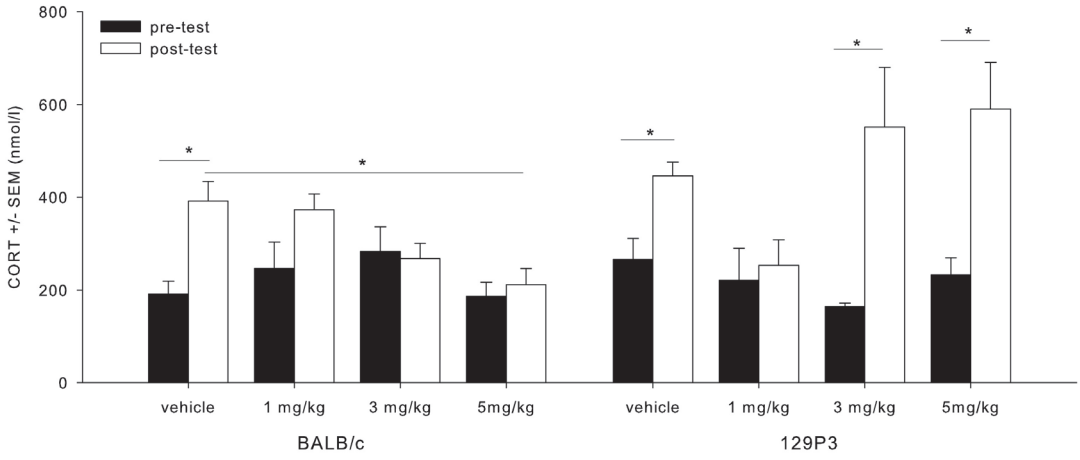


Figure 4: Mean (± SEM) CORT levels after diazepam treatment. CORT data are displayed before (basal) and after (non-basal) behavioural testing in the OF for BALB/c mice (left) and 129P3 mice (right). * = P<0.0057.

Fig. 5

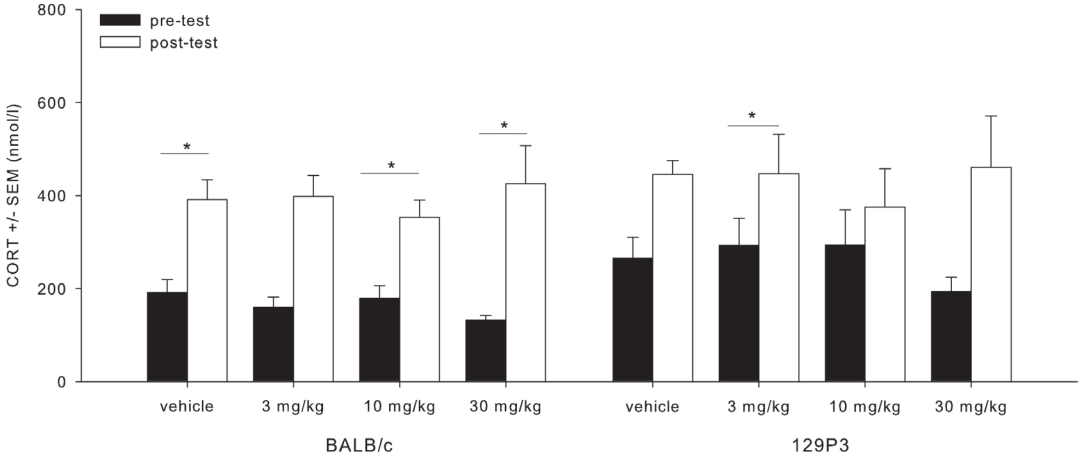


Figure 5: Mean (± SEM) CORT levels after MPEP treatment. CORT data are displayed before (basal) and after (non-basal) behavioural testing in the OF for BALB/c mice (left) and 129P3 mice (right). * = P<0.0057.

c-Fos immunohistochemistry

Only brains from the OF and EPM (vehicle, 1 mg/kg diazepam and 10 mg/kg MPEP, n=8 per group) were processed for c-Fos immunohistochemistry.

OF *[see Table S6]*: Significant strain differences were observed in the PreL ($F(1,79) = 18.377$, $P<0.001$, *[Fig 6a]*), LSD ($F(1,79) = 15.999$, $P<0.001$), DG ($F(1,79) = 32.194$, $P<0.001$), PVN ($F(1,79) = 18.377$, $P<0.001$, *[Fig 6b]*), DMH ($F(1,79) = 8.588$, $P<0.002$) and the BLA ($F(1,79) = 10.287$, $P<0.002$). Post hoc testing revealed significantly higher c-Fos expression in vehicle-treated BALB/c mice in the PreL, LSD and DG ($P<0.0057$) compared to vehicle-treated 129P3 mice. After 1 mg/kg diazepam treatment, significantly higher c-Fos expression was found in the PreL, DG and BLA in BALB/c mice compared to 129P3 mice ($P<0.0057$). MPEP-treated BALB/c mice showed a significantly higher c-Fos expression in the PVN compared to 129P3 mice ($P<0.0057$).

Significant treatment effects were found in the PreL ($F(2,79) = 5.066$, $P<0.009$), LSV ($F(2,79) = 5.725$, $P<0.005$), BSTMA ($F(2,79) = 3.837$, $P<0.026$), BSTMV ($F(2,79) = 3.142$, $P<0.032$), DMH ($F(2,79) = 9.034$, $P<0.001$), BLA ($F(2,79) = 7.008$, $P<0.001$), lPAG- ($F(2,79) = 3.574$, $P<0.032$) and vlPAG ($F(2,79) = 8.827$, $P<0.002$).

Vehicle-treated BALB/c mice showed a higher c-Fos expression in the PreL, PVN, LSD, and LSV compared to diazepam-treated BALB/c mice ($P<0.0057$). Lower c-Fos expression was found after MPEP-treatment compared to vehicle treatment in BALB/c mice in the PreL, PVN, DMH, LSD, LSV, lPAG and vlPAG. Diazepam-treated 129P3 mice showed lower c-Fos expression in the PVN, BLA and DMH compared to vehicle-treated 129P3 mice. MPEP treatment in 129P3 mice induced lower c-Fos expression in the PVN, DMH, LSV, BLA and higher c-Fos expression in the PreL, DG, BSTMV and BSTMA compared to vehicle-treated 129P3 mice.

EPM *[see Table S7]*: Significant strain differences were observed in the PreL ($F(1,73) = 12.820$, $P<0.001$, Fig 7a), LSD ($F(1,73) = 8.861$, $P<0.004$), BSTL ($F(1,73) = 13.395$, $P<0.001$) and DG ($F(1,73) = 4.416$, $P<0.033$). Higher c-Fos expression in BALB/c mice was found after vehicle treatment in PreL, LSD and DG compared to vehicle-treated 129P3 mice. In contrast, diazepam-treated BALB/c mice showed a lower c-Fos expression in the PreL and BSTLP compared to diazepam-treated 129P3 mice. After MPEP treatment only in the BSTLP less c-Fos expression was observed in BALB/c mice compared to 129P3 mice.

Significant treatment effects were observed in the PreL ($F(2,73) = 10.887$, $P<0.001$), LSD ($F(2,73) = 12.904$, $P<0.001$), LSI ($F(2,73) = 12.582$, $P<0.001$), BSTMA ($F(2,73) = 4.433$, $P<0.016$), BSTMV ($F(2,73) = 5.470$, $P<0.006$), PVN ($F(2,73) = 8.364$, $P<0.001$, *[Fig 7b]*) and CeA ($F(2,73) = 6.142$, $P<0.004$). Diazepam and MPEP treatment in BALB/c mice decreased c-Fos expression in the PVN ($P<0.0057$)

compared to vehicle-treated BALB/c mice. Diazepam treatment increased c-Fos expression in the PreL compared to vehicle-treated BALB/c mice. In 129P3, MPEP and diazepam increased c-Fos activity in the PreL, LSD, and LSI compared to vehicle- treated 129P3 mice. After diazepam treatment in 129P3 mice also higher c-Fos activity was observed in the CeA (P<0.0057) compared to vehicle. MPEP-treated 129P3 mice showed more c-Fos expression in the BSTMV and lower c-Fos expression in the PVN compared to vehicle treatment.

Fig. 6 a+b

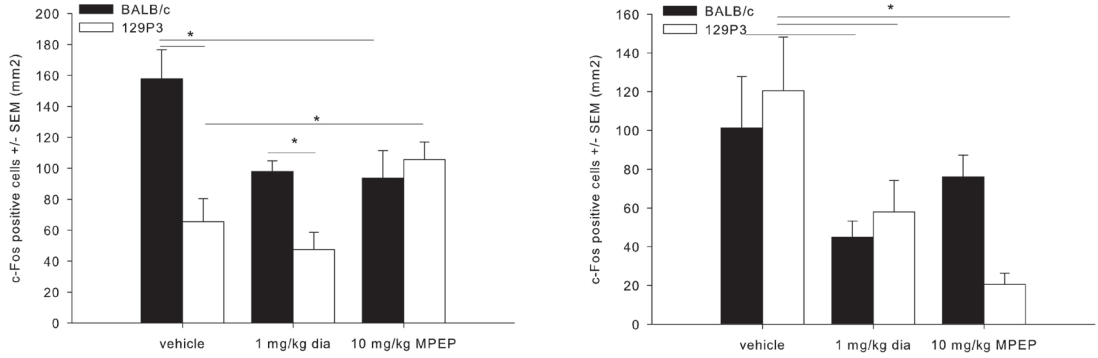


Figure 6: Mean number of c-Fos positive cells (± SEM) after pre-treatment with vehicle, 1 mg/kg diazepam or 10 mg/kg MPEP treatment in the OF. Data are displayed for the PreL (left) and the PVN (right). * P<0.0057.

Fig. 7 a+b

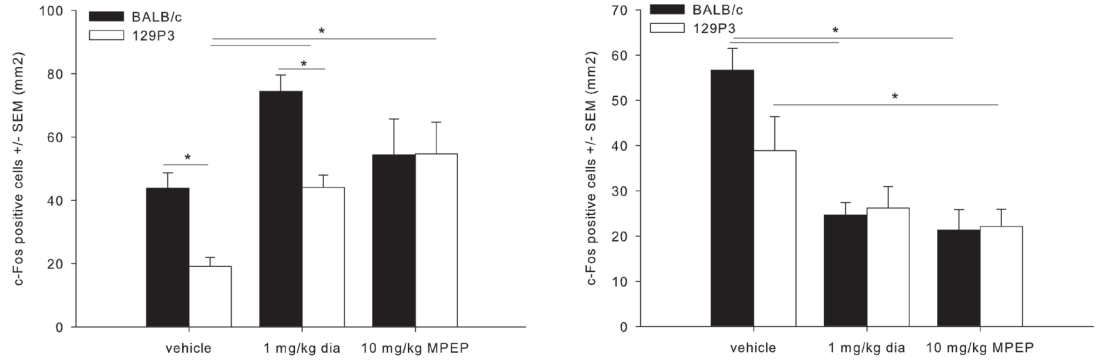


Figure 7: Mean number of c-Fos positive cells (± SEM) after pre-treatment with vehicle, 1 mg/kg diazepam or 10 mg/kg MPEP treatment in the EPM. Data are displayed for the PreL (left) and the PVN (right). *P<0.0057.

Discussion

Previous studies showed that 129P3 mice are characterized by impaired inter-session habituation when compared to BALB/c animals. This characteristic is accompanied by lower neural c-Fos expression after behavioural testing in brain areas involved in the integration of emotional and cognitive processes (Salomons et al., 2010b; Salomons et al., 2010c). From these results, we hypothesized that 129P3 mice may represent an interesting mouse model to investigate non-adaptive, i.e. pathological anxiety.

Results from the present study confirm that BALB/c mice are characterized by high initial anxiety, followed by a decrease in anxiety-related behaviour within one trial, i.e. intrasession habituation. This corresponds to findings from for example Tang (Tang et al., 2002) who revealed that BALB/c mice spent increased time in the centre of an open field during a 30 minutes trial.

These findings strengthen our conclusion that BALB/c mice are characterized by an initially high but adaptive, i.e. non-pathological anxiety profile. Notably, and in contrast to BALB/c mice, 129P3 individuals failed to show intra-session habituation. On the contrary, their avoidance behaviour increased within the test session, confirming previous results found by Tang (Tang and Sanford, 2005). Apparently, the non-adaptive habituation phenotype in 129P3 mice determines their behaviour between as well as within sessions in different test systems, suggesting that this lack of habituation is a general characteristic in this inbred strain.

The strain differences identified at the behavioural level are further extended by the results of the pharmacological treatment: while diazepam treatment acted as an anxiolytic in BALB/c mice, which is in accordance with previous studies (Griebel et al., 2000; Griebel et al., 1993; Ohl et al., 2001b), the same treatment induced sedation in 129P3 individuals. This difference in sensitivity towards diazepam treatment is likely to be due to strain characteristics in benzodiazepine receptor densities or sensitivity. BALB/c mice have been shown to exhibit a five-fold decrease in the density of benzodiazepine binding sites as compared to C57BL/6 mice (Robertson, 1979). While there are no reports on diazepam treatment in 129P3 mice, some pharmacological studies however have reported sedative effects of benzodiazepines in other 129 substrains.

For example Rodgers (Rodgers et al., 2002b) found sedation in 129S2 mice after chlordiazepoxide treatment and suggested that 129 mice have an abnormal benzodiazepine/GABAA receptor function.

The same might be true for 129P3 mice as well. It is of note that the higher doses (3 and 5 mg/kg) of diazepam not only produced sedation in 129P3 animals but increased their post-testing CORT levels as well when compared to both vehicle-treated 129P3 mice and diazepam-treated BALB/c mice. This effect suggests either an activating effect on the HPA axis (Mikkelsen et al., 2005), or an

emotionally aversive effect of the treatment-induced sedation in 129P3 animals (Makowska and Weary, 2009).
Notably, a different picture emerged after treatment with MPEP in the two strains. While behaviour in BALB/c remained almost unaffected by treatment with MPEP, 129P3 mice showed significantly reduced avoidance and risk assessment behaviour during OF testing.
Again, in contrast to diazepam MPEP had no sedative effects in either strain, which corresponds to the existing literature on MPEP (Ballard et al., 2005; Spooren et al., 2000), demonstrating that mGlu5 receptors antagonists seem to lack the side effects of benzodiazepine treatment (Busse et al., 2004). The anxiolytic properties of MPEP in the present study were complemented with a decrease in post-testing CORT levels at all MPEP doses in 129P3 mice, whereas only the lowest dose of MPEP resulted in a decrease of CORT levels in BALB/c mice.
It is of note that the lack of habituation found in 129P3 mice is not paralleled by a one trial sensitization in the EPM. While, consistent with previous findings (Espejo, 1997; Holmes and Rodgers, 1998; Holmes and Rodgers, 1999; Rodgers et al., 1997; Treit et al., 1993), undrugged BALB/c mice showed sensitization during the second trial, behavioural signs of sensitization in 129P3 mice remained non-significant. However, the increase in blood corticosterone levels after the second trial in 129P3 mice was more pronounced than in BALB/c animals, indicating a pronounced hormonal stress response in the 129P3 strain. Further, all visible effects of sensitization in BALB/c mice were ameliorated by pharmacological treatment with either diazepam or MPEP, while both the increased hormonal stress-response and the slightly increased immobility time in 129P3 animals remained unaffected by the same treatment. Thus, the effects of pharmacological treatment on EPM behaviour contrast the results found in OF testing. This contrast, however, is in line with literature which shows that EPM testing often produces contradictory results when compared to behavioural testing in for example the OF (Carola et al., 2002). Furthermore, it is unclear whether a behavioural profile characterized by a lack of habituation should result in increased EPM-sensitization at the behavioural level.
Regardless, the increased CORT-response in 129P3 mice indicates that re-testing in the EPM is highly stressful for these animals, irrespective of anxiolytic pre-treatment.
The strain-specific findings were further extended by the results of c-Fos expression in different brain areas after behavioural testing. Brain areas involved in the integration of emotional and cognitive processes, such as the PreL, BST and LS (Goldman-Rakic, 1995; Herman et al., 2005; Makino et al., 2002; Sheehan et al., 2004) were found to be more active after MPEP treatment in 129P3 mice, whereas the BLA and PVN, brain areas primarily involved in emotional processes (McNaughton and Corr, 2004; Singewald

et al., 2003), showed lower c-Fos activity as compared to vehicle-treated 129P3 mice. In contrast, diazepam treatment in BALB/c mice especially resulted in decreased c-Fos expression in emotionally related brain areas, while no effects were found on higher cortical anxiety-related brain areas. Notably, strain differences in the PreL, DG and LSD in vehicle treated animals could not be found in MPEP treated animals.

A likely explanation for the strains-specific diazepam or MPEP treatment effects, respectively, is that diazepam and MPEP exert their anxiolytic effects via different mechanisms (George et al., 2009). Given the fact that the two strains are further characterized by specific anxiety-phenotypes, distinct regulatory systems, underlying the specific phenotypes, may be suggested: diazepam is known to act inhibitory via the GABAergic system. In diazepam-treated BALB/c mice, we found c-Fos expression in the PVN, known to regulate HPA-axis activity, and the BLA, known to process anxiety-responses, to be reduced; these effects could not be shown in 129P3 mice.

The latter, in contrast, appeared to be sensitive for the mGlu5 receptor antagonist MPEP. mGlu5 receptors are highly expressed in several limbic structures such as the hippocampus, amygdala and septum (Romano et al., 1995; Shigemoto et al., 1993) and activation of these receptors leads to excitatory effects in the brain, i.e. an increase of glutamatergic transmission (Benquet et al., 2002; Gerber et al., 2007; Pintor et al., 2000). A controlled glutamatergic transmission is known to be critical for higher order mental processes, whereas excessive glutamatergic transmission can lead to impairment of normal neural processes and even cell death (Kemp and McKernan, 2002; Rothstein et al., 1993). Notably, it has been suggested that anxiety disorders may arise through excessive excitatory neurotransmission in response to stress (Simon and Gorman, 2006). For MPEP it has been shown that glutamate release is effectively reduced after acute treatment (Thomas et al., 2001). Interestingly, MPEP increased c-Fos expression in the PreL, BST and DG in 129P3 mice, when compared to vehicle treated animals, suggesting that cognitive processes regulated by the prefrontal cortex are affected in addition to primarily emotional processes.

From the present results as well as from our previous studies in 129P3 mice we conclude that the prelimbic cortex seems to play an important role in the phenotype of 129P3 mice. This is further supported by the notion that 129P3 mice display impaired fear extinction (Camp et al., 2009), a process which relies on a reciprocal connection between the prelimbic cortex and the amygdala (Quirk et al., 2006). The prelimbic cortex has been shown to act as the cognitive control system in emotional processing (Davidson, 2002; Goldman-Rakic, 1995). Given the fact that both vehicle and diazepam treated 129P3 individuals show a significantly lower neuronal activity of the prelimbic cortex than their BALB/c

counterparts, and that this strain difference is ameliorated by MPEP treatment, it may be hypothesized that it is primarily the cognitive control of their emotionality, which determines the behavioural profile of the 129P3 strain.

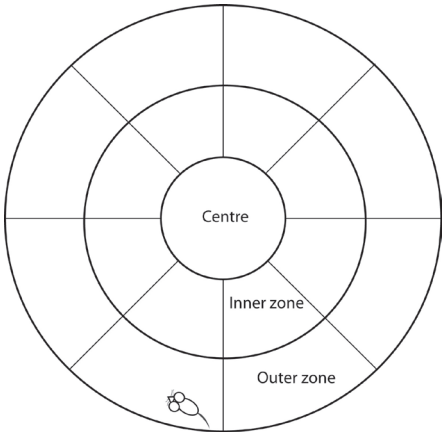
It has repeatedly been suggested that pathological, i.e. non-adaptive anxiety may primarily be a specific, but not general, cognitive dysfunction (Beuzen and Belzung, 1995; McNaughton, 1997).

To control for general cognitive functioning, we investigated the effects of the (putative) anxiolytic pre-treatment in a one trial object recognition test in both strains as well. As found earlier (Salomons et al., 2010c), BALB/c mice revealed discrimination between the novel and familiar object. The fact that after diazepam treatment no positive discrimination index was found in this strain is likely to be explained by diazepam induced amnesic effects, which have been extensively reported (Kuribara and Asahi, 1997; Tomaz et al., 1992). In contrast to our earlier findings, vehicle-treated 129P3 mice showed no positive discrimination index, a discrepancy which may be explained by differences in experimental design: in our previous study, but not in the present one, animals were habituated to the test environment, which might be of impact on exploration behaviour especially in 129P3 mice. Notably, MPEP treatment increased the discrimination index and a significant positive discrimination index was observed in 129P3 mice. This effect is especially interesting as in literature either no effects of MPEP are found on retrieval of object memory (Ballard et al., 2005; Barker et al., 2006) or even impaired object recognition (Christoffersen et al., 2008). Again, the apparent improvement of object recognition in this study in 129P3 mice after MPEP treatment in contrast to these other studies mentioned is likely to be explained by different setups.

However, the finding that MPEP affects cognitive processing in 129P3 animals underlines the suggestion that the behavioural profile of these animals may be the result of a dysfunctional integration of cognitive and emotional processes. Thus, the 129P3 mouse strain seems to be an interesting model to investigate the non-adaptive phenotype of these animals, which may arise through impaired neural processing between the prelimbic cortex and emotional brain areas regulated by glutamatergic neurotransmission.

Supplementary material

Fig. S1 a+b



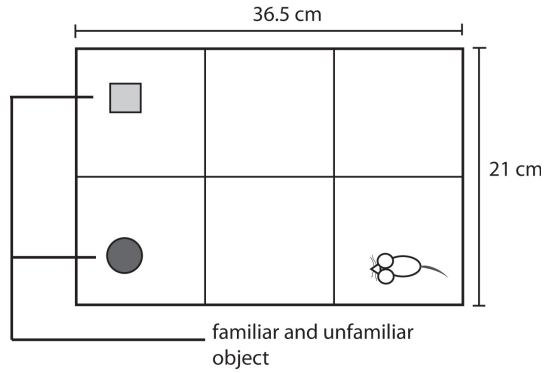


Figure S1: Schematic drawing of the OF *(a)* and the ORT *(b)*. *a)* Centre, inner and outer zone were divided by lines on the floor area. Extra lines were added to measure locomotor activity. *b)* After 24h familiarisation with one of the two objects, the familiar and an unfamiliar object were placed opposite the starting position.

Table S1: Overview of the behavioural parameters measured in the OF after diazepam or vehicle treatment in both strains. Data are presented for the first time interval (1) and the last time interval (6).

	strain	vehicle		1 mg/kg diazepam		3 mg/kg diazepam		5 mg/kg diazepam		Significance
Time interval		**1**	**6**	**1**	**6**	**1**	**6**	**1**	**6**	
Latency centre (sec)	BALB/c	684.9 ± 221.2	-	311.5 ± 215.0	-	395.9 ± 130.8	-	274.0 ± 119.0	-	Tr
	129P3	487.1 ± 228.5	-	89.6 ± 21.7	-	598.4 ± 265.3	-	248.1 ± 107.8	-	Tr
Centre duration (%)	BALB/c	0.8 ± 0.7	2.3 ± 0.6	1.8 ± 0.7	1.6 ± 0.6	0.9 ± 0.6	2.0 ± 0.5	0.5 ± 0.2	1.3 ± 0.4	T, T*S, S
	129P3	2.0 ± 0.9	3.1 ± 1.1	1.8 ± 0.6	1.9 ± 0.8	0.5 ± 0.3	0.2 ± 0.1	0.5 ± 0.2	9.5 ± 4.9	Tr, T, T*S, S
Centre entries (nr)	BALB/c	1 ± 2	7 ± 6	5 ± 6	5 ± 6	1 ± 3 a	7 ± 7 a. b	2 ± 3	3 ± 3	T, T*S, S
	129P3	3 ± 6	4 ± 4	3 ± 2	5 ± 1	1 ± 3	1 ± 1 b	2 ± 2	1 ± 2	Tr, T, T*S, S
Stretched attends (nr)	BALB/c	18 ± 22 agh	0 ± 0 a	5 ± 6 d	0 ± 1 d	5 ± 7 eg	0 ± 0 e	4 ± 2 fh	0 ± 0 f	Tr, T
	129P3	14 ± 35	0 ± 0	7 ± 6 b	0 ± 0 b	6 ± 7	0 ± 1	1 ± 3 c	0 ± 0 c	Tr, T
Line crossings (nr)	BALB/c	146 ± 153	230 ± 75	286 ± 104	287 ± 140	134 ± 89	363 ± 163 b	217 ± 82	240 ± 87 c	T, T*S, S
	129P3	105 ± 153	116 ± 86	181 ± 44	117 ± 73 a	53 ± 153	14 ± 52 b	41 ± 104	51 ± 48 ac	Tr, T, T*S, S
Immobility duration (%)	BALB/c	5.0 ± 4.3	0.5 ± 0.3	0.0 ± 0.0	0.4 ± 0.4	5.7 ± 2.9	4.1 ± 4.0	7.7 ± 5.0	4.2 ± 2.7	T, S
	129P3	0.3 ± 0.3	5.3 ± 4.9	0.6 ± 0.3	0.6 ± 0.4	30.1 ± 10.8	38.5 ± 12.1	32.6 ± 9.2	33.5 ± 11.5	Tr, T, S
Latency immobility	BALB/c	778.2 ± 240.9	-	1286.3 ± 34.7	-	300.9 ± 218.1	-	596.7 ± 231.2	-	Tr
	129P3	830.7 ± 212.6	-	600.7 ± 217.4	-	343.6 ± 222.9	-	141.5 ± 60.9	-	Tr
Rearings (nr)	BALB/c	2 ± 3 a	37 ± 36 a	6 ± 17	45 ± 43	1 ± 3 b	9 ± 21 b	1 ± 2 c	13 ± 15 c	T
	129P3	2 ± 9	16 ± 23	5 ± 6	31 ± 9	0 ± 0	2 ± 10	1 ± 1	2 ± 11	Tr, T
Grooming duration (%)	BALB/c	1.5 ± 0.5	2.4 ± 1.0	1.6 ± 1.3	0.1 ± 0.1 a	0.4 ± 0.2	6.7 ± 2.4	2.8 ± 1.3	8.2 ± 1.9 a	Tr, T, S
	129P3	1.6 ± 0.6	5.3 ± 2.2	0.0 ± 0.0	3.8 ± 3.2	0.3 ± 0.3	10.4 ± 3.3	2.9 ± 0.9	7.7 ± 2.5	Tr, T, S
Latency groom (sec)	BALB/c	372.9 ± 56.2	-	652.1 ± 135.7	-	521.6 ± 172.2	-	393.4 ± 83.6	-	ns
	129P3	443.5 ± 73.0	-	439.6 ± 41.0	-	754.7 ± 213.2	-	757.1 ± 181.6	-	ns
Defecations	BALB/c	13 ± 3	-	5 ± 7	-	4 ± 2	-	9 ± 5	-	S, Tr, S*Tr
	129P3	8 ± 2	-	9 ± 4	-	5 ± 4	-	4 ± 5	-	S, Tr, S*Tr

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR. Duration and ordinal data were analysed with repeated measures ANOVA with within factor: time interval (T) and between factors: strain (S) and treatment (Tr). The latency and defecation data were analysed using an univariate ANOVA with dependent variables: latency data and number of defecations resp. and fixed variables: strain and treatment (Tr). T*S = time interval × strain interaction, T*Tr = time interval × treatment interaction, S*Tr = strain × treatment interaction. Post hoc analysis was done using the non-paired Student t-test for continuous data and the Mann-Whitney U test for ordinal data. Values with the same letters in the same row (or two rows, when strain differences are found) are significantly different (P≤0.00512).
ns = non-significant.

Table S2: Overview of the behavioural parameters in the OF after MPEP or vehicle treatment in both strains. Data are presented for the first time interval (1) and the last time interval (6).

	strain	vehicle		3 mg/kg MPEP		10 mg/kg MPEP		30 mg/kg MPEP		Significance
Time interval		**1**	**6**	**1**	**6**	**1**	**6**	**1**	**6**	
Latency centre (sec)	BALB/c	684.9 ± 221.2	-	453.6 ± 154.9	-	1250.5 ± 106.1 a	-	525.5 ± 279.0	-	S
	129P3	487.1 ± 228.5	-	565.5 ± 272.0	-	184.5 ± 120.3 a	-	125.1 ± 39.4	-	S
Centre duration (%)	BALB/c	0.8 ± 0.7	2.3 ± 0.6	0.7 ± 0.4	3.0 ± 0.7	0.6 ± 0.4	1.4 ± 0.8	1.9 ± 0.9	3.2 ± 1.2	T, T*S, S, T*Tr
	129P3	2.0 ± 0.9	3.1 ± 1.1	1.4 ± 0.6	2.6 ± 1.6	2.1 ± 0.6	2.1 ± 0.5	1.1 ± 0.2	2.6 ± 0.5	T, T*S, S
Centre entries (nr)	BALB/c	1 ± 2	7 ± 6	0 ± 4 c	6 ± 2 c	0 ± 1	2 ± 6	3 ± 5	8 ± 4	T
	129P3	3 ± 6	4 ± 4	2 ± 3	4 ± 9	6 ± 4	5 ± 3	5 ± 4	9 ± 2	Tr, T
Stretched attends (nr)	BALB/c	18 ± 22 a	0 ± 0 a	26 ± 21 j	0 ± 0 j	23 ± 14 k	0 ± 7 k	16 ± 31	0 ± 5	T, T*S, S
	129P3	14 ± 35	0 ± 0	11 ± 20	0 ± 8	5 ± 10	0 ± 0	4 ± 3 i	0 ± 0 i	T, T*S, S
Line crossings (nr)	BALB/c	146 ± 153	230 ± 75	105 ± 68	192 ± 35	89 ± 115	174 ± 129	189 ± 127	221 ± 130	T, T*S, T*Tr
	129P3	105 ± 153	116 ± 86	140 ± 210	133 ± 96	199 ± 46	131 ± 63	204 ± 42 d	138 ± 52 d	T, T*S
Immobility duration (%)	BALB/c	5.0 ± 4.3	0.5 ± 0.3	1.8 ± 1.8	0.3 ± 0.3	0.0 ± 0.0	1.5 ± 1.1	0.2 ± 0.2	0.4 ± 0.2	ns
	129P3	0.3 ± 0.3	5.3 ± 4.9	5.6 ± 2.7	3.0 ± 1.9	0.0 ± 0.0	0.3 ± 0.3	0.4 ± 0.4	0.0 ± 0.0	Tr
Latency immobility	BALB/c	778.2 ± 240.9	-	1087.3 ± 251.2	-	1559.2 ± 164.8	-	832.9 ± 212.6	-	Tr
	129P3	830.7 ± 212.6	-	526.0 ± 206.0 a	-	1531.7 ± 175.7 a	-	1070.6 ± 248.7	-	Tr
Rearings (nr)	BALB/c	2 ± 3 a	37 ± 36 a	1 ± 2 f	52 ± 16 f	2 ± 4	12 ± 27	2 ± 14	30 ± 17	T, T*S, T*Tr
	129P3	2 ± 9	16 ± 23	5 ± 6	20 ± 43	4 ± 4 d	27 ± 22 d	6 ± 9 e	17 ± 16 e	T, T*S
Grooming duration (%)	BALB/c	1.5 ± 0.5	2.4 ± 1.0	2.8 ± 1.5	2.8 ± 1.0	9.7 ± 5.7	5.9 ± 2.5	1.0 ± 0.4	3.6 ± 1.3	Tr, T, S, T*Tr
	129P3	1.6 ± 0.6	5.3 ± 2.2	9.5 ± 3.3	4.3 ± 1.9	1.9 ± 0.6	5.5 ± 1.6	4.0 ± 1.2	10.2 ± 3.8	T, S
Latency groom (sec)	BALB/c	372.9 ± 56.2	-	479.5 ± 119.0	-	415.0 ± 47.0	-	414.7 ± 33.3 b	-	S*Tr
	129P3	443.5 ± 73.0	-	539.5 ± 44.9 a	-	448.0 ± 62.3	-	226.1 ± 38.4 ab	-	S*Tr
Defecations	BALB/c	13 ± 3	-	7 ± 4	-	10 ± 4	-	12 + 9	-	Sr
	129P3	8 ± 2	-	8 ± 7	-	8 ± 2	-	6 ± 3	-	S

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR. Duration and ordinal data were analysed with repeated measures ANOVA with within factor: time interval (T) and between factors: strain (S) and treatment (Tr). The latency and defecation data were analysed using an univariate ANOVA with dependent variables: latency data and number of defecations resp. and fixed variables: strain and treatment (Tr). T*S = time interval × strain interaction, T*D = time interval × dose interaction, S*Tr = strain × treatment interaction. Post hoc analysis was done using the non-paired Student t-test for continuous data and the Mann-Whitney U test for ordinal data. Values with the same letters in the same row (or two rows, when strain differences are found) are significantly different (P≤0.00512).
ns = non-significant.

Table S3: Overview of behavioural parameters in the EPM. Data are presented for the first (undrugged) trial (Trial 1) and the second trial (Trial 2, pre-treatment with vehicle, 1mg/kg diazepam or 10 mg/kg MPEP).

	strain	vehicle		1 mg/kg diazepam		10 mg/kg diazepam		Significance
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	
Time open arm (%)	BALB/c	53.3 ± 4.7	39.5 ± 7.8	46.7 ± 2.9	35.0 ± 4.2	48.4 ± 3.3	35.7 ± 6.5	T, S
	129P3	56.2 ± 4.3	51.4 ± 4.5	56.6 ± 3.3	46.9 ± 4.0	55.0 ± 2.6	50.6 ± 4.4	T, S
Open arm entries (nr)	BALB/c	16 ± 10	7 ± 14	16 ± 6	11 ± 9	11 ± 3 a	13 ± 7	T, T*S, S
	129P3	22 ± 9	15 ± 5	26 ± 8 b	13 ± 13 b	24 ± 8 a	18 ± 6	T, T*S, S
Stretched attends (nr)	BALB/c	35 ± 23	16 ± 13	25 ± 18	15 ± 20	33 ± 20	21 ± 17	T
	129P3	23 ± 6	14 ± 10	28 ± 14	17 ± 12	30 ± 15	25 ± 10	T
Total arm entries (nr)	BALB/c	28 ± 14	19 ± 12	35 ± 7 c	36 ± 23	24 ± 8 ac	30 ± 14	T, T*S, S, Tr
	129P3	35 ± 10	28 ± 9	43 ± 16	31 ± 19	45 ± 7 ab	33 ± 6 b	T, T*S, S
Immobility duration (%)	BALB/c	1.8 ± 1.3	18.1 ± 7.4	0.5 ± 0.3	2.3 ± 2.1	2.8 ± 1.7	0.9 ± 0.7	T, T*S
	129P3	1.2 ± 0.4	3.3 ± 1.7	0.6 ± 0.3	8.1 ± 2.4	0.3 ± 0.1	4.2 ± 2.2	T, T*S
Latency immobility	BALB/c	229.3 ± 39.3	152.4 ± 37.8	244.6 ± 28.4	182.4 ± 31.6	173.5 ± 44.1	235.3 ± 34.1	T
	129P3	248.8 ± 19.1	213.0 ± 37.0	287.4 ± 12.6	134.7 ± 34.6	223.2 ± 39.3	174.0 ± 27.6	T
Rearings (nr)	BALB/c	21 ± 10 a	7 ± 13	15 ± 19	20 ± 5	13 ± 8	15 ± 12	T, S
	129P3	6 ± 6 a	5 ± 6	11 ± 6	11 ± 5	14 ± 9	10 ± 4	T, S, Tr
Head dips (nr)	BALB/c	23 ± 13	7 ± 18	18 ± 8	16 ± 14	18 ± 12	12 ± 16	T
	129P3	20 ± 9 a	10 ± 4 a	24 ± 10 b	10 ± 12 b	21 ± 5	13 ± 13	T
End explorations (nr)	BALB/c	1 ± 4	0 ± 1	0 ± 4	0 ± 10	0 ± 2	0 ± 1	T*S
	129P3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 2	T*S, T*Tr
Grooming duration (%)	BALB/c	0.7 ± 0.3	1.5 ± 0.6	2.4 ± 1.1	4.7 ± 1.4	0.5 ± 0.4 a	3.8 ± 1.0 a	T
	129P3	0.8 ± 0.4	0.9 ± 0.4	0.5 ± 0.3	1.8 ± 1.0	0.4 ± 0.3	1.5 ± 0.7	T
Latency groom (sec)	BALB/c	269.0 ± 17.2	245.2 ± 24.5	224.9 ± 13.8	166.6 ± 28.8a	294.0 ± 4.6	193.4 ± 20.9	T, S
	129P3	286.7 ± 7.9	246.4 ± 27.3	293.4 ± 5.1	267.0 ± 19.8a	289.0 ± 8.2	269.7 ± 15.5	T, S
Defecations (nr)	BALB/c	6 ± 4 e	3 ± 3 f	8 ± 1 a	7 ± 5 b. f	9 ± 2 cde	4 ± 4 d	T, T*S, S, Tr
	129P3	4 ± 4	4 ± 2	4 ± 3 a	3 ± 3 b	4 ± 3 c	3 ± 3	T, T*S, S

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR. The data was analysed using repeated measures ANOVA with within factor: "Trial 1/trial 2" (T) and between factors: strain (S) and treatment (Tr). T*S = "Trial 1/trial 2" × strain interaction, T*Tr = "Trial 1/trial 2" × treatment interaction.
Post hoc analysis was done using the non-paired Student t-test for continuous data and the Mann-Whitney U test for ordinal data. Values with the same letters in the same row are significantly different (P≤ 0.00851).

Table S4: Overview of results from the object recognition test after pretreatment with vehicle, diazepam (1mg/kg) or MPEP (10 mg/kg).

	BALB/c			**129P3**			
	vehicle	**diazepam**	**MPEP**	**vehicle**	**diazepam**	**MPEP**	**Sig**
Discrimination Index (DI)	0.28 ± 0.06a	0.20 ± 0.08	0.31 ± 0.04	-0.05 ± 0.15a	0.06 ± 0.05	0.16 ± 0.08	S
Latency novel object [sec]	23.1 ± 4.4	21.2 ± 2.6	51.4 ± 8.1	18.0 ± 3.3	24.8 ± 5.2	28.5 ± 4.7	ns
Latency familiar object [sec]	23.5 ± 5.1	18.9 ± 2.4	43.7 ± 9.6	23.8 ± 5.4	31.4 ± 5.6	32.3 ± 6.8	ns
Time novel object [%]	7.1 ± 1.3	5.8 ± 1.2	5.5 ± 0.9	4.3 ± 0.9	5.9 ± 0.7	5.6 ± 0.6	ns
Time familiar object [%]	4.0 ± 0.7	4.1 ± 0.9	2.8 ± 0.4	4.1 ± 0.5	5.0 ± 0.4	4.1 ± 0.5	ns
Stretched attends [nr]	5.5 ± 4.5	6.0 ± 6.5	17.5 ± 12.5	11.0 ± 11.0	12.0 ± 5.5	5.0 ± 6.5	Tr
Line crossings [nr]	159.5 ± 68.0 a	176.0 ± 35.5b	149.5 ± 38.5c	91.0 ± 94.0a	60.0 ± 67.5b	69.0 ± 56.0c	S
Immobility duration [%]	0.5 ± 0.3a	0.3 ± 0.1b	0.3 ± 0.2c	11.8 ± 4.5a	23.4 ± 5.4b	11.7 ± 3.4c	S
Latency immobility	425.7 ± 75.5a	405.3 ± 76.5b	479.8 ± 74.5c	169.7 ± 37.3a	140.3 ± 29.8b	190.7 ± 30.4c	S
Rearings [nr]	85.5 ± 30.5 a	126.0 ± 38.0	77.5 ± 74.0	30.0 ± 75.0	10.0 ± 27.5	19.0 ± 28.0	S
Grooming [%]	2.2 ± 0.5	1.3 ± 0.4	1.6 ± 0.4	2.1 ± 0.4	1.1 ± 0.3	1.8 ± 0.3	ns
Latency groom [sec]	189.5 ± 20.4a	300.9 ± 37.5a	172.7 ± 11.2	223.7 ± 44.4	302.6 ± 41.4b	188.2 ± 27.7b	Tr

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR.
Two-way ANOVA was performed using strain and treatment as main factors. A P value less than 0.05 was considered significant. nr = number, sec=seconds, S = trial effect, Tr = treatment effect, S x Tr = strain x treatment interaction, ns=non-significant. The same letters in one row represent significant differences revealed by post hoc testing (P<0.0127).

Table S5: Overview of CORT levels before (basal) and after behavioural testing (non-basal).

	BALB/c		**129P3**	
	basal	**non-basal**	**basal**	**non-basal**
Open Field				
vehicle	191.8 ± 27.4a	391.5 ± 35.4a	265.3 ± 45.0b	445.9 ± 29.5b
1 mg/kg diazepam	245.8 ± 57.5	372.3 ± 34.8	221.4 ± 67.9	253.3 ± 54.4
3 mg/kg diazepam	282.6 ± 53.6	263.8 ± 32.4	163.7 ± 7.8a	551.1 ± 128.2a
5 mg/kg diazepam	183.6 ± 30.4	211.3 ± 34.6b	232.3 ± 36.9a	589.5 ± 100.7ab
3 mg/kg MPEP	159.6 ± 22.3	398.0 ± 44.6	293.0 ± 53.8	446.8 ± 85.0
10 mg/kg MPEP	179.4 ± 26.7a	353.0 ± 37.8a	293.7 ± 75.5	375.4 ± 82.5
30 mg/kg MPEP	131.8 ± 10.6a	425.3 ± 81.6a	194.3 ± 30.3	460.3 ± 110.7
Elevated Plus maze				
vehicle	156.4 ± 24.0a	438.7 ± 69.6a	226.5 ± 55.8b	704.3 ± 98.8b
1 mg/kg diazepam	165.5 ± 13.4	177.2 ± 35.9	310.2 ± 52.0	552.3 ± 86.9
10 mg/kg MPEP	252.9 ± 51.5	298.3 ± 475.4	255.5 ± 36.0a	563.3 ± 50.2a
Object recognition test				
vehicle	136.4 ± 16.6a	402.6 ± 136.7ac	270.9 ± 45.3b	1059.3 ± 159.8bc
1 mg/kg diazepam	172.3 ± 20.7	438.9 ± 82.7	325.4 ± 86.1	774.2 ± 164.1
10 mg/kg MPEP	153.0 ± 14.3	389.5 ± 69.9	382.3 ± 109.8	108.9 ± 47.3

Data are presented as mean nmol/l ± SEM. Data were analysed using a repeated measure ANOVA with strain and basal/non-basal CORT levels as main factors. Post hoc analyses were done using a paired (basal/non-basal) or unpaired (strain) Student t-test. Same letters in the same row represent significant differences as found by post hoc testing (P<00512).

Table S6: Overview of c-Fos positive cells after behavioural testing in the OF. PreL (prelimbic cortex), LSD (dorsal lateral septum), LSI (intermediary lateral septum), V (ventral lateral septum), BSTMA (bed nucleus of the stria terminalis, medial anterior part), BSTLP (bed nucleus of the stria terminalis, lateral posterior part), BSTMV (bed nucleus of the stria terminalis, medial ventral part), DG (dentate gyrus), PVN (paraventricular nucleus), DMH (dorsal medial hypothalamus), BLA (basolateral amygdala), CeA (central nucleus of the amygdala), dlPAG (dorsolateral part of periaqueductal grey), dmPAG (dorsomedial part of the periaqueductal grey), lPAG (lateral part of the periaqueductal grey) and the vlPAG (ventrolateral part of the periaqueductal grey).

	BALB/c			**129P3**			
	vehicle	**1 mg/kg diazepam**	**10 mg/kg MPEP**	**vehicle**	**1 mg/kg diazepam**	**10 mg/kg MPEP**	**Sig**
PreL	157.8 ± 18.5ac	97.9 ± 6.8b	93.6 ± 17.6c	65.4 ± 14.9ad	47.4 ± 11.2b	105.7 ± 11.3d	S,Tr, S*Tr
LSD	47.6 ± 10.8a	18.8 ± 10.8	21.9 ± 9.9	11.8 ± 7.4a	4.0 ± 3.0	6.3 ± 2.3	S, S*Tr
LSI	35.4 ± 9.7	22.0 ± 5.4	20.4 ± 3.8	19.1 ± 5.7	18.4 ± 4.9	16.9 ± 4.5	ns
LSV	64.9 ± 20.2ab	26.6 ± 4.5a	22.9 ± 7.6b	48.4 ± 14.8c	36.9 ± 15.3	17.7 ± 9.5c	Tr
BSTMA	62.3 ± 7.6	37.9 ± 12.2	67.5 ± 11.0	40.6 ± 7.7a	48.8 ± 5.6	82.9 ± 14.3a	Tr
BSTLP	62.6 ± 11.1	38.5 ± 6.2	51.0 ± 8.0	29.0 ± 5.0	58.4 ± 10.5	71.6 ± 11.1	S*Tr
BSTMV	23.2 ± 2.9	27.6 ± 6.2	22.9 ± 5.2	19.8 ± 5.4a	30.6 ± 6.6	58.0 ± 18.0a	Tr, S*Tr
DG	72.3 ± 12.2a	73.2 ± 10.9b	73.8 ± 10.2	25.0 ± 6.0a	25.5 ± 4.3b	48.2 ± 14.7	S
PVN	101.3 ± 20.6b	44.8 ± 8.5b	75.9 ± 11.4a	120.5 ± 20.7cd	58.0 ± 16.0c	20.6 ± 5.8ad	S, S*Tr
DMH	138.8 ± 33.4b	114.9 ± 19.4a	53.9 ± 17.4b	89.9 ± 8.0cd	61.5 ± 8.2ac	40.0 ± 5.8d	S, Tr
BLA	51.9 ± 9.1	39.2 ± 5.9a	41.1 ± 5.2	42.9 ± 4.9b	18.1 ± 2.6ab	31.3 ± 3.2	S, Tr
CeA	35.5 ± 8.6	34.2 ± 5.8	28.8 ± 8.0	40.7 ± 5.6	18.7 ± 3.4	21.9 ± 4.7	ns
dlPAG	119.9 ± 13.9	77.6 ± 8.9	56.0 ± 14.1	63.4 ± 10.3	63.9 ± 7.9	69.9 ± 6.7	S*Tr
dmPAG	72.2 ± 26.3	57.7 ± 22.1	31.8 ± 12.7	35.8 ± 14.0	52.6 ± 18.9	43.5 ± 15.2	S*Tr
lPAG	108.9 ± 14.1a	85.7 ± 12.3	66.1 ± 17.1a	106.2 ± 19.9	105.5 ± 13.8	76.7 ± 4.5	Tr
vlPAG	85.4 ± 13.7a	60.2 ± 5.8	46.9 ± 7.0a	98.9 ± 21.9	70.6 ± 7.1	56.5 ± 4.4	Tr

Results are represented as mean number of c-Fos positive cells per mm^2 (± SEM). Sig = significance, S = significant for strain, Tr = significant for treatment condition, S*Tr = strain * treatment interaction, =ns = non-significant. The same letters in one row represent significant differences revealed by post hoc testing (P<0.0127).

Table S7: Overview of c-Fos positive cells after behavioural testing in the EPM. PreL (prelimbic cortex), LSD (dorsal lateral septum), LSI (intermediary lateral septum), V (ventral lateral septum), BSTMA (bed nucleus of the stria terminalis, medial anterior part), BSTLP (bed nucleus of the stria terminalis, lateral posterior part), BSTMV (bed nucleus of the stria terminalis, medial ventral part), DG (dentate gyrus), PVN (paraventricular nucleus), DMH (dorsal medial hypothalamus), BLA (basolateral amygdala), CeA (central nucleus of the amygdala), dlPAG (dorsolateral part of periaqueductal grey), dmPAG (dorsomedial part of the periaqueductal grey), lPAG (lateral part of the periaqueductal grey) and the vlPAG (ventrolateral part of the periaqueductal grey).

	BALB/c			**129P3**			
	vehicle	**1 mg/kg diazepam**	**10 mg/kg MPEP**	**vehicle**	**1 mg/kg diazepam**	**10 mg/kg MPEP**	**Sig**
PreL	43.8 ± 4.9ac	74.4 ± 5.2bc	54.3 ± 11.4	19.1 ± 2.8ade	44.1 ± 3.9bd	54.6 ± 10.1e	S, Tr, S*Tr
LSD	19.5 ± 6.0ac	14.6 ± 2.4	50.1 ± 7.7bc	4.6 ± 2.6ade	21.0 ± 7.7d	24.1 ± 4.8be	S, Tr, S*Tr
LSI	28.4 ± 4.7	17.1 ± 3.8	46.2 ± 10.6	10.4 ± 2.0ab	21.8 ± 3.6a	36.9 ± 5.0b	Tr
LSV	64.9 ± 14.2	34.8 ± 7.8	69.1 ± 12.3	16.2 ± 5.4	52.7 ± 17.0	56.1 ± 14.4	S*Tr
BSTMA	36.3 ± 4.3	32.5 ± 10.9	36.6 ± 7.3	54.6 ± 15.8	15.4 ± 2.9a	50.1 ± 7.6a	Tr
BSTLP	27.3 ± 4.9	15.9 ± 4.4a	21.7 ± 2.8b	26.5 ± 4.5	35.2 ± 6.2a	40.2 ± 3.7b	S, S*Tr
BSTMV	45.5 ± 9.8	31.5 ± .8	47.8 ± 9.5	32.4 ± 5.7a	25.9 ± 6.1	52.9 ± 7.3a	Tr
DG	45.5 ± 7.2a	40.4 ± 7.3	74.3 ± 18.5	28.0 ± 2.6a	35.6 ± 14.1	43.0 ± 10.9	S
PVN	56.6 ± 4.7ab	24.7 ± 2.7a	21.3 ± 5.6b	38.8 ± 7.6c	26.2 ± 4.7	22.1 ± 3.8c	Tr
DMH	32.2 ± 4.8	17.6 ± 3.1	28.8 ± 10.5	22.1 ± 9.1	38.4 ± 9	14.5 ± 2.8	S*Tr
BLA	22.1 ± 2.1	15.4 ± 2.9	15.1 ± 3.0	13.5 ± 2.4	22.2 ± 4.6	10.7 ± 3.6	S*Tr
CeA	19.4 ± 3.9	15.1 ± 4.4	9.9 ± 3.5	18.0 ± 2.5a	31.0 ± 5.3a	10.2 ± 3.8	Tr, S*Tr
dlPAG	46.2 ± 11.6	51.7 ± 12.1	52.4 ± 10.1	56.9 ± 9.4	42.5 ± 11.4	47.5 ± 7.2	ns
dmPAG	73.4 ± 13.2	95.4 ± 20.0	91.4 ± 14.2	94.1 ± 10.7	60.6 ± 15.3	58.2 ± 9.6	ns
lPAG	70.4 ± 13.9	55.9 ± 13.1	73.8 ± 10.4	78.8 ± 5.3	86.0 ± 13.4	90.7 ± 9.7	ns
vlPAG	52.5 ± 13.5	47.7 ± 12.6	52.2 ± 8.8	68.6 ± 13.8	42.5 ± 6.3	85.4 ± 11.1	ns

Results are represented as mean number of c-Fos positive cells per mm^2 (± SEM). A two-way ANOVA was performed with strain and treatment as main factors. A P value less than 0.05 was considered significant (Sig = significance, S = significant for strain, Tr = significant for treatment condition, S*Tr = strain * treatment interaction, =ns = non-significant. The same letters in one row represent significant differences revealed by post hoc testing (P<0.0127).

Chapter

7

Expression of corticotropin-releasing factor receptor type 1 and metabotropic glutamate receptor 5 mRNA following repeated testing in mice that differ in anxiety-related behaviour

manuscript in preparation

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Abstract

Previous studies showed a profound lack of habituation in 129P3/J mice compared to the rapidly habituating BALB/cJ mice after repeated exposure to an initially novel environment, which was accompanied by strain-specific c-Fos expression in emotional and cognitive brain areas.
The metabotropic glutamate receptor 5 (mGlu5) antagonist MPEP, was found to improve habituation in 129P3/J mice and increased c-Fos expression in the prelimbic cortex and dentate gyrus, brain areas related to cognition while it decreased c-Fos expression in the amygdala, a brain area primarily involved in emotion.
We hypothesised that the non-adaptive phenotype of 129P3/J mice arises through impaired neural processing between the prelimbic cortex and emotional brain areas regulated by glutamatergic neurotransmission. Since glutamate is most abundant in the brain, we hypothesised that a specific modulation of glutamate transmission is needed. Corticotropin-releasing factor (CRF) and especially its receptor CRFR1 are believed to modulate glutamatergic neurotransmission and both CRFR1 and mGlu5 are expressed in limbic brain areas. In the present study we investigated mRNA expression of CRFR1 and mGlu5 in the prelimbic cortex and amygdala in naïve and behaviourally tested BALB/cJ and 129P3/J mice to further investigate the role of these receptors in the habituation profiles.
Behavioural results replicated our previous findings i.e. habituating BALB/cJ and non-habituating 129P3/J mice, while similar strain-specific c-Fos expression was found in brain areas controlling cognitive and emotional processes. Furthermore we showed that a decrease in CRFR1 in the prelimbic cortex is associated with habituation, while no clear differences in mGlu5 mRNA expression were observed. We conclude that it is primarily impaired cognitive processes which influence the non-adaptive phenotype of 129P3/J mice, and CRFR1 seems to play an important role in this, although the functional relationship between mGlu5 and CRFR1 remain to be investigated.

Introduction

Habituation during repeated exposure to a novel environment is thought to be indicative for an adaptive anxiety response in mice (Leussis and Bolivar, 2006; Salomons et al., 2010c). Consequently, a lack of habituation would indicate a non-adaptive anxiety response. We have recently shown that the BALB/cJ inbred mouse strain initially shows high anxiety, followed by rapid habituation, whereas the 129P3/J mouse strain was characterized by impaired habituation after repeated exposure to a test environment (Salomons et al., 2010b; Salomons et al., 2010c). Strain specific c-Fos expression in the prelimbic cortex and dentate gyrus, brain areas related to emotionally and cognitive processes which integrate information to guide subsequent behaviour suggested that 129P3/J mice show a deficit in this processing leading to their non-adaptive phenotype. Remarkably, the habituation profile of 129P3/J mice was improved after acute treatment with the metabotropic glutamate 5 receptor (mGlu5) antagonist 2-methyl-6-(phenylethynyl)pyridine MPEP, thereby suggesting a role for the mGlu5 receptor.

Based on these findings we hypothesised that the non-adaptive phenotype of 129P3/J mice may be resulting from an impaired integration of emotional and cognitive processes, more precisely, an impaired neural processing between the prelimbic cortex and emotional brain areas regulated by glutamatergic transmission. However, glutamate is widely distributed in the CNS and the effects of glutamatergic neurotransmission are multiple and widespread. Glutamate transmission can be influenced and regulated by a multitude of factors and it is therefore unlikely that glutamate alone exert its effect on the phenotype of 129P3/J mice. At a higher cortical level, one key factor in coordinating behavioural responses to stressors is corticotropin-releasing factor (CRF): for example, increased levels of anxiety behaviour were found in CRF over-expressing mice (Stenzel-Poore et al., 1994). More specifically, the CRF receptor 1 (CRFR1) is thought to be directly involved in mediating anxiogenic behaviours (Heinrichs et al., 1997; Liebsch et al., 1999) but also in cognitive processing (Contarino et al., 1999). Further, Liu (Liu et al., 2004) found that CRF related peptides play a role as modulators of in vitro glutamatergic neurotransmission in the central amygdala and septum that express CRF receptors.

It was suggested by the authors that CRF related peptides modulate or perhaps regulate excitatory glutamate transmission under both normal and stressful conditions. Following this reasoning and based on our previously found results, we suggested that altered CRFR1 expression in 129P3/J mice affects glutamatergic transmission, more specifically mGlu5 regulation, since the mGlu5 receptor antagonist MPEP improved habituation in 129P3/J mice.

Therefore, the present study was aimed to investigate whether strain-specific CRFR1 and mGlu5 mRNA expression differences can be

demonstrated in the prelimbic cortex and amygdala after repeated testing. Additionally CRFR1, mGlu5 receptor mRNA and c-Fos expression were investigated under baseline conditions.

Materials and methods

Ethical note

The experimental protocols were peer reviewed by the scientific committee of the Department of Animals in Science and Society (Utrecht University, The Netherlands) and subsequently were reviewed and approved by the Animal Experiments Committee of the Academic Biomedical Centre Utrecht, The Netherlands. The Animal Experiments Committee based its decision on the Dutch implementation of the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Further, all animal experiments followed the national 'Code on laboratory animal care and welfare' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003).

Animals and housing

Experiments were performed with naive, male BALB/cJ (BALB/c, stock number 000651, n=30) and male 129P3/J (129P3, stock number 000690, n=30) mice obtained from the Jackson Laboratory (Bar Harbor, Maine, USA) and randomly assigned into two groups, naïve control animals (n=15 per strain) and non-naïve tested animals (n=15 per strain). All animals were 6-7 weeks old at arrival and individually housed in Euro-standard Type II cages (size: 26.7 x 20.7 x 14 cm, Tecniplast, Buguggiate, Italy) provided with bedding material (Lignocel®, J. Rettenmaier & Söhne GmbH, Germany), a tissue (KLEENEX® Facial Tissue, Kimberly-Clark Professional BV, Ede, The Netherlands) and a shelter for cage enrichment. Mice chow (CRM, Expanded, Special Diets Services Witham, UK) and tap water were available ad libitum. The mice were acclimated to their housing room for 17 days at the animal facilities of the Netherlands Vaccine Institute (Bilthoven, The Netherlands) under a reversed dark/light cycle (lights on between 18.00h and 6.00h) and a radio played constantly as background noise. During this period the animals were handled three times a week by the person who also performed the behavioural tests. All behavioural testing took place in this room and equipment was installed before the animals arrived. Behavioural testing was done between 9.00h and 13.00h, during the early activity phase of the animals. Naïve control animals were kept in the experimental room and decapitated on the same day as their tested counterparts. Relative humidity was kept at a constant level of approximately 50%, room temperature was sustained at 22°C ± 2 and ventilation rate was 15-20 air changes per hour.

The modified hole board (mHB)

The mHB represents a combination of an open field and a hole board (Ohl et al., 2001a). The experimental setup consisted of an opaque grey PVC box (100 x 50 x 50 cm) with a board (60 x 20 x 2 cm), which was made of the same material as the box, positioned in the middle of the box, thus representing the unprotected area comparable with the centre of an open field. On the board 20 cylinders (15 x 15 mm) were staggered in three lines. The area around the board was divided by black lines into 10 rectangles (20 x 15 cm) and 2 squares (20 x 20 cm). The board was illuminated with an additional stage light (white light 120 lx), though the surrounding box was only illuminated with red light (1-5 lx). For the investigation of food intake inhibition, the animals were familiarized with a small piece of almond (45 mg, given with a forceps) in their home cage on three consecutive days (at 9.00h) prior to behavioural testing. During testing, the familiar (almonds) and unfamiliar food (Dustless Precision Pellets, 45 mg, Bio-Serv, Frenchtown, USA) objects were always placed in the same corner of the mHB, either one was positioned at the same distance from the wall.
For testing, one animal at the time was directly transferred from its home cage and always placed in the same corner diametrically opposed to the corner in which the food objects were placed and allowed to explore the mHB for 5 minutes per trial (4 trials per day, on 5 consecutive days, total of 20 trials). After each trial, the animal was placed back in its home cage and the mHB was carefully cleaned with tap water and a damp towel. All tests were videotaped for raw data storage and behaviour was directly scored by a trained observer using the program Observer 5.0 (Noldus Information Technology, Wageningen, The Netherlands). The following behavioural parameters were measured and assigned to different behavioural categories according to previous studies (Ohl et al., 2001b); *(a)* avoidance behaviour directed towards the unprotected area: the latency until the first board entry (latency board), the percentage of time spent on the board (time board) and the total number of board entries (board entries); *(b)* risk assessment, measured as the number of stretched attend postures (stretched attends) and the latency until the first stretched attend (latency stretched attend); *(c)* locomotor activity: the total number of line crossings (line crossings), the latency until the first line crossing (latency line crossing), the total time spent immobile (time immobile) and the latency until the first immobility event (latency immobility); *(d)* general exploration: the total number of rearings in the box (rearings box) and on the board (rearings board), the latency until the first rearing in the box (latency rear box) and on the board (latency rearings board), the total number of hole explorations (hole explorations) and the latency until the first hole exploration (latency hole exploration) (a hole was counted as explored when the animal's nose was directed to a

hole; direct contact with the hole was not necessary); *(e)* directed exploration: the total number of holes visited (holes visited, a hole was counted as visited when the mouse dipped the nose below the rim of the hole) and the latency until the first hole visit (latency hole); *(f)* food intake inhibition: the latency until the first exploration of the unfamiliar (latency unfamiliar food) and familiar food object (latency familiar food); *(g)* arousal or de-arousal: the percentage of time spent self-grooming (time grooming), the latency until the first self-grooming event (latency grooming), the total number of self-grooming events (self-groomings) and the total number of fecal boli (boli); *(h)* escape behaviour: the total number of jumps (jumps).

Corticosterone

Basal blood samples from all animals were collected 4 days before the start of mHB testing to determine basal plasma levels of corticosterone (CORT) (pre-test). Basal samples were taken at least 3 hours after the onset of the dark period and 5 hours before the light period to minimize variations in CORT levels due to circadian rhythmicity (Oshima et al., 2003). Trunk blood was collected 2h after the last mHB trial to determine CORT levels after behavioural testing (post-test). All blood sampling and decapitation took place in a separate room adjacent to the experimental room. One animal at the time was transferred in its home cage to this room and in order not to disturb circadian rhythm of the mice, the hallway and rooms were kept under red light conditions. Basal blood samples were collected (± 50µl) using tail vein incision and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Trunk blood after decapitation was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 12.000 rpm, 4°C) and stored at -20°C until analysis. CORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

Brain analyses

Brains were removed from the tested animals two hours after the last mHB trial. Mice not exposed to the test paradigm (n=15 per strain, naïve control) were treated identically immediately after removal from their home cages at the same time as tested animals.
Brains were frozen in -80°C 2-methyl-butane which was cooled with dry ice and stored at -20°C. Coronal sections of 20 µm were cut and mounted on Menzel SuperFrost Plus slides (Menzel GmbH&Co, Braunschweig, Germany) and stored at -80°C. Five serial sections were cut which allowed us to perform c-Fos immunohistochemistry and in situ hybridisation in all tested and naïve control animals.

The anatomical localisation of the c-Fos positive cells and in situ probes was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas (Franklin and Paxinos, 1997). For each region at least two overt landmarks were used.
The following brain areas, which are known to be involved in anxiety (Arzt and Holsboer,2006; Muigg et al., 2007; Nguyen et al., 2006), were processed for c-Fos immunohistochemistry (numbers correspond with Bregma levels); medial prefrontal cortex (prelimbic, +1.78), lateral septum (dorsal, intermediary and ventral, +0.86), bed nucleus of the stria terminalis (medial ventral, +0.38, medial anterior and lateral posterior, + 0.14), dentate gyrus (granular layer, -1.35), paraventricular nucleus (-0.82), dorsal medial hypothalamus (-1.58), amygdala (basolateral nucleus and central nucleus, -1.58) and periaqueductal gray (dorsolateral, dorsomedial, lateral and ventrolateral, -4.72). For in situ hybridisation only the prelimbic cortex and amygdala were investigated.
c-Fos immunohistochemistry: the sections were processed for c-Fos immunohistochemistry as described previously (Salomons et al., 2010c). First, dilution with a polyclonal primary antibody (1:1000, SC-52 Santa Cruz Biotechnology, Santa Cruz, USA), and a donkey-anti-rabbit IgG Biotin SP conjugated secondary antibody (1:400, Jackson ImmunoResearch Laboratories, Inc, USA) took place.
For visualisation of the c-Fos positive cells, the substrate H2O2 (30%, 1:2000) was added to the diaminobenzidine tetrahydrochloride solution (DAB) containing nickel sulphate. Cells containing a nuclear brown-black reaction product were considered as c-Fos positive cells.
For quantitative analysis of c-Fos positive cells, the program Leica QWin (image processing and analysis software, Cambridge, United Kingdom) was used. Left and right hemispheres were analysed in one section separately, calculated for stained neurons per mm2 and averaged for each animal.
In situ hybridisation: the probes used for in situ hybridisation were complementary to the cDNA sequence coding for mouse mGluR5 (Genbank NM 001081414) and CRFR1 (kindly provided by M. Schmidt). The synthesis of the mGlu5 probe was performed as follows: a region of the cDNA corresponding with nucleotides 3535-4024 was amplified using primers 5'- TGCAGCTGGTGCTGG TAG CG-3' (forward primer) and 5' CTTCAGCTCCTGCG GCGTC-3' (reverse primer). Following agarose gel electrophoresis, PCR products were cloned into a suitable cloning vector pCR®II-TOPO® (Invitrogen GmbH, Darmstadt, Germany.). The probes were labelled with digoxigenin (Roche Pharmaceuticals, Switzerland) according to the manufacturer's instructions. Brain sections were first fixated with 4% paraformaldehyde and permeabilized in 0.25% acetic anhydride in triethanolamine (pH 8.0), followed by several dehydration steps. The hybridisation mix contained 50 % formamide, 4x sodium saline citrate buffer (SSC), 0.25 mg/µl tRNA, 50x Denhards reagent and

10% Dextran (pH 7.0). The sections then were incubated overnight at 60°C with 140 µl hybridisation mix, 8 µl DNA (Sonificated Salmon Sperm, Stratagene) and Dig-mGlu5 (0.09 ng/µl) or Dig-CRFR1 (0.04 ng/µl) labelled probe. Following hybridisation, the sections were rinsed in SSC and in SSC containing RNAseA (0.04 %).
Next, the sections were incubated overnight at 4°C with anti-Dig-AP (Roche Pharmaceuticals) in a blocking buffer (1:5000).
The Dig-labelled probes were visualised in a buffer containing 0.1 M Tris, 0.1 M NaCl, 0.1 M MgCl2 plus NBT/BCIP (pH 9.5) and incubated overnight in a light-tight box. The reaction was stopped by a buffer containing 0.25 M Tris, 0.15 M NaCl and 0.015 M EDTA (pH 7.5). After rinsing twice, the slides were dried and coverslipped with Merckoglass (Merck, Darmstadt, Germany).
Images of brain sections were first digitalized using an Olympus BX microscope interfaced with a computer. Quantitative analyses were done using the program Cell^D (Olympus Europa GmbH, Hamburg, Germany). A calibrated grid (150µm by 150µm) was used to count cells containing mRNA and determine mean colour intensity.
All cells that were visible and only when they had a clearly visible nucleus were counted by an observer blind to treatment and strain. For each animal, background staining was measured in an area with no specific mRNA expression. Dependent on the target area, a number of squares were analysed for their number of cells which was averaged for each animal. Analyses were done on the number of cells containing mRNA.

Statistics

Statistical analyses were performed using the software program SPSS for Windows (version 16.0.2; SPSS Inc., IL, USA). Continuous data (CORT, latency and relative duration of behavioural parameters) were summarized as means with standard error of the mean (SEM), whereas discrete data on the ordinal scale (total number of behavioural parameters) were represented as medians with the interquartile range (IQR). The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data. Group analyses using the Kolmogorov-Smirnov one sample test revealed a non-parametric distribution of several continuous parameters. These parameters, as well as the total number of behavioural parameters, were rank transformed (Conover and Iman, 1982). Repeated measures ANOVA was done on the (transformed) behavioural data with trial as within factor and strain as between factor, Huyn-Feldt adjustment was applied. C-Fos positive cells and receptor mRNA expression were analysed by a two-way ANOVA using strain and control vs. test- exposed as main factors.
If ANOVA revealed a $P < 0.05$, post hoc analyses were done using a Student-t test and the P value was corrected for the number of comparisons by a Dunn-Sidak correction ($\alpha = 1 - 0.951/q$, q = number of comparisons, (Ludbrook, 1991).

Results

mHB

A summary of the mHB results for the first trial and the last trial can be found in [Table S1].

Avoidance behaviour: Significant strain ($F(1,26) = 10.931$, $P<0.003$), trial ($F(19,494) = 2.590$, $P<0.001$) and strain x trial ($F(19,342) = 19.824$, $P<0.001$) effects were found for the latency until the first board entry *[Fig 1a]*. Across trials, BALB/c mice showed a decrease while 129P3 mice showed a general increase in the latency until the first board entry. Significant trial and strain x trial effects were observed for the total time spent on the board ($F(19,342) = 7.800$, $P<0.001$, $F(19,342) = 7.631$, $P<0.001$ resp.) and the number of board entries ($F(19,342) = 9.916$, $P<0.001$, $F(19,342) = 12.170$, $P<0.001$). Although no significant strain effects were observed, BALB/c mice spent more time on the board, whereas 129P3 mice almost showed no change in total time spent on the board and board entries across the experimental trials.

Risk assessment: Both strains showed a decrease in the number of stretched attends (trial: $F(19,342) = 31.017$, $P<0.001$; strain x trial: $F(19,342) = 1.814$, $P<0.019$) and an increase in the latency until the first stretched attend (trial: $F(19,342) = 30.680$, $P<0.001$) during the experimental period.

Locomotor activity: Significant strain ($F(1,26) = 39.770$, $P<0.003$), trial ($F(19,494) = 10.133$, $P<0.001$) and strain x trial ($F(19,342) = 6.278$, $P<0.001$) effects were found for the total number of line crossings. Both strains initially showed a similar number of line crossings, but this increased for BALB/c mice while no change was observed in 129P3 mice across trials. For the latency until the first line crossing a significant trial ($F(19,494) = 12.284$, $P<0.001$) and strain x trial ($F(19,342) = 3.671$, $P<0.001$) effect was found.

Both strains displayed a decrease in the latency until the first line crossing. 129P3 mice spent more time immobile than BALB/c mice ($F(1,26) = 5.908$, $P<0.022$) and showed a significant decrease in the latency until the first immobility event across trials ($F(1,26) = 6.445$, $P<0.017$).

General exploration: For the number of rearings in the box and the latency until the first rearing in the box, a significant strain ($F(1,26) = 17.550$, $P<0.00$; $F(1,26) = 12.048$, $P<0.002$ resp.), trial ($F(19,494) = 19.467$, $P<0.001$; $F(19,494) = 5.549$, $P<0.001$ resp.) and strain x trial ($F(19,342) = 3.532$, $P<0.001$; $F(19,342) = 3.620$, $P<0.001$) effect was found. BALB/c displayed more rearings in the box and showed a decrease in the latency until the first rearing in the box compared to 129P3 mice, who displayed less rearings and no change in the latency until the first rearing during the experimental period.

Both strains showed a small increase in the number of rearings on the board (trial: $F(19,494) = 1.646$, $P<0.042$) and a decrease in the latency until the first rearing on the board (trial: $F(19,494) = 1.663$,

P<0.39), though both strains hardly displayed this behaviour on the board. Significant trial (F (19,494) = 5.028, P<0.001) and strain x trial (F (19,342) = 5.175, P<0.001) effects were found for the number of hole explorations. BALB/c initially explored fewer holes compared to 129P3 mice but this increased across trials, whereas 129P3 mice showed no change in the number of hole explorations.
Additionally, a significant strain (F (1,26) = 5.754, P<0.00), trial (F (19,342) = 2.441, P<0.001) and trial x strain (F (19,342) = 12.372, P<0.001) effect was found for the latency until the first hole exploration. A decrease in latency until the first hole exploration was observed in BALB/c mice across trials in contrast to 129P3 mice.
Directed exploration: Both strains showed a significant increase in the number of hole visits (trial: F (19,494) = 21.908, P<0.001; strain x trial: F (19,494) = 3.833, P<0.001) and a decrease in the latency until the first hole visit (trial: F (19,494) = 19.197, P<0.001; strain x trial: F (19,494) = 3.073, P<0.001) during the experimental period, though BALB/c mice displayed more hole visits (F (1,26) = 27.403, P<0.003) and a lower latency until the first hole visit (F (1,26) = 32.902, P<0.003) compared to 129P3 mice.
Food intake inhibition: Both strains showed a significant decrease in the latency until the first intake of the familiar (F (19,494) = 21.007, P<0.001) and unfamiliar (F (19,494) = 1.904, P<0.012) food across the experimental trials.
Arousal or de-arousal: Both strains showed an increase in the time spent grooming (F (19,494) = 6.230, P<0.001) during the experimental period, though BALB/c mice spent significantly more time grooming than 129P3 mice (F (1,26) = 5.679, P<0.025). The latency until the first grooming event decreased across trials in both strains (trial: F (19,494) = 8.331, P<0.001; strain x trial (F (19,494) = 2.024, P<0.007), but less in 129P3 mice (F (1,26) = 18.910, P<0.001).
Additionally, BALB/c mice displayed more grooming events than 129P3 mice (F (1,26) = 12.671, P<0.001), while the number of events increased in both strains across trials (trial: F (19,494) = 7.174, P<0.001). Significant strain differences were observed for the total number of fecal boli (F (1,26) = 31.430, P<0.001). BALB/c mice dropped more boli than 129P3 mice, while no difference in the number of fecal boli across the trials were observed.
Escape: BALB/c mice showed in an increase in the number of jumps across trials (trial: F (19,494) = 7.610, P<0.001) and jumped more than 129P3 mice (F(1,26) = 23.188, P<0.001)

Fig. 1 a+b

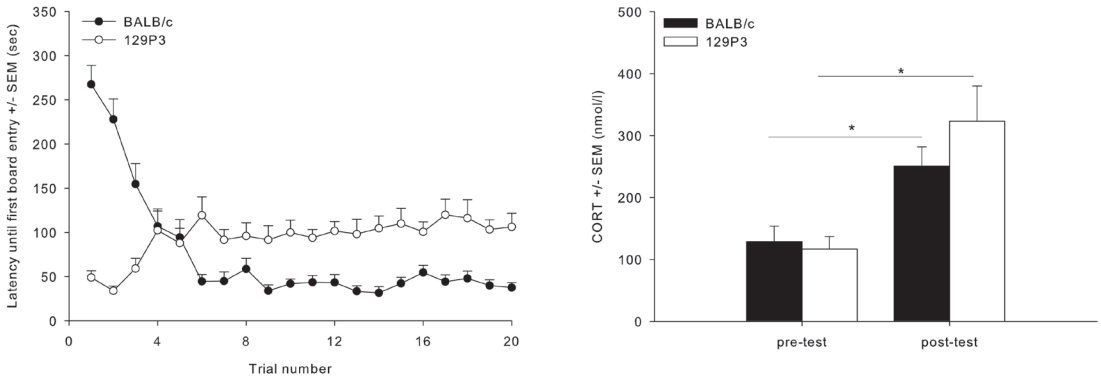


Figure 1: Mean latency until the first board entry (left) and mean plasma CORT levels (right) before (pre-test) and after behavioural testing (post-test). * P <0.001 (b).

CORT

No significant strain differences were found in the pre-tested or post-tested CORT level. BALB/c mice showed a significantly higher post-testing CORT level compared to pre-testing levels ($t = -2.815$, $P<0.0057$). Behaviourally tested 129P3 mice had significantly higher CORT levels compared to baseline levels ($t = -4.218$, $P <0.0057$ *[Fig 1b]*).

c-Fos immunohistochemistry

A significant strain ($F(1,99) = 1.782$, $P = 0.039$) and control vs. test-exposed ($F(1,99) = 6.038$, $P = 0.016$) effect was found for the number of c-Fos positive cells in the prelimbic cortex *[Fig 2a]*. Post hoc testing revealed more c-Fos expression in behaviourally tested BALB/c mice compared to naïve control BALB/c mice ($t = -2.209$, $P = 0.030$) and compared to behaviourally tested 129P3 mice ($t = -2.025$, $P = 0.048$). Significant strain differences ($F(1,100) = 8.405$, $P = 0.005$) were found in the medial ventral part of the bed nucleus stria terminalis (BST). Behaviourally tested 129P3 mice showed more c-Fos expression than tested BALB/c mice ($t = 2841$, $P = 0.007$) in the medial ventral BST, though no strain differences in naïve control animals were observed. In the lateral posterior part of the BST a significant control vs. test-exposed effect ($F(1,99) = 9.383$, $P = 0.003$) was found. Both strains showed more c-Fos positive cells than their naïve control counterparts (BALB/c: $t = 2.257$, $P = 0.029$; 129P3: $t = 2.110$, $P = 0.040$). A significant strain x control vs. test exposed interaction was found in the dentate gyrus ($F(1,103) = 10.046$, $P = 0.002$). Post hoc testing revealed more c-Fos expression in behaviourally tested BALB/c mice compared to naïve control BALB/c mice ($t = -2.851$, $P = 0.006$) and compared to behaviourally tested 129P3 mice ($t = 3.266$, $P = 0.002$).

In the dorsomedial hypothalamus a significant strain (F (1,103) = 4.303, P = 0.041) and control vs. test-exposed effect (F (1,103) = 6.332, P = 0.013) was found, though post hoc testing only showed significantly more c-Fos expression in behaviourally tested 129P3 mice compared to naive control 129P3 mice (t = 2.860, P = 0.042). Significant strain effects were found in the basolateral amygdala (F (1,94) = 4.437, P = 0.038), and in the lateral periaqueductal grey (F (1,103) = 6.033, P = 0.016). Post hoc testing revealed significantly more c-Fos positive cells in naïve BALB//c mice compared to naïve control 129P3 mice *[Fig 2b]*. No significant differences were found in the other investigated brain areas *[see Table S2]*.

Fig. 2 a+b

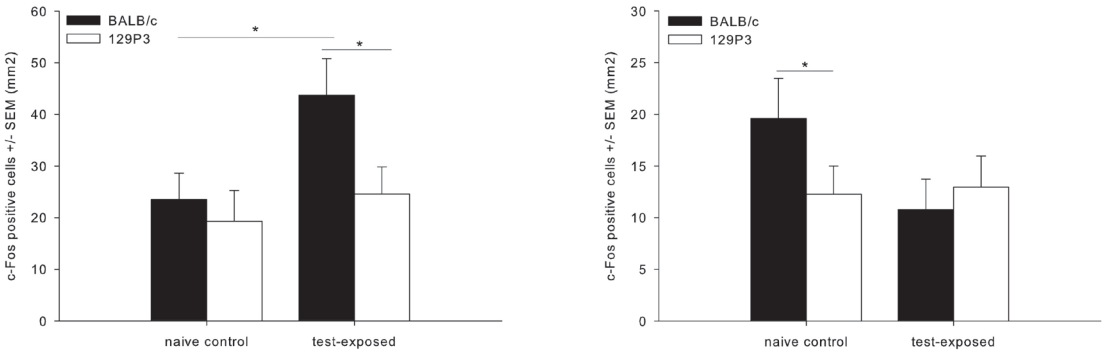


Figure 2: Mean number of c-Fos positive cells in the in naïve control animals and test-exposed animals in the prelimbic cortex (left) and in the basolateral amygdala (right). * P <0.001

In situ hybridization

A general overview of in situ results can be found in [Table S3].

CRFR1: A significant control vs. test exposed (F (1,49) = 5.049, P = 0.029) and an interaction effect (F (1,49) = 11.049, P < 0.001) was found for the number of mRNA containing cells in the prelimbic cortex. Post hoc testing revealed a higher number of CRFR1 mRNA containing cells in naïve BALB/c animals compared to test exposed animals (t = 3.592, P <0.002). Moreover, test exposed 129P3 mice showed more CRFR1 mRNA containing cells than test exposed BALB/c mice (t = -3.204, P < 0.004). No significant differences were found in CRFR1 mRNA containing cells and in the corrected mean colour intensity level in the basolateral amygdala. mGlu5: no significant differences were found in the number of mGlu5 receptor mRNA containing cells.

Fig. 3 a+b

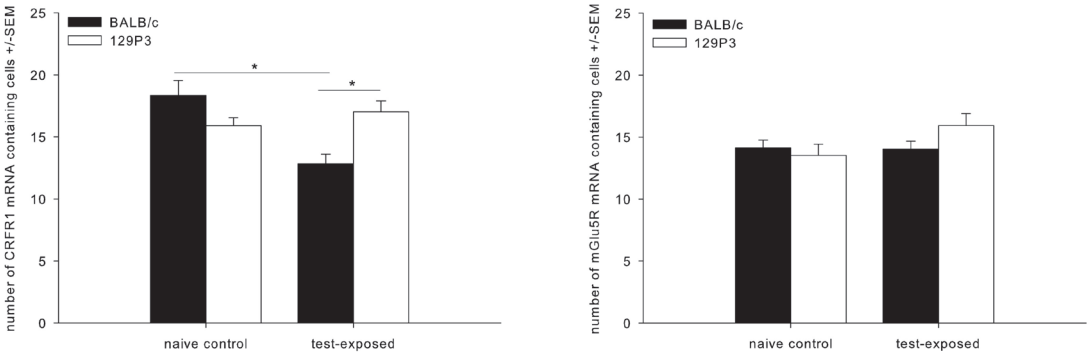


Figure 3: Mean number of CRFR1 (left) or mGlu5 (right) containing cells in the prelimbic cortex in naïve and test-exposed animals. * P< 0.0041

Discussion

In general, behavioural results of the present experiment replicated our previous findings (Salomons et al., 2010c). Following high initial anxiety, BALB/c mice showed a rapid decrease in avoidance behaviour after the first trials, paralleled by an increase in locomotor activity and exploration, demonstrating a rapid habituation to the test situation i.e. adaptive anxiety. In contrast, 129P3 mice showed low initial anxiety, not followed by habituation but even by a slight sensitization in avoidance behaviour. This lack of behavioural habituation to novelty was further underlined by other parameters *[see Table 1]*. Notably, increased CORT levels were observed after behavioural testing in both strains *[Fig 1b]*, although in our previous study we did not find this effect. A likely explanation for this discrepancy between the studies is the different location of the experimental setting. It is well known that exact the same experimental set-up can result in different results across laboratories (Crabbe et al., 1999). Still, behavioural findings were similar to our previous studies and it therefore may be assumed that the non-adaptive phenotype of 129P3 mice is a general characteristic of this strain.

The present experiment complemented previous findings by investigating basal c-Fos expression in several anxiety-related brain areas in naïve control animals in comparison to behaviourally tested animals. In both the prelimbic cortex and dentate gyrus, we found an increase in c-Fos expression in BALB/c mice after testing, which was not the case in 129P3 animals. These structures are known for their involvement in processes of cognitive control on

emotional processes. The prefrontal cortex integrates information to guide subsequent behaviour (Goldman-Rakic, 1995) and processes emotional stimuli and behavioural flexibility (Davidson, 2002). Anxiogenic stimuli and drugs are known to stimulate c-Fos expression in this brain area (Hebb et al., 2004; Morrow et al., 2000; Singewald et al., 2003). The hippocampus, including the dentate gyrus is involved in memory formation and plays a role in the distinction between novel and familiar stimuli (Finn et al., 2003). Moreover, the hippocampus is involved in the stress-induced response of the Hypothalamic-Pituitary-Axis (HPA) system (Herman et al., 2005). Together, testing effects in c-Fos expression in amygdala, prelimbic cortex and dentate gyrus in BALB/c animals may therefore reflect an increased cognitive control on less pronounced emotional processing after habituation. As these testing effects could not be found in behaviourally tested 129P3 mice, we feel our hypothesis to be supported that 129P3 mice are not able to correctly integrate cognitive and emotional processes.

Interestingly, the only significant strain difference in naïve animals was a higher c-Fos expression in BALB/c mice compared to naïve 129P3 mice in the basolateral amygdala. Emotional processes are primarily regulated by the amygdala and several anxiogenic drugs as well as exposure to novelty have been found to induce c-Fos expression in this brain area (Hale et al., 2006; Muigg et al., 2009; Singewald et al., 2003). However, under baseline conditions c-Fos expression in general is very low, but some brain areas such as the amygdala express c-Fos responses to very mild stimuli (Cullinan et al., 1995; Hughes et al., 1992) and there are different sorts of challenges that can induce c-Fos expression such as depolarization and neurotransmitters (Kovacs,1998). This strain difference between naïve control animals might therefore reflect a different mechanisms or threshold level regulating c-Fos expression under baseline conditions (Herdegen et al., 1995). The fact that the strain difference in c-Fos expression in the basolateral amygdala is ameliorated after habituation nevertheless suggests a functional relation with behavioural habituation, such as a lowered emotional response to the test environment in BALB/c mice.

Overall, the habituation procedure resulted in quite some strain differences in c-Fos expression. For example, higher c-Fos expression was found in the dorsomedial hypothalamus in behaviourally tested 129P3 mice compared to their control counterparts, while no such difference was found in BALB/c mice. The dorsomedial hypothalamus plays a crucial role in the regulation of anxiety states (Shekhar, 1994) and several stressors have been found to induce c-Fos expression in this area (Singewald et al., 2003). Thus, the higher c-Fos expression in 129P3 mice after testing supports our hypothesis that the behavioural profile, which is accompanied by increased CORT levels, mirrors non-adaptive anxiety.

Repeated testing and habituation in BALB/c mice, but not in 129P3 animals, was accompanied by a decrease in CRFR1 mRNA expression in the prelimbic cortex *[Fig 3a]*. This effect in BALB/c mice is likely to indicate a functional involvement of CRFR1 in their anxiety profile as it is in accordance with studies showing that CRFR1 antagonists have significant anixolytic effects (for review see (Takahashi, 2001), while for example CRFR1 knockdown mice reveal a less anxious profile when compared with their wildtype counterpart (Heinrichs et al., 1997). However, the increased c-Fos expression in the prelimbic cortex in BALB/c mice obviously is indicative of CRFR1-independent mechanisms or neurons that become activated. For example, increased c-Fos expression in the prelimbic cortex might be associated with increased GABAergic activity, which could induce an inhibitory effect of the prelimbic cortex on downstream emotional targets and thus eventually promote adaptation and cognitive control (Quirk and Gehlert, 2003; Shah et al., 2004).
The fact that no testing effect was observed in 129P3 mice anyhow supports the suggestion that a (down) regulation of CRFR1 mRNA in the prelimbic cortex is involved in behavioural habituation.
No significant differences were found in the number of cells containing mGlu5 mRNA in both BALB/c and 129P3 mice. The fact that MPEP treatment exerted an anxiolytic effect in 129P3 mice while no effects of MPEP were observed in BALB/c mice, can therefore not be explained based on the present findings. It is of course easily possible that MPEP exerts its effect via other brain areas. The functional role of the mGlu5 receptor system in behavioural adaptation thus remains to be investigated and further brain areas, such as the hippocampus and lateral septum, still are under investigation.
From the present results we still can hypothesise, that it is primarily the cognitive control of emotional responses which is impaired in 129P3 mice. It has been suggested that pathological anxiety in general may be a primarily cognitive dysfunction, disabling the sufferer to attune or integrate information on a higher cognitive level, which finally results in inappropriate emotional responses (McNaughton, 1997). The non-adaptive phenotype of 129P3 mice offers a promising model to investigate the neural constructs underlying this specific phenotype.

Supplementary material

Table S1: Overview of significant main effects found by repeated measures ANOVA and average per behavioural parameter for trial 1 and 20 (± SEM) in the mHB. Behavioural parameters are assigned to different behavioural categories.

			BALB/c		129P3/J	
Behavioural category	**Behavioural parameter**	**Significance**	**Trial 1**	**Trial 20**	**Trial 1**	**Trial 20**
Avoidance	Latency until first board entry [sec]	T, S, T x S	267.4 ± 21.4	37.5 ± 5.7	48.8 ± 7.9	106.3 ± 15.3
	Total time spent on board [%]	T, T x S	0.2 ± 0.1	12.8 ± 1.9	10.0 ± 0.8	8.4 ± 1.9
	Total number of board entries [nr.]	T, T x S	0.0 ± 0.0	11.0 ± 6.5	9.5 ± 6.0	7.5 ± 8.0
Risk assessment	Total number of stretched attends [nr.]	T, T x S	26.5 ± 17.5	0.0 ± 0.0	10.5 ± 13.0	0.0 ± 1.0
	Latency until first stretched attend [sec]	T	4.9 ± 0.7	300.0 ± 0.0	6.6 ± 1.2	293.5 ± 6.2
Locomotion	Total number of line crossings [nr.]	T, S, T x S	55.0 ± 57.5	181.0 ± 32.5	99.0 ± 79.0	121.0 ± 39.0
	Latency until first line crossing [sec]	T, T x S	25.3 ± 5.4	1.7 ± 0.1	15.6 ± 3.7	4.1 ± 1.5
	Total time spent immobile [%]	S	0.1 ± 0.1	0.2 ± 0.2	0.0 ± 0.0	0.4 ± 0.4
	Latency until first immobility [sec]	S	285.0 ± 9.8	287.4 ± 12.2	300.0 ± 0.0	277.6 ± 19.8
General exploration	Total number of rearings in the box [nr.]	T, S, T x S	3.5 ± 4.0	24.0 ± 16.0	4.5 ± 10.0	17.5 ± 11.5
	Latency until first rearing in the box [sec]	T, S, T x S	146.4 ± 18.1	47.6 ± 6.0	134.5 ± 26.6	72.2 ± 7.4
	Total number of rearings on the board [nr.]	T	0.0 ± 0.0	0.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.5
	Latency until first rearing board [sec]	ns	300.0 ± 0.0	286.8 ± 8.8	300.0 ± 0.0	297.4 ± 1.8
	Total number of hole explorations [nr.]	T, T x S	1.0 ± 2.5	9.0 ± 7.0	10.0 ± 5.5	8.0 ± 10.5
	Latency until first hole exploration [sec]	T, S, T x S	220.0 ± 26.2	41.2 ± 8.3	36.5 ± 7.7	90.9 ± 11.5
Directed exploration	Total number of hole visits [nr.]	T,S, T x S	0.0 ± 0.0	6.0 ± 3.5	0.0 ± 0.0	2.0 ± 6.0
	Latency until first hole visit [sec]	T,S, T x S	300.0 ± 0.0	98.0 ± 15.3	297.8 ± 2.1	186.8 ± 26.5
Food intake inhibition	Latency first exploration unfamiliar food [sec]	T	283.8 ± 11.7	199.3 ± 32.1	265.0 ± 21.7	192.1 ± 33.5
	Latency first exploration familiar food [sec]	T, T x S	214.0 ± 25.5	9.4 ± 1.7	155.1 ± 25.8	11.7 ± 3.6
Arousa	Total time spent grooming [%]	T, S	0.0 ± 0.0	0.8 ± 0.1	0.0 ± 0.0	0.4 ± 0.1
	Latency until first self groom [sec]	T, S, T x S	298.9 ± 1.1	152.7 ± 22.7	293.2 ± 6.6	272.0 ± 12.2
	Total number of self-groomings [nr.]	T, S	0.0 ± 0.0	1.0 ± 0.5	0.0 ± 0.0	0.0 ± 1.0
	Total number of boli [nr.]	T, S	5.0 ± 4.0	4.5 ± 2.0	1.0 ± 3.0	1.5 ± 1.5
Escape	Total number of jumps [nr.]	T, S, T x S	0.0 ± 0.0	2.5 ± 2.0	0.0 ± 0.0	0.0 ± 0.5

Data are presented as mean ± SEM for continuous data, for discrete data on the ordinal scale the results are presented as median ± IQR. Repeated measures ANOVA was performed using trial as within factor and strain as between factor. A P value less than 0.05 was considered significant. nr, number; sec, seconds; T, trial effect; S, strain effect; T x S, trial x strain interaction; ns, non-significant.

Table S2: Overview of the number of c-Fos positive cells in naïve control animals and in animals after behavioural testing. PreL (prelimbic cortex), LSD (dorsal lateral septum), LSI (intermediary lateral septum), V (ventral lateral septum), BSTMA (bed nucleus of the stria terminalis, medial anterior part), BSTLP (bed nucleus of the stria terminalis, lateral posterior part), BSTMV (bed nucleus of the stria terminalis, medial ventral part), DG (dentate gyrus), PVN (paraventricular nucleus), DMH (dorsal medial hypothalamus), BLA (basolateral amygdala), CeA (central nucleus of the amygdala), dlPAG (dorsolateral part of periaqueductal grey), dmPAG (dorsomedial part of the periaqueductal grey), lPAG (lateral part of the periaqueductal grey) and the vlPAG (ventrolateral part of the periaqueductal grey).

	Naïve control		**Non-naïve tested**		
	BALB/c	**129P3**	**BALB/c**	**129P3**	**Significance**
PreL	23.5 ± 4.1a	23.3 ± 4.4	39.1 ± 5.7ab	24.7b	S, T
LSD	10.4 ± 2.1	13.9 ± 4.2	9.5 ± 2.6	6.7 ± 2.0	ns
LSI	10.1 ± 2.3	13.2 ± 2.1	13.7 ± 5.2	14.9 ± 3.4	ns
LSV	18.2 ± 7.6	13.7 ± 3.7	14.4 ± 6.5	20.2 ± 5.4	ns
BSTMV	8.6 ± 2.3	11.1 ± 2.4	7.2 ± 2.3a	17.7 ± 4.4a	S
BSTMA	13.8 ± 3.2	13.6 ± 2.4	14.1 ± 2.7	18.9 ± 3.9	ns
BSTLP	6.9 ± 1.5a	6.1 ± 1.6b	12.9 ± 3.2a	12.0 ± 3.5b	T
PVN	3.5 ± 0.8	4.0 ± 1.2	3.0 ± 0.9	3.6 ± 1.3	ns
DG	4.1 ± 1.0a	5.4 ± 1.4	8.4 ± 1.7ab	3.3 ± 1.0b	S x T
DMH	18.4 ± 3.5	24.4 ± 4.8a	24.6 ± 6.1	36.5 ± 6.1a	S, T
BLA	19.6 ± 4.9a	12.3 ± 2.7a	10.8 ± 2.9	12.9 ± 3.0	S
CeA	17.1 ± 7.2	9.2 ± 2.6	15.9 ± 5.4	6.9 ± 2.6	ns
dlPAG	7.7 ± 1.9a	13.0 ± 2.8a	11.7 ± 2.4	11.3 ± 2.1	ns
dmPAG	20.9 ± 4.1	10.7 ± 2.1	21.2 ± 3.3	19.2 ± 3.7	ns
lPAG	10.5 ± 2.0	14.6 ± 1.9	11.2 ± 2.3	15.2 ± 2.0	S
vlPAG	12.6 ± 2.4	15.1 ± 2.4	11.9 ± 2.7	13.1 ± 2.1	ns

Results are represented as mean number of c-Fos positive cells per mm^2 (± SEM). ns, non-significant, S = strain effect, T = treatment (i.e. control vs. tested), S x T = strain x treatment interaction.

Table S3: Overview of CRFR1 and mGlu5 mRNA containing cells in naïve control and behaviourally tested BALB/c and 129P3 mice.

		Naïve control		**Non-naïve tested**		
		BALB/c	**129P3**	**BALB/c**	**129P3**	**Significance**
CRFR1	PreL	18.3 ± 1.2a	15.9 ± 0.6	12.8 ± 0.8ab	17.0 ± 0.9b	S, S x T
	BLA	17.8 ± 0.8	21.8 ± 1.2	18.1 ± 1.3	17.5 ± 1.3	ns
mGlu5	PreL	14.2 ± 0.6	13.5 ± 0.9	14.0 ± 0.6	15.9 ± 1.0	ns
	BLA	18.8 ± 0.7	21.1 ± 0.6	18.1 ± 1.2	18.2 ± 1.0	ns

Data are represented as the mean number of CRFR1 or mGlu5 mRNA positive cells (± SEM) in the prelimbic cortex (PreL) or in the basolateral amygdala (BLA). S = strain effect, T = treatment (i.e. control vs. tested), S x T = strain x treatment interaction.

Chapter

8

General Discussion

General discussion

This thesis consists of multiple studies characterizing the adaptive capacity of two inbred mouse strains during repeated exposure to an initially novel environment. The main aim of these studies was to evaluate behavioural habituation as an indicator of non-adaptive, i.e. pathological anxiety in mice. Furthermore, stress hormone levels were measured and the immediate early gene c-Fos, as a marker for neural activity was investigated in brain areas related to anxiety. This chapter describes the main results of these studies, integrating these findings and discussing future prospects.

Habituation as indicator of non-adaptive anxiety

'Pathological anxiety' in animals might be distinguished from 'normal anxiety' as a persistent and uncontrollable emotion lacking adaptive value (Ohl et al., 2008). In rodents, testing for anxiety is regularly based on their natural characteristic to initially avoid novelty (Belzung and LePape, 1994; Ohl, 2005; Treit, 1985).
When being confronted with novelty, the typical rodent behaviour shows a conflict between the drive to explore the novel stimulus, or environment, and to avoid a potentially harmful situation at the same time. For example, when introduced to a novel arena, rodents will first explore the 'protected' area (for example surrounded by walls), while 'unprotected' areas will be avoided. This behaviour is termed thigmotaxis (Treit and Fundytus, 1988), and it is often accompanied by risk assessment behaviour, i.e. carefully exploring the closer surroundings such as stretching of the body. Once an animal has learned about its surroundings and perceives that there is no immediate danger, it will decrease its avoidance and risk assessment behaviour, in the meantime levels of locomotor and exploratory behaviour will usually increase, thereby showing adaptation to the environment (Leussis and Bolivar, 2006).
These processes of behavioural habituation indicate adaptation to the environment whereas non-adaptive avoidance behaviour would be mirrored by a lack of habituation. The main focus in this thesis was to identify this lack of habituation in commonly used mouse strains.

Numerous mouse strains have been characterized for their anxiety profile and several of them have been labelled 'pathologically anxious' (for review: Belzung and Griebel, 2001). However, the characterization of their anxiety profile is usually based only on a single acute exposure to a novel environment. Under these conditions, high avoidance behaviour can be considered normal, i.e. adaptive anxious behaviour according to our hypothesis.
To investigate whether a lack of habituation to novelty can be found in highly anxious mice during repeated testing, we chose to investigate two mouse strains which are reported to show different

innate anxiety levels: BALB/c and 129P3 mice. BALB/c mice are known to initially show high anxiety and high risk assessment behaviour and have repeatedly been reported as representative animal model for pathological anxiety (Griebel et al., 1993; Montkowski et al., 1997; Sheehan et al., 2004). 129P3 mice have not been reported to show behavioural extremes nor have they been reported to be pathologically anxious (Bothe et al., 2005) and were therefore chosen to function as a control strain.

After testing male and female individuals of both strains repeatedly for their habituation capacity and under different conditions, it was evident that BALB/c mice showed rapid habituation towards novelty, that is 'adaptive anxiety'. Surprisingly, 129P3 mice displayed a profound lack of habituation both between trials and within one trial (Salomons et al., 2010c, Chapter 3; Chapter 6). As locomotor and exploratory behaviours were comparable in both strains, we concluded that the lack of habituation in 129P3 mice indicates 'non-adaptive' anxiety. This characteristic appeared to be gender-independent (Salomons et al., 2010a, Chapter 5) and sensitive to chronic mild stress (Salomons et al., 2010b, Chapter 4). Notably, the sustained high level of avoidance behaviour in 129P3 mice seemed to be effective under artificial testing conditions, since no increased hormonal stress response was observed in 129P3 mice after repeated testing. In contrast, acute exposure to a more aversive environment increased corticosterone (CORT) levels dramatically in 129P3 mice. It is important to realize that sustained avoidance behaviour, although it may be effective in avoiding stress under these specific experimental conditions, cannot be understood as being adaptive in the biological sense, since it would probably not promote survival under natural conditions. It is of note that our results contrast findings in literature who reported no specific pathological anxiety-phenotype of 129P3 mice. We propose that single test exposure might be insufficient to identify distinct behavioural characteristics and that non-adaptive anxiety can only be identified by behavioural changes over time and, thus, after repeated test exposure. We thus concluded that the 129P3 strain is of high value as a potential mouse model to further investigate habituation as indicator for (non)-adaptive anxiety.

Neural mechanisms of adaptation

All behavioural experiments in this thesis were complemented with immunohistochemical investigations of the immediate early gene (IEG) c-Fos. C-Fos is the most widely used anatomical mapping tool to identify neural activity since c-Fos is quickly upregulated after various stimuli such as administration of anxiogenic compounds or exposure to novel environments, while basal c-Fos expression is generally very low (Greenberg and Ziff, 1984; Sagar et al., 1988).

Since a large number of brain areas are involved in different aspects of anxiety (Kent and Rauch, 2003; Shin and Liberzon, 2010; Wu et al., 2008), we first screened a variety of brain areas potentially involved in the regulation of the non-adaptive behavioural phenotype of 129P3 mice. We found strain-specific differences in the prelimbic cortex and dentate gyrus before and after behavioural testing (Chapter 3 and 7). Both areas are involved in higher cognitive processes regulating behavioural flexibility and responses to stress (Goldman-Rakic, 1995; McEwen, 1999). Moreover, no strain differences were observed in brain areas known to specifically regulate emotional processes, such as the amygdala (Phelps and LeDoux, 2005; Shin and Liberzon, 2010). We concluded that the behavioural phenotype of 129P3 mice was not the result of a purely emotional process but rather of higher cognitive functioning, which would be in accordance with previous hypotheses by McNaughton and Gray (McNaughton,1997), who suggested that pathological anxiety in humans is primarily a cognitive dysfunction.

Based on the phenotypical and c-Fos results in 129P3 mice up to this point, we hypothesised that the cognitive control (processed by the prelimbic cortex) of emotions (processed by the amygdala) was different between the two strains. While in BALB/c mice an increase in prelimbic neuronal activity could be observed after habituation (Chapter 3, *[Fig 3a]*), probably reflecting a learning process in controlling emotional input during repeated testing, this phenomenon was absent in 129P3 mice. To further evaluate the ability of 129P3 mice to process emotional stimuli, as a next step we attempted to modulate the emotional component of avoidance behaviour by treatment with two anxiolytic compounds. Interestingly, the metabotropic glutamate receptor 5 (mGluR5) antagonist MPEP improved habituation in 129P3 mice.
On the contrary, only a low dose of the protypic anxiolytic benzodiazepine diazepam had an effect in 129P3 mice, while higher doses caused marked sedation. In contrast, BALB/c mice showed the typical dose-dependent anxiolytic response after treatment with diazepam, while MPEP hardly had an effect (Chapter 6,*[Fig 1a]*). Moreover, MPEP increased c-Fos expression in the prelimbic cortex and dentate gyrus only in 129P3 animals and decreased c-Fos expression in the amygdala and paraventricular nucleus of the hypothalamus, again suggesting a decisive role of the prelimbic cortex in the anxiety-profile of the 129P3 strain.

Our findings are in good accordance with a study of Camp and colleagues (Camp et al., 2009), who showed a lack of fear extinction in 129P3 mice, a process based on the reciprocal connection between the prelimbic cortex and the amygdala (Herry and Mons, 2004; Quirk et al., 2006) and a known characteristic of anxiety

disorders. Since treatment with MPEP is known to effectively reduce glutamate transmission (Thomas et al., 2001), we further specified our hypothesis by suggesting that the non-adaptive phenotype in 129P3 mice arises through impaired neural processing between the prelimbic cortex and emotional brain areas regulated by glutamatergic neurotransmission. However, glutamate is the most abundant excitatory neurotransmitter in the brain, being involved in a variety of processes and it is regulated by a multitude of factors (Rothstein et al., 1993). A specific involvement of glutamatergic neurotransmission in habituation processes would therefore demand a specific modulation of the glutamatergic system. CRF would fulfil this role as it is known to be a neuromodulator involved in emotional processes (Gallagher et al., 2008; Heinrichs and Koob, 2004; Koob et al., 1993). Further, the CRFR1 system and mGlu5 receptors are both expressed in brain areas known to be crucial for emotional and cognitive processing such as the prefrontal cortex and hippocampus (Arzt and Holsboer, 2006; Spooren et al., 2001). Moreover, it was suggested that CRF-related peptides can modulate glutamatergic transmission under normal and stressful conditions (Liu et al., 2004). Our preliminary results of CRFR1 mRNA expression indeed suggest that habituation in BALB/c animals is functionally regulated by the CRFR1 system in the prelimbic cortex, which is in accordance with earlier findings on this receptor system in which increased CRFR1 was associated with anxiogenic behaviour (Heinrichs et al., 1997;Takahashi, 2001). Notably, the absence of behavioural adaptation in 129P3 animals is paralleled by a lack of change in CRFR1 mRNA expression. Although other brain areas are still under investigation, these findings are consistent with our hypothesis about a decisive role of CRFR1-regulated cognitive control in the non-adaptive phenotype of 129P3 mice is further supported by these findings. Further studies are necessary to draw conclusions on the role of the mGlu5 system in the process of habituation.

Implications and future directions

A valid mouse model for pathological anxiety would be of high use for both translational research on human anxiety disorders and investigations of animal welfare aspects.

Valid animal models for human diseases have to fulfil three criteria (McKinney, Jr. and Bunney, Jr., 1969): 1) face validity: physiological or behavioural responses or symptoms as observed in human patients should be the same; 2) predictive validity: this implies that the animal model should be sensitive to clinically effective pharmacological compounds and; 3) construct validity: which means that there must be a similarity between the mechanisms regulating the modelling characteristics and the human symptoms to be modelled.

Face validity in 129P3 mice as animal model for pathological anxiety.

Human anxiety disorders can be classified into six different types of anxiety disorders (DSM IV, 2000), and diagnosis depends on the symptoms involved (or previous exposure to trauma). Although some symptoms are specific to the type of anxiety disorder, most of them are characterized by their own irrational fear. In fact, all anxiety disorders are based on the misinterpretation of non-threatening situations, which has also been described as a lack of habituation (Beck et al., 1985). Beck noted that the average individual grows more confident and less anxious after repeated exposure to possible threatening situations, while the more anxious person grows even more anxious and is unable to make the distinction between what is safe and what is not. In 129P3 mice we found a comparable behavioural symptom, i.e. increased anxiety-related behaviour over time within a novel but non-harmful situation. We thus conclude that 129P3 mice show face validity for a lack of habituation, although being aware of the fact that the behavioural phenotype in 129P3 mice has to be further validated, especially with respect to their cognitive abilities.

Predictive validity in 129P3 mice as animal model for pathological anxiety.

The second criterion implies that 129P3 mice should be sensitive to clinically validated anxiolytic agents. In chapter 6 we show that 129P3 mice show an anxiolytic response to a low dose of diazepam, a gold standard to investigate the predictive validity of an animal model of anxiety (Treit, 1985). Higher doses of diazepam caused pronounced sedation in 129P3 mice, a side effect which is also observed in humans (Woods, 1998). Remarkably, the anxiety-phenotype in 129P3 mice was more responsive to the anxiolytic properties of the mGlu5 receptor antagonist MPEP. Although MPEP has not been tested in clinical trials yet, several animal studies point towards the potency of mGlu5 receptor antagonists as new approach in the treatment of anxiety disorders (Pietraszek et al., 2005; Spooren et al., 2001; Tatarczynska et al., 2001). 129P3 mice thus show predictive validity when acutely treated with anxiolytic compounds. However, since patients suffering from anxiety disorders usually are treated for longer time-periods, the further evaluation of predictive validity should include the effect of chronic anxiolytic treatment on the non-adaptive phenotype of 129P3 mice and other (putative) anxiolytics.

Construct validity in 129P3 mice as animal model for pathological anxiety.

The underlying constructs of psychiatric disorders are still largely unknown. As a matter of fact research on psychiatric disorders in animal models is predominantly used to unravel the underlying construct. Thus, the construct validity of animal models for psychiatric disorders can hardly be demonstrated.
Nevertheless, in case of anxiety disorders several hypotheses regarding central nervous mechanisms have been developed.
For example, there is evidence that abnormal HPA-axis functioning may characterize a subset of anxiety disorders (Holsboer, 2003), whereas others may be based on the weakened inhibition of amygdala functions caused by excessive excitatory amino acid neurotransmission (via the hippocampus or prefrontal cortex) in response to stress (Simon and Gorman, 2006). It has as well been suggested that pathological anxiety in general may be a primarily cognitive dysfunction, which disables the sufferer to attune or integrate information on a higher cognitive level, finally resulting in inappropriate emotional responses (McNaughton, 1997).
We suggested that the underlying mechanisms of the non-adaptive phenotype in 129P3 mice are based on the impaired integration of cognitive and emotional processes. More specifically, we hypothesise that it is primarily the impaired cognitive control exerted by the prelimbic cortex which is responsible for the non-adaptive anxiety phenotype in 129P3 mice. Still, it remains to be investigated whether modulation of cognitive processes affect the habituation profile of 129P3 mice. Further, it would be of interest whether for example lesioning of the prelimbic cortex in BALB/c mice leads to the same specific non-adaptive behavioural profile. Moreover, more specific evaluation of projections from and to the prelimbic cortex would shed more light on the underlying construct of the 129P3 anxiety-phenotype.

Animal welfare considerations

129P3 mice are not only a promising translational model for human anxiety disorders, but the fact that these animals show a possible pathological phenotype also suggests that their welfare may be compromised. Animal welfare can be described as the physiological and behavioural ability of an animal to adapt to environmental challenges (Korte et al., 2007; Salomons et al., 2009).
Furthermore, it has been argued that welfare is compromised when an animal is constrained to behave outside its adaptive boundaries which have evolved during the course of evolution of its species (Barnard, 2007). This would imply that under laboratory circumstances, animal welfare can be promoted by defining species-specific housing conditions using the corresponding wild-type as reference value. Domestic mice, which have later been bred into laboratory mouse strains as well, have a common ancestor

(Morse, 1978). A variety of specific strains were selected and bred with respect to distinct characteristics. This artificial breeding process may have had an unintended impact on different characteristics, including the animal's adaptive abilities. Consequently, the definition of 'housing' and 'treatment conditions' for mice on a species-level would probably not be adequate for all mouse strains, but would have to be adjusted to strain-specific (emotional) phenotypes. In case of 129P3 mice, the standard mouse housing conditions as were applied in our studies, probably do not benefit their welfare, however strain- or even sub strain specific requirements in terms of housing and treatment conditions probably exist for 129P3 mice. For example, it is known that 129P3 mice are challenged breeders and show a high percentage of litter death (Millstein et al., 2006; Millstein and Holmes, 2007; unpublished observations from our lab). Jackson Laboratory, the mice supplier, in fact states that breeding 129P3 mice requires specific maintenance, such as minimizing handling and noise, maximize darkness and providing enough nesting material (The Jackson Laboratory, 2010).

From these considerations, the question arises whether the 129P3 phenotype is the result of the interaction between the genetic background of this strain and housing and treatment conditions as applied in our studies, or, whether their phenotype is the result of developmental conditions or, possibly, an interaction of both. Poor maternal care and a high percentage of litter death as observed in 129P3 mice may explain the fact that 129P3 mice seem unable to adapt to unthreatening situations. Other factors, such as changing of the social structure after weaning, handling procedures and transport from the supplier or within the institute, may also contribute to the non-adaptive behavioural profile of 129P3 mice. Factors that occur during development or events that happen later in life can all contribute to altered neuro-behavioural processing, which eventually can result in pathological anxiety (Barros et al., 2006; Chorpita and Barlow, 1998). Therefore, characterizing such factors and resulting emotional phenotypes in laboratory animals is necessary and would lead to a better understanding of potentially confounding factors on animal welfare and their underlying mechanisms.

Chapter

9

References

[A]

Arndt SS, Laarakker MC, van Lith HA, van der Staay FJ, Gieling E, Salomons AR et al. (2009). Individual housing of mice--impact on behaviour and stress responses. Physiol Behav 97, 385-393.

Arzt E and Holsboer F (2006). CRF signaling: molecular specificity for drug targeting in the CNS. Trends Pharmacol Sci 27, 531-538.

Auer JA, Goodship A, Arnoczky S, Pearce S, Price J, Claes L et al. (2007). Refining animal models in fracture research: seeking consensus in optimising both animal welfare and scientific validity for appropriate biomedical use. BMC Musculoskelet Disord 8, 72.

[B]

Ballard TM, Woolley ML, Prinssen E, Huwyler J, Porter R, Spooren W (2005). The effect of the mGlu5 receptor antagonist MPEP in rodent tests of anxiety and cognition: a comparison. Psychopharmacology (Berl) 179, 218-229.

Balogh SA, McDowell CS, Stavnezer AJ, Denenberg VH (1999). A behavioral and neuroanatomical assessment of an inbred substrain of 129 mice with behavioral comparisons to C57BL/6J mice. Brain Res 836, 38-48.

Barker GR, Bashir ZI, Brown MW, Warburton EC (2006). A temporally distinct role for group I and group II metabotropic glutamate receptors in object recognition memory. Learn Mem 13, 178-186.

Barlow DH and Wincze J (1998). DSM-IV and beyond: what is generalized anxiety disorder? Acta Psychiatr Scand Suppl 393, 23-29.

Barnard CJ (2007). Ethical regulation and animal science: why animal behaviour is special. Anim Behav 74: 5-13.

Barros VG, Rodriguez P, Martijena ID, Perez A, Molina VA, Antonelli MC (2006). Prenatal stress and early adoption effects on benzodiazepine receptors and anxiogenic behavior in the adult rat brain. Synapse 60, 609-618.

Bartolomucci A, Parmigiani S, Gioiosa L, Ceresini G, Palanza P (2009). Effects of housing social context on emotional behaviour and physiological responses in female Mice. Scandinavian Journal of Laboratory Animal Science 36, 87-95.

Baumans V (1997). Environmental Enrichment: Practical Applications. In:.Zutphen LFM , Balls M (eds). Animal Alternatives, Welfare and Ethics. Elsevier BV., The Netherlands, 187-191.

Beck AT, Emery G, Greenberg RL (1985). Anxiety disorders and phobias; A cognitive perspective. Basic Books: New York.

Behbehani MM (1995). Functional characteristics of the midbrain periaqueductal gray. Prog Neurobiol 46, 575-605.

Belzung C and 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.

Belzung C and Griebel G (2001). Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res 125, 141-149.

Belzung C and LePape G. (1994). Comparison of different behavioral test situations used in psychopharmacology for measurement of anxiety. Physiol Behav 56, 623-628.

Benquet P, Gee CE, Gerber U (2002). Two distinct signaling pathways upregulate NMDA receptor responses via two distinct metabotropic glutamate receptor subtypes. J Neurosci 22, 9679-9686.

Bernard C (1865). Introduction a l'Etude de la Medecine Experimentale (Introduction to the study of experimental medicine). Dover Publications (Published 1957): New York.

Bernard JF and Bandler R (1998). Parallel circuits for emotional coping behaviour: new pieces in the puzzle. J Comp Neurol 401, 429-436.

Beuzen A and Belzung C (1995). Link between emotional memory and anxiety states: a study by principal component analysis. Physiol Behav 58, 111-118.

Bisazza A (1981). Social organization and territorial behaviour in three strains of mice. Bolletino Zoologica 48, 157-167.

Blanchard DC, Shepherd JK, De Padua CA, Blanchard RJ (1991). Sex effects in defensive behavior: baseline differences and drug interactions. Neurosci Biobehav Rev 15, 461-468.

Blanchard DC, Griebel G, Blanchard RJ (1995). Gender Bias in the Preclinical Psychopharmacology of Anxiety - Male Models for (Predominantly) Female Disorders. Journal of Psychopharmacology 9, 79-82.

Blanchard DC, Griebel G, Blanchard RJ (2001). Mouse defensive behaviors: pharmacological and behavioral assays for anxiety and panic. Neurosci Biobehav Rev 25, 205-218.

Bolivar VJ, Caldarone BJ, Reilly AA, Flaherty L (2000). Habituation of activity in an open field: A survey of inbred strains and F1 hybrids. Behav Genet 30, 285-293.

Bolivar VJ (2009). Intrasession and intersession habituation in mice: from inbred strain variability to linkage analysis. Neurobiol Learn Mem 92, 206-214.

Bothe GW, Bolivar VJ, Vedder MJ, Geistfeld JG (2004). Genetic and behavioral differences among five inbred mouse strains commonly used in the production of transgenic and knockout mice. Genes Brain Behav 3, 149-157.

Bothe GW, Bolivar VJ, Vedder MJ, Geistfeld JG (2005). Behavioral differences among fourteen inbred mouse strains commonly used as disease models. Comp Med 55, 326-334.

Bouwknecht JA, Spiga F, Staub DR, Hale MW, Shekhar A, Lowry CA (2007). Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. Brain Res Bull 72, 32-43.

Brain P and Parmigiani S (2008). Variation in aggressiveness in house mouse populations. Biological Journal of the Linnean Society 41, 257-269.

Brambell FWR (1965). Report of the technical committee to inquire into the welfare of animals kept under intensive livestock and husbandry. London: Her Majesty's Stationary Office.

Brooks SP, Pask T, Jones L, Dunnett SB (2005). Behavioural profiles of inbred mouse strains used as transgenic backgrounds. II: cognitive tests. Genes Brain Behav 4, 307-317.

Broom DM (1986). Indicators of poor welfare. Br Vet J 142, 524-526.

Busse CS, Brodkin J, Tattersall D, Anderson JJ, Warren N, Tehrani L et al. (2004). The behavioral profile of the potent and selective mGlu5 receptor antagonist 3-[(2-methyl1,3-thiazol–4-yl) ethynyl] pyridine (MTEP) in rodent models of anxiety. Neuropsychopharmacology 29, 1971-1979.

[C]

Camp M, Norcross M, Whittle N, Feyder M, D'Hanis W, Yilmazer-Hanke D et al. (2009). Impaired Pavlovian fear extinction is a common phenotype across genetic lineages of the 129 inbred mouse strain. Genes Brain Behav 8, 744-752.

Cannon WB (1932). The wisdom of the body. W.W. Norton: New York.

Carey MP, Deterd CH, de KJ, Helmerhorst F, de Kloet ER (1995). The influence of ovarian steroids on hypothalamic-pituitary-adrenal regulation in the female rat. J Endocrinol 144, 311-321.

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 behaviour in inbred mice. Behav Brain Res 134, 49-57.

Chamove AS (1989). Cage design reduces emotionality in mice. Lab Anim 23, 215-219.

Chapillon P, Patin V, Roy V, Vincent A, Caston J (2002). Effects of pre- and postnatal stimulation on developmental, emotional, and cognitive aspects in rodents: a review. Dev Psychobiol 41, 373-387.

Chen X and Herbert J (1995). Regional changes in c-fos expression in the basal forebrain and brainstem during adaptation to repeated stress: correlations with cardiovascular, hypothermic and endocrine responses. Neuroscience 64, 675-685.

Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS (2002). Identification and ranking of genetic and laboratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. Neurosci Biobehav Rev 26, 907-923.

Chorpita BF and Barlow DH (1998). The development of anxiety: the role of control in the early environment. Psychol Bull 124, 3-21.

Christoffersen GR, Simonyi A, Schachtman TR, Clausen B, Clement D, Bjerre VK et al. (2008). MGlu5 antagonism impairs exploration and memory of spatial and non-spatial stimuli in rats. Behav Brain Res 191, 235-245.

Chrousos GP (1998). Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture. Ann N Y Acad Sci 851, 311-335.

Chung S, Son GH, Park SH, Park E, Lee KH, Geum D et al. (2005). Differential adaptive responses to chronic stress of maternally stressed male mice offspring. Endocrinology 146, 3202-3210.

Conover WJ and Iman RL (1982). Analysis of covariance using the rank transformation. Biometrics 38, 715-724.

Contarino A, Dellu F, Koob GF, Smith GW, Lee KF, Vale W et al. (1999). Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1. Brain Res 835, 1-9.

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.

Crabbe JC, Wahlsten D, Dudek BC (1999). Genetics of mouse behavior: interactions with laboratory environment. Science 284, 1670-1672.

Crusio WE and Schwegler H (1987). Hippocampal mossy fiber distribution covaries with open-field habituation in the mouse. Behav Brain Res 26, 153-158.

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.

Cunha JM and Masur J (1978). Evaluation of psychotropic drugs with a modified open field test. Pharmacology 16, 259-267.

[D]

D'Aquila PS, Brain P, Willner P (1994). Effects of chronic mild stress on performance in behavioural tests relevant to anxiety and depression. Physiol Behav 56, 861-867.

Davidson RJ (2002). Anxiety and affective style: role of prefrontal cortex and amygdala. Biol Psychiatry 51, 68-80.

Davis M (2006). Neural systems involved in fear and anxiety measured with fear-potentiated startle. Am Psychol 61, 741-756.

de Kloet ER, Joels M, Holsboer F (2005). Stress and the brain: from adaptation to disease. Nat Rev Neurosci 6, 463-475.

Dere E, de Souza Silva MA, Topic B, Fiorillo C, Li JS, Sadile AG et al. (2002). Aged endothelial nitric oxide synthase knockout mice exhibit higher mortality concomitant with impaired open-field habituation and alterations in forebrain neurotransmitter levels. Genes Brain Behav 1, 204-213.

DSM-IV (2000). The Diagnostic and Statistical Manual of Mental Disorders, fourth edition. American Psychiatric Association.

Dimitsantos E, Escorihuela RM, Fuentes S, Armario A, Nadal R (2007). Litter size affects emotionality in adult male rats. Physiol Behav 92, 708-716.

Dubovicky M and Jezova D (2004). Effect of chronic emotional stress on habituation processes in open field in adult rats. Ann N Y Acad Sci 1018, 199-206.

Ducottet C, Aubert A, Belzung C (2004). Susceptibility to subchronic unpredictable stress is related to individual reactivity to threat stimuli in mice. Behav Brain Res 155, 291-299.

Ducottet C and Belzung C (2005). Correlations between behaviours in the elevated plus-maze and sensitivity to unpredictable subchronic mild stress: evidence from inbred strains of mice. Behav Brain Res 156, 153-162.

Duncan GE, Knapp DJ, Breese GR (1996). Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res 713, 79-91.

[E]

Eisenstein, EM, Eisenstein , Smith JC (2001). The Evolutionary Significance of Habituation and Sensitization Across Phylogeny: A Behavioral Homeostasis Model. Integrative Physiological and Behavioral Science 256-265. New York, Springer New York.

Eisenstein EM and Eisenstein D (2006). A behavioral homeostasis theory of habituation and sensitization: II. Further developments and predictions. Rev Neurosci 17, 533-557.

Espejo EF (1997). Effects of weekly or daily exposure to the elevated plus-maze in male mice. Behav Brain Res 87, 233-238.

[F]

Faravelli C, Rosi S, Truglia E (2003). Treatments: Benzodiazepines. In:.Nutt DJ , Ballenger JC (eds). Anxiety Disorders. Blackwell Science: Oxford. pp. 315-338.

Ferrari PF, Palanza P, Parmigiani S, Rodgers RJ (1998). Interindividual variability in Swiss male mice: relationship between social factors, aggression, and anxiety. Physiol Behav 63, 821-827.

Festing MF, Simpson EM, Davisson MT, Mobraaten LE (1999). Revised nomenclature for strain 129 mice. Mamm Genome 10, 836.

Finn DA, Rutledge-Gorman MT, Crabbe JC (2003). Genetic animal models of anxiety. Neurogenetics 4, 109-135.

Franklin KGB and Paxinos G (1997). The mouse brain in stereotaxic coordinates. Academic Press.

[G]

Gallagher JP, Orozco-Cabal LF, Liu J, Shinnick-Gallagher P (2008). Synaptic physiology of central CRH system. Eur J Pharmacol 583, 215-225.

Gasparini F, Lingenhohl K, Stoehr N, Flor PJ, Heinrich M, Vranesic I et al. (1999). 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. Neuropharmacology 38, 1493-1503.

George SA, Hutson PH, Stephens DN (2009). Differential effects of MPEP and diazepam in tests of conditioned emotional response and Pavlovian-to-instrumental transfer suggests 'anxiolytic' effects are mediated by different mechanisms. Psychopharmacology (Berl) 204, 499-509.

Gerber U, Gee CE, Benquet P (2007). Metabotropic glutamate receptors: intracellular signaling pathways. Curr Opin Pharmacol 7, 56-61.

Girotti M, Pace TW, Gaylord RI, Rubin BA, Herman JP, Spencer RL (2006). Habituation to repeated restraint stress is associated with lack of stress-induced c-fos expression in primary sensory processing areas of the rat brain. Neuroscience 138, 1067-1081.

Goldman-Rakic PS (1995). Architecture of the prefrontal cortex and the central executive. Ann N Y Acad Sci 769, 71-83.

Gray JA (1982). The Neuropsychology of Anxiety: an Enquiry in To the Functions of the Septo-hippocampal System. Oxford University Press: Oxford.

Gray JA and McNaughton N (1983). Comparison between the behavioural effects of septal and hippocampal lesions: a review. Neurosci Biobehav Rev 7, 119-188.

Greenberg ME and Ziff EB (1984). Stimulation of 3T3 cells induces transcription of the c-fos proto-oncogene. Nature 311, 433-438.

Greenberg PE, Sisitsky T, Kessler RC, Finkelstein SN, Berndt ER, Davidson JR et al. (1999). The economic burden of anxiety disorders in the 1990s. J Clin Psychiatry 60, 427-435.

Griebel G, Belzung C, Misslin R, Vogel E (1993). The free-exploratory paradigm: an effective method for measuring neophobic behaviour in mice and testing potential neophobia-reducing drugs. Behav Pharmacol 4, 637-644.

Griebel G, Perrault G, Sanger DJ (1998). Characterization of the behavioral profile of the non-peptide CRF receptor antagonist CP-154,526 in anxiety models in rodents. Comparison with diazepam and buspirone. Psychopharmacology (Berl) 138, 55-66.

Griebel G, Belzung C, Perrault G, Sanger DJ (2000). Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology (Berl) 148, 164-170.

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.

[H]

Halbreich U and Kahn LS (2001). Role of estrogen in the aetiology and treatment of mood disorders. CNS Drugs 15, 797-817.

Hale MW, Bouwknecht JA, Spiga F, Shekhar A, Lowry CA (2006). Exposure to high- and low-light conditions in an open-field test of anxiety increases c-Fos expression in specific subdivisions of the rat basolateral amygdaloid complex. Brain Res Bull 71, 174-182.

Hall CS (1936). Emotional behavior in the rat. III. The relationship between emotionality and ambulatory activity. Journal of Comparative Psychology 22, 345-352.

Hall FS, Huang S, Fong GW, Sundstrom JM, Pert A (2000). Differential basis of strain and rearing effects on open-field behavior in Fawn Hooded and Wistar rats. Physiol Behav 71, 525-532.

Hebb AL, Zacharko RM, Gauthier M, Trudel F, Laforest S, Drolet G (2004). Brief exposure to predator odor and resultant anxiety enhances mesocorticolimbic activity and enkephalin expression in CD-1 mice. Eur J Neurosci 20, 2415-2429.

Hefner K, Whittle N, Juhasz J, Norcross M, Karlsson RM, Saksida LM et al. (2008). Impaired fear extinction learning and cortico-amygdala circuit abnormalities in a common genetic mouse strain. J Neurosci 28, 8074-8085.

Heinrichs SC and Koob GF (2004). Corticotropin-releasing factor in brain: a role in activation, arousal, and affect regulation. J Pharmacol Exp Ther 311, 427-440.

Heinrichs SC, Lapsansky J, Lovenberg TW, De Souza EB, Chalmers DT (1997). Corticotropin-releasing factor CRF1, but not CRF2, receptors mediate anxiogenic-like behavior. Regul Pept 71, 15-21.

Henniger MS, Ohl F, Holter SM, Weissenbacher P, Toschi N, Lorscher P et al. (2000). Unconditioned anxiety and social behaviour in two rat lines selectively bred for high and low anxiety-related behaviour. Behav Brain Res 111, 153-163.

Herdegen T, Kovary K, Buhl A, Bravo R, Zimmermann M, Gass P (1995). Basal expression of the inducible transcription factors c-Jun, JunB, JunD, c-Fos, FosB, and Krox-24 in the adult rat brain. J Comp Neurol 354, 39-56.

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.

Herry C and Mons N (2004). Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala. Eur J Neurosci 20, 781-790.

Hewson CJ (2003). Can we assess welfare? Can Vet J 44, 749-753.

Hinks GL, Brown P, Field M, Poat JA, Hughes J (1996). The anxiolytics CI-988 and chlordiazepoxide fail to reduce immediate early gene mRNA stimulation following exposure to the rat elevated X-maze. Eur J Pharmacol 312, 153-161.

Hoehn-Saric R and McLeod DR (1988). The peripheral sympathetic nervous system. Its role in normal and pathologic anxiety. Psychiatr Clin North Am 11, 375-386.

Hoehn-Saric R, McLeod DR, Zimmerli WD (1989). Somatic manifestations in women with generalized anxiety disorder. Psychophysiological responses to psychological stress. Arch Gen Psychiatry 46, 1113-1119.

Holmes A and Rodgers RJ (1998). Responses of Swiss-Webster mice to repeated plus-maze experience: further evidence for a qualitative shift in emotional state? Pharmacol Biochem Behav 60, 473-488.

Holmes A and Rodgers RJ (1999). Influence of spatial and temporal manipulations on the anxiolytic efficacy of chlordiazepoxide in mice previously exposed to the elevated plus-maze. Neurosci Biobehav Rev 23, 971-980.

Holsboer F (2003). Corticotropin-releasing hormone modulators and depression. Curr Opin Investig Drugs 4, 46-50.

Homberg JR, Raaso HS, Schoffelmeer AN, De Vries TJ (2004). Individual differences in sensitivity to factors provoking reinstatement of cocaine-seeking behavior. Behav Brain Res 152, 157-161.

Huang YH, Cheng CY, Hong CJ, Tsai SJ (2004). Expression of c-Fos-like immunoreactivity in the brain of mice with learned helplessness. Neurosci Lett 363, 280-283.

Hughes P, Lawlor P, Dragunow M (1992). Basal expression of Fos, Fos-related, Jun, and Krox 24 proteins in rat hippocampus. Brain Res Mol Brain Res 13, 355-357.

Humble M (1987). Aetiology and mechanisms of anxiety disorders. Acta Psychiatr Scand Suppl 335, 15-30.

[K]

Kalueff AV and Tuohimaa P (2004). Grooming analysis algorithm for neurobehavioural stress research. Brain Res Brain Res Protoc 13, 151-158.

Kalueff AV (2007). Neurobiology of memory and anxiety: from genes to behavior. Neural Plast 78171.

Kasahara M, Groenink L, Breuer M, Olivier B, Sarnyai Z (2007). Altered behavioural adaptation in mice with neural corticotrophin-releasing factor overexpression. Genes Brain Behav 6, 598-607.

Kemp JA and McKernan RM (2002). NMDA receptor pathways as drug targets. Nat Neurosci 5 Suppl, 1039-1042.

Kempermann G, Kuhn HG, Gage FH (1997). More hippocampal neurons in adult mice living in an enriched environment. Nature 386, 493-495.

Kent JM, Mathew SJ, Gorman JM (2002). Molecular targets in the treatment of anxiety. Biol Psychiatry 52, 1008-1030.

Kent JM and Rauch SL (2003). Neurocircuitry of anxiety disorders. Curr Psychiatry Rep 5, 266-273.

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.

Killcross S, Robbins TW, Everitt BJ (1997). Different types of fear-conditioned behaviour mediated by separate nuclei within amygdala. Nature 388, 377-380.

Kim D, Chae S, Lee J, Yang H, Shin HS (2005). Variations in the behaviors to novel objects among five inbred strains of mice. Genes Brain Behav 4, 302-306.

Koob GF, Heinrichs SC, Pich EM, Menzaghi F, Baldwin H, Miczek K et al. (1993). The role of corticotropin-releasing factor in behavioural responses to stress. Ciba Found Symp 172, 277-289.

Koob GF and Le MM (2001). Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology 24, 97-129.

Koolhaas JM, Korte SM, De Boer SF, Van D, V, Van Reenen CG, Hopster H et al. (1999). Coping styles in animals: current status in behavior and stress-physiology. Neurosci Biobehav Rev 23, 925-935.

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.

Korte SM, Olivier B, Koolhaas JM (2007). A new animal welfare concept based on allostasis. Physiol Behav 92, 422-428.

Kovacs KJ (1998). c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Int 33, 287-297.

Kuribara H and Asahi T (1997). Assessment of the anxiolytic and amnesic effects of three benzodiazepines, diazepam, alprazolam and triazolam, by conflict and non-matching to sample tests in mice. Nihon Shinkei Seishin Yakurigaku Zasshi 17, 1-6.

[L]

Lader MH, Gelder MG, Marks IM (1967). Palmar skin conductance measures as predictors of response to desensitization. J Psychosom Res 11, 283-290.

Lang PJ, Bradley MM, Cuthbert BN (1998). Emotion, motivation, and anxiety: brain mechanisms and psychophysiology. Biol Psychiatry 44, 1248-1263.

Leonardo ED and Hen R (2006). Genetics of affective and anxiety disorders. Annu Rev Psychol 57, 117-137.

Leussis MP and Bolivar VJ (2006). Habituation in rodents: A review of behavior, neurobiology, and genetics. Neurosci Biobehav Rev 30, 1045-1064.

Li S, Wang C, Wang W, Dong H, Hou P, Tang Y (2008). Chronic mild stress impairs cognition in mice: from brain homeostasis to behavior. Life Sci 82, 934-942.

Liebsch G, Landgraf R, Engelmann M, Lorscher P, Holsboer F (1999). Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. J Psychiatr Res 33, 153-163.

Lim LW, Temel Y, Sesia T, Vlamings R, Visser-Vandewalle V, Steinbusch HW et al. (2008). Buspirone induced acute and chronic changes of neural activation in the periaqueductal gray of rats. Neuroscience 155, 164-173.

Litvin Y, Pentkowski NS, Blanchard DC, Blanchard RJ (2007). CRF type 1 receptors in the dorsal periaqueductal gray modulate anxiety-induced defensive behaviors. Horm Behav 52, 244-251.

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.

Liu RC, Linden JF, Schreiner CE (2006). Improved cortical entrainment to infant communication calls in mothers compared with virgin mice. Eur J Neurosci 23, 3087-3097.

Livesey PJ (1986). Learning and emotion: a biological synthesis. Evolutionary Processes 1. Lawrence Erlbaum Associates.

Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005). Modulation of anxiety circuits by serotonergic systems. Stress 8, 233-246.

Ludbrook J (1991). On making multiple comparisons in clinical and experimental pharmacology and physiology. Clin Exp Pharmacol Physiol 18, 379-392.

Lyte M, Opitz N, Goehler LE, Gaykema RP, Overmier JB (2005). Recommended housing conditions and test procedures can interact to obscure a significant experimental effect. Behav Res Methods 37, 651-656.

[M]

Makino J, Kato K, Maes FW (1991). Temporal Structure of Open-Field Behavior in Inbred Strains of Mice. Japanese Psychological Research 33, 145-152.

Makino S, Hashimoto K, Gold PW (2002). Multiple feedback mechanisms activating corticotropin-releasing hormone system in the brain during stress. Pharmacol Biochem Behav 73, 147-158.

Makowska IJ and Weary DM (2009). Rat aversion to induction with inhalant anaesthetics. Applied Animal Behaviour Science 119, 229-235.

Mao QQ, Ip SP, Ko KM, Tsai SH, Che CT (2009). Peony glycosides produce antidepressant-like action in mice exposed to chronic unpredictable mild stress: Effects on hypothalamic-pituitary-adrenal function and brain-derived neurotrophic factor. Prog Neuropsychopharmacol Biol Psychiatry 33, 1211-1216.

Marin MT, Cruz FC, Planeta CS (2007). Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. Physiol Behav 90, 29-35.

Mathew SJ, Coplan JD, Schoepp DD, Smith EL, Rosenblum LA, Gorman JM (2001). Glutamate-hypothalamic-pituitary-adrenal axis interactions: implications for mood and anxiety disorders. CNS Spectr 6, 555-561.

McEwen BS and Stellar E (1993). Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153, 2093-2101.

McEwen BS (1999). Stress and hippocampal plasticity. Annu Rev Neurosci 22, 105-122.

McEwen BS (2000). The neurobiology of stress: from serendipity to clinical relevance. Brain Res 886, 172-189.

McKinney WT, Jr. and Bunney WE, Jr. (1969). Animal model of depression. I. Review of evidence: implications for research. Arch Gen Psychiatry 21, 240-248.

McNaughton N (1997). Cognitive dysfunction resulting from hippocampal hyperactivity--a possible cause of anxiety disorder? Pharmacol Biochem Behav 56, 603-611.

McNaughton N and Corr PJ (2004). A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance. Neurosci Biobehav Rev 28, 285-305.

Melia KR, Ryabinin AE, Schroeder R, Bloom FE, Wilson MC (1994). Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. J Neurosci 14, 5929-5938.

Mikkelsen JD, Soderman A, Kiss A, Mirza N (2005). Effects of benzodiazepines receptor agonists on the hypothalamic-pituitary-adrenocortical axis. Eur J Pharmacol 519, 223-230.

Mills DS (2008). Recognising the nature of problem behaviour. Vet J 176, 127-128.

Millstein RA, Ralph RJ, Yang RJ, Holmes A (2006). Effects of repeated maternal separation on prepulse inhibition of startle across inbred mouse strains. Genes Brain Behav 5, 346-354.

Millstein RA and Holmes A (2007). Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. Neurosci Biobehav Rev 31, 3-17.

Mineur YS, Belzung C, Crusio WE (2006). Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. Behav Brain Res 175, 43-50.

Momose K, Inui A, Asakawa A, Ueno N, Nakajima M, Fujimiya M et al. (1999). Intracerebroventricularly administered corticotropin-releasing factor inhibits food intake and produces anxiety-like behaviour at very low doses in mice. Diabetes Obes Metab 1, 281-284.

Mongeau R, Miller GA, Chiang E, Anderson DJ (2003). Neural correlates of competing fear behaviors evoked by an innately aversive stimulus. J Neurosci 23, 3855-3868.

Montkowski A, Poettig M, Mederer A, Holsboer F (1997). Behavioural performance in three substrains of mouse strain 129. Brain Res 762, 12-18.

Morgan MA and LeDoux JE (1995). Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. Behav Neurosci 109, 681-688.

Morrow BA, Elsworth JD, Lee EJ, Roth RH (2000). Divergent effects of putative anxiolytics on stress-induced fos expression in the mesoprefrontal system of the rat. Synapse 36, 143-154.

Morse HC (1978). Origins of inbred mice. Academic Press.

Muigg P, Hoelzl U, Palfrader K, Neumann I, Wigger A, Landgraf R et al. (2007). Altered Brain Activation Pattern Associated With Drug-Induced Attenuation of Enhanced Depression-Like Behavior in Rats Bred for High Anxiety. Biol Psychiatry 61, 782-796.

Muigg P, Scheiber S, Salchner P, Bunck M, Landgraf R, Singewald N (2009). Differential stress-induced neuronal activation patterns in mouse lines selectively bred for high, normal or low anxiety. PLoS ONE 4, e5346.

Muller U, Cristina N, Li ZW, Wolfer DP, Lipp HP, Rulicke T et al. (1994). Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. Cell 79, 755-765.

[N]

Nemeroff CB (1988). The role of corticotropin-releasing factor in the pathogenesis of major depression. Pharmacopsychiatry 21, 76-82.

Nguyen NK, Keck ME, Hetzenauer A, Thoeringer CK, Wurst W, Deussing JM et al. (2006). Conditional CRF receptor 1 knockout mice show altered neuronal activation pattern to mild anxiogenic challenge. Psychopharmacology (Berl) 188, 374-385.

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.

[O]

O'Keefe J (1999). Do hippocampal pyramidal cells signal non-spatial as well as spatial information? Hippocampus 9, 352-364.

Ohl F, Holsboer F, Landgraf R (2001a). The modified hole board as a differential screen for behavior in rodents. Behav Res Methods Instrum Comput 33, 392-397.

Ohl F, Sillaber I, Binder E, Keck ME, Holsboer F (2001b). Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test. J Psychiatr Res 35, 147-154.

Ohl F, Toschi N, Wigger A, Henniger MS, Landgraf R (2001c). Dimensions of emotionality in a rat model of innate anxiety. Behav Neurosci 115, 429-436.

Ohl F, Roedel A, Storch C, Holsboer F, Landgraf R (2002). Cognitive performance in rats differing in their inborn anxiety. Behav Neurosci 116, 464-471.

Ohl F, Roedel A, Binder E, Holsboer F (2003). Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. Eur J Neurosci 17, 128-136.

Ohl F (2005). Animal models of anxiety. Handb Exp Pharmacol 35-69.

Ohl F, Arndt SS, van der Staay FJ (2008). Pathological anxiety in animals. Vet J 175, 18-26.

Oshima A, Flachskamm C, Reul JM, Holsboer F, Linthorst AC (2003). Altered serotonergic neurotransmission but normal hypothalamic-pituitary-adrenocortical axis activity in mice chronically treated with the corticotropin-releasing hormone receptor type 1 antagonist NBI 30775. Neuropsychopharmacology 28, 2148-2159.

[P]

Palanza P (2001). Animal models of anxiety and depression: how are females different? Neurosci Biobehav Rev 25, 219-233.

Paylor R, Morrison SK, Rudy JW, Waltrip LT, Wehner JM (1992). Brief exposure to an enriched environment improves performance on the Morris water task and increases hippocampal cytosolic protein kinase C activity in young rats. Behav Brain Res 52, 49-59.

Pellow S, Chopin P, File SE, Briley M (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14, 149-167.

Pham TM, Ickes B, Albeck D, Soderstrom S, Granholm AC, Mohammed AH (1999). Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. Neuroscience 94, 279-286.

Phelps EA and LeDoux JE (2005). Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48, 175-187.

Piazza PV, Deminiere JM, Maccari S, Mormede P, Le MM, Simon H (1990). Individual reactivity to novelty predicts probability of amphetamine self-administration. Behav Pharmacol 1, 339-345.

Pietraszek M, Sukhanov I, Maciejak P, Szyndler J, Gravius A, Wislowska A et al. (2005). Anxiolytic-like effects of mGlu1 and mGlu5 receptor antagonists in rats. European Journal of Pharmacology 514, 25-34.

Pintor A, Pezzola A, Reggio R, Quarta D, Popoli P (2000). The mGlu5 receptor agonist CHPG stimulates striatal glutamate release: possible involvement of A2A receptors. Neuroreport 11, 3611-3614.

Platel A and Porsolt RD (1982). Habituation of exploratory activity in mice: a screening test for memory enhancing drugs. Psychopharmacology (Berl) 78, 346-352.

Pryce C, Mohammed A, Feldon J (2002). Environmental manipulations in rodents and primates. Insights into pharmacology, biochemistry and behaviour. Pharmacol Biochem Behav 73, 1-5.

[Q]

Quirk GJ and Gehlert DR (2003). Inhibition of the amygdala: key to pathological states? Ann N Y Acad Sci 985, 263-272.

Quirk GJ, Garcia R, Gonzalez-Lima F (2006). Prefrontal mechanisms in extinction of conditioned fear. Biol Psychiatry 60, 337-343.

[R]

Robertson HA (1979). Benzodiazepine receptors in "emotional" and "non-emotional" mice; comparison of four strains. Eur J Pharmacol 56, 163-166.

Rodgers RJ and Cole JC (1993). Influence of social isolation, gender, strain, and prior novelty on plus-maze behaviour in mice. Physiol Behav 54, 729-736.

Rodgers RJ, Johnson NJ, Carr J, Hodgson TP (1997). Resistance of experientially-induced changes in murine plus-maze behaviour to altered retest conditions. Behav Brain Res 86, 71-77.

Rodgers RJ, Boullier E, Chatzimichalaki P, Cooper GD, Shorten A (2002a). Contrasting phenotypes of C57BL/6JOlaHsd, 129S2/SvHsd and 129/SvEv mice in two exploration-based tests of anxiety-related behaviour. 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 behavioural baseline. Genes Brain Behav 1, 242-251.

Romano C, Sesma MA, McDonald CT, O'Malley K, van den Pol AN, Olney JW (1995). Distribution of metabotropic glutamate receptor mGluR5 immunoreactivity in rat brain. J Comp Neurol 355, 455-469.

Rosenzweig MR (1998). Proceedings of the 6th conference on the neurobiology of learning and memory. Brain and memory: from genes to behavior. Some historical background of topics in this conference. Neurobiol Learn Mem 70, 3-13.

Rossler AS, Joubert C, Chapouthier G (2000). Chronic mild stress alleviates anxious behaviour in female mice in two situations. Behav Processes 49, 163-165.

Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW (1993). Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci U S A 90, 6591-6595.

Ryabinin AE, Wang YM, Finn DA (1999). Different levels of Fos immunoreactivity after repeated handling and injection stress in two inbred strains of mice. Pharmacol Biochem Behav 63, 143-151.

[S]

Sagar SM, Sharp FR, Curran T (1988). Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240, 1328-1331.

Salomons AR, Arndt SS, Ohl F (2009). Anxiety in relation to animal enviornment and welfare. Scandinavian Journal of Laboratory Animal Science 36: 37-45.

Salomons AR, Bronkers G, Kirchhoff S, Arndt SS, Ohl F (2010a). Behavioural habituation to novelty and brain area specific immediate early gene expression in female mice of two inbred strains. Behav Brain Res 215, 95-101.

Salomons AR, Kortleve T, Reinders NR, Kirchhoff S, Arndt SS, Ohl F (2010b). Susceptibility of a potential animal model for pathological anxiety to chronic mild stress. Behav Brain Res 209, 241-248.

Salomons AR, van Luijk JA, Reinders NR, Kirchhoff S, Arndt SS, Ohl F (2010c). Identifying emotional adaptation: behavioural habituation to novelty and immediate early gene expression in two inbred mouse strains. Genes Brain Behav 9, 1-10.

Sapolsky RM (2003). Stress and plasticity in the limbic system. Neurochem Res 28, 1735-1742.

Schmidt MV, Oitzl MS, Muller MB, Ohl F, Wurst W, Holsboer F et al. (2003). Regulation of the developing hypothalamic-pituitary-adrenal axis in corticotropin releasing hormone receptor 1-deficient mice. Neuroscience 119, 589-595.

Schweizer MC, Henniger MS, Sillaber I (2009). Chronic mild stress (CMS) in mice: of anhedonia, 'anomalous anxiolysis' and activity. PLoS ONE 4, e4326.

Shader RI and Greenblatt DJ (1993). Use of benzodiazepines in anxiety disorders. N Engl J Med 328, 1398-1405.

Shah AA, Sjovold T, Treit D (2004). Inactivation of the medial prefrontal cortex with the GABAA receptor agonist muscimol increases open-arm activity in the elevated plus-maze and attenuates shock-probe burying in rats. Brain Res 1028, 112-115.

Sheehan TP, Chambers RA, Russell DS (2004). Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res Brain Res Rev 46, 71-117.

Shekhar A (1994). Effects of treatment with imipramine and clonazepam on an animal model of panic disorder. Biol Psychiatry 36, 748-758.

Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, Mizuno N (1993). Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. Neurosci Lett 163, 53-57.

Shin LM and Liberzon I (2010). The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology 35, 169-191.

Sik A, van NP, Prickaerts J, Blokland A (2003). Performance of different mouse strains in an object recognition task. Behav Brain Res 147, 49-54.

Simon AB and Gorman JM (2006). Advances in the treatment of anxiety: targeting glutamate. NeuroRx 3, 57-68.

Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE, Sharp JJ (1997). Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nat Genet 16, 19-27.

Singewald N, Salchner P, Sharp T (2003). Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. Biol Psychiatry 53, 275-283.

Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH et al. (1998). Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 20, 1093-1102.

Soffie M, Hahn K, Terao E, Eclancher F (1999). Behavioural and glial changes in old rats following environmental enrichment. Behav Brain Res 101, 37-49.

Somers JM, Goldner EM, Waraich P, Hsu L (2006). Prevalence and incidence studies of anxiety disorders: a systematic review of the literature. Can J Psychiatry 51, 100-113.

Spooren WP, Gasparini F, Salt TE, Kuhn R (2001). Novel allosteric antagonists shed light on mglu(5) receptors and CNS disorders. Trends Pharmacol Sci 22, 331-337.

Spooren WP, Vassout A, Neijt HC, Kuhn R, Gasparini F, Roux S et al. (2000). Anxiolytic-like effects of the prototypical metabotropic glutamate receptor 5 antagonist 2-methyl-6-(phenylethynyl) pyridine in rodents. J Pharmacol Exp Ther 295, 1267-1275.

Steciuk M, Kram M, Kramer GL, Petty F (1999). Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats. Brain Res 822, 256-259.

Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW (1994). Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci 14, 2579-2584.

Sterling P & Eyer J (1988) Allostasis: a new paradigm to explain arousal pathology. New York, Wiley.

Strekalova T, Spanagel R, Dolgov O, Bartsch D (2005). Stress-induced hyperlocomotion as a confounding factor in anxiety and depression models in mice. Behav Pharmacol 16, 171-180.

Sullivan RM and Gratton A (2002). Behavioral effects of excitotoxic lesions of ventral medial prefrontal cortex in the rat are hemisphere-dependent. Brain Res 927, 69-79.

[T]

Takahashi LK (2001). Role of CRF(1) and CRF(2) receptors in fear and anxiety. Neurosci Biobehav Rev 25, 627-636.

Tang X, Orchard SM, Sanford LD (2002). Home cage activity and behavioral performance in inbred and hybrid mice. Behav Brain Res 136, 555-569.

Tang X and Sanford LD (2005). Home cage activity and activity-based measures of anxiety in 129P3/J, 129X1/SvJ and C57BL/6J mice. Physiol Behav 84, 105-115.

Tatarczynska E, Klodzinska A, Chojnacka-Wojcik E, Palucha A, Gasparini F, Kuhn R et al. (2001). Potential anxiolytic- and antidepressant-like effects of MPEP, a potent, selective and systemically active mGlu5 receptor antagonist. Br J Pharmacol 132, 1423-1430.

Ter Horst GJ, Wichmann R, Gerrits M, Westenbroek C, Lin Y (2009). Sex differences in stress responses: focus on ovarian hormones. Physiol Behav 97, 239-249.

The Jackson Laboratory (2010). Tips for poor breeders.http://jaxmice.jax.org/support/husbandry/poor-breeders.html .

Thiel CM, Muller CP, Huston JP, Schwarting RK (1999). High versus low reactivity to a novel environment: behavioural, pharmacological and neurochemical assessments. Neuroscience 93, 243-251.

Thomas LS, Jane DE, Gasparini F, Croucher MJ (2001). Glutamate release inhibiting properties of the novel mGlu(5) receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP): complementary in vitro and in vivo evidence. Neuropharmacology 41, 523-527.

Thompson RF and Spencer WA (1966). Habituation: a model phenomenon for the study of neuronal substrates of behavior. Psychol Rev 73, 16-43.

Tomaz C, ckinson-Anson H, McGaugh JL (1992). Basolateral amygdala lesions block diazepam-induced anterograde amnesia in an inhibitory avoidance task. PROC NATL ACAD SCI U S A 89, 3615-3619.

Treit D (1985). Animal models for the study of anti-anxiety agents: a review. Neurosci Biobehav Rev 9, 203-222.

Treit D and Fundytus M (1988). Thigmotaxis as a test for anxiolytic activity in rats. Pharmacol Biochem Behav 31, 959-962.

Treit D, Menard J, Royan C (1993). Anxiogenic stimuli in the elevated plus-maze. Pharmacol Biochem Behav 44, 463-469.

Tuli JS, Smith JA, Morton DB (1995). Stress measurements in mice after transportation. Lab Anim 29, 132-138.

[V]

Valle FP (1970). Effects of strain, sex, and illumination on open-field behavior of rats. Am J Psychol 83, 103-111.

van de Weerd HA and Baumans V (1995). Environmental Enrichment in Rodents. In:. Environmental enrichment information resources for laboratory animals. AWIC Resource series 2, 145-149.

van de Weerd HA, Van Loo PL, van Zutphen LF, Koolhaas JM, Baumans V (1997). Nesting material as environmental enrichment has no adverse effects on behavior and physiology of laboratory mice. Physiol Behav 62, 1019-1028.

van der Staay FJ, Arndt SS, Nordquist RE (2009). Evaluation of animal models of neurobehavioral disorders. Behav Brain Funct 5, 11.

van Loo PL, Van Zutphen LF, Baumans V (2003). Male management: Coping with aggression problems in male laboratory mice. Lab Anim 37, 300-313.

van Oortmerssen GA (1971). Biological significance, genetics and evolutionary origin of variability in behaviour within and between inbred strains of mice (Mus musculus). A behaviour genetic study. Behaviour 38, 1-92.

Voikar V, Polus A, Vasar E, Rauvala H (2005). Long-term individual housing in C57BL/6J and DBA/2 mice: assessment of behavioral consequences. Genes Brain Behav 4, 240-252.

[W]

Whitaker J, Moy SS, Saville BR, Godfrey V, Nielsen J, Bellinger D et al. (2007). The effect of cage size on reproductive performance and behavior of C57BL/6 mice. Lab Anim (NY) 36, 32-39.

Wilensky AE, Schafe GE, Kristensen MP, LeDoux JE (2006). Rethinking the fear circuit: The central nucleus of the amygdala is required for the acquisition, consolidation, and expression of pavlovian fear conditioning. Journal of Neuroscience 26, 12387-12396.

Willner P (1997). Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. Psychopharmacology (Berl) 134, 319-329.

Willner P (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology 52, 90-110.

Winocur G (1998). Environmental influences on cognitive decline in aged rats. Neurobiol Aging 19, 589-597.

Woods JH (1998). Problems and opportunities in regulation of benzodiazepines. J Clin Pharmacol 38, 773-782.

Wu LJ, Kim SS, Zhuo M (2008).
Molecular targets of anxiety: from membrane to nucleus. Neurochem Res 33, 1925-1932.

[Z]

Zender R and Olshansky E (2009).
Women's mental health: depression and anxiety. Nurs Clin North Am 44, 355-364.

Chapter

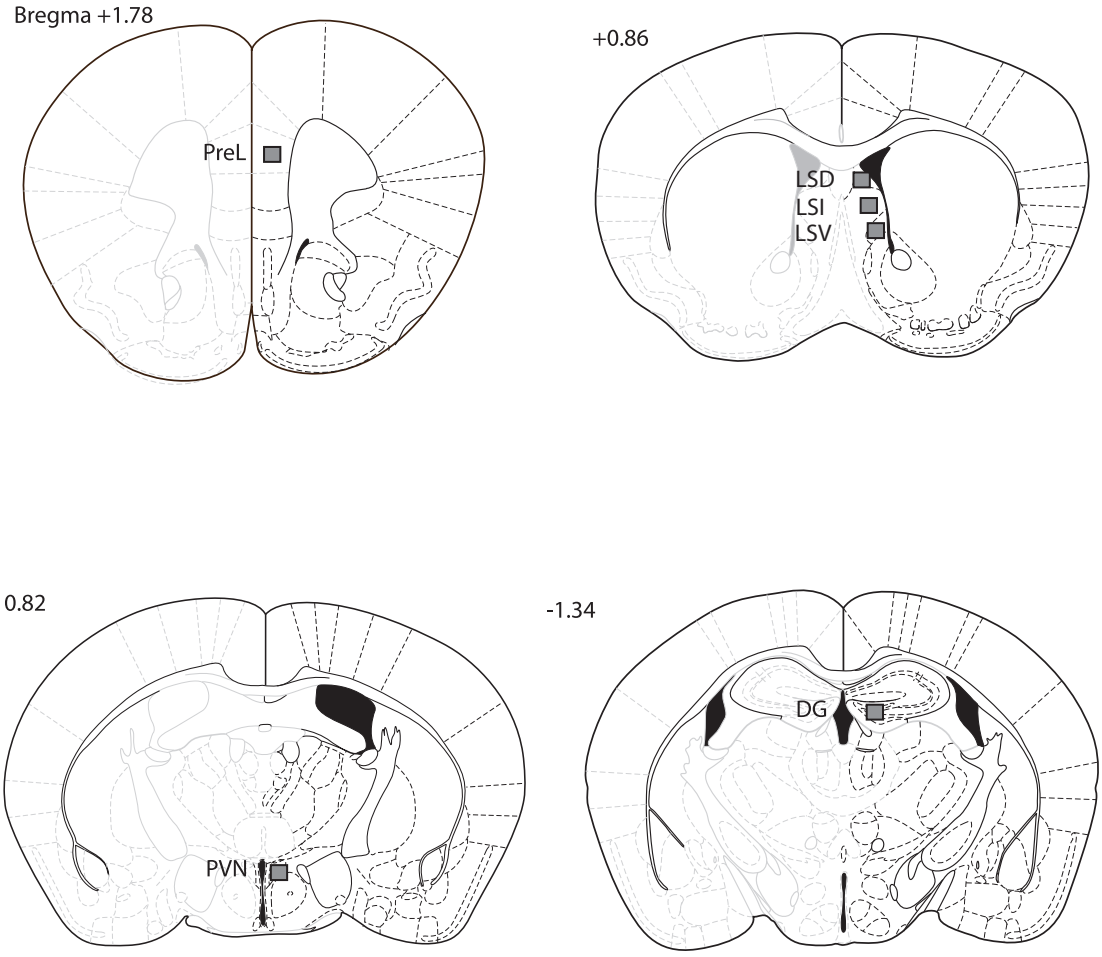
10

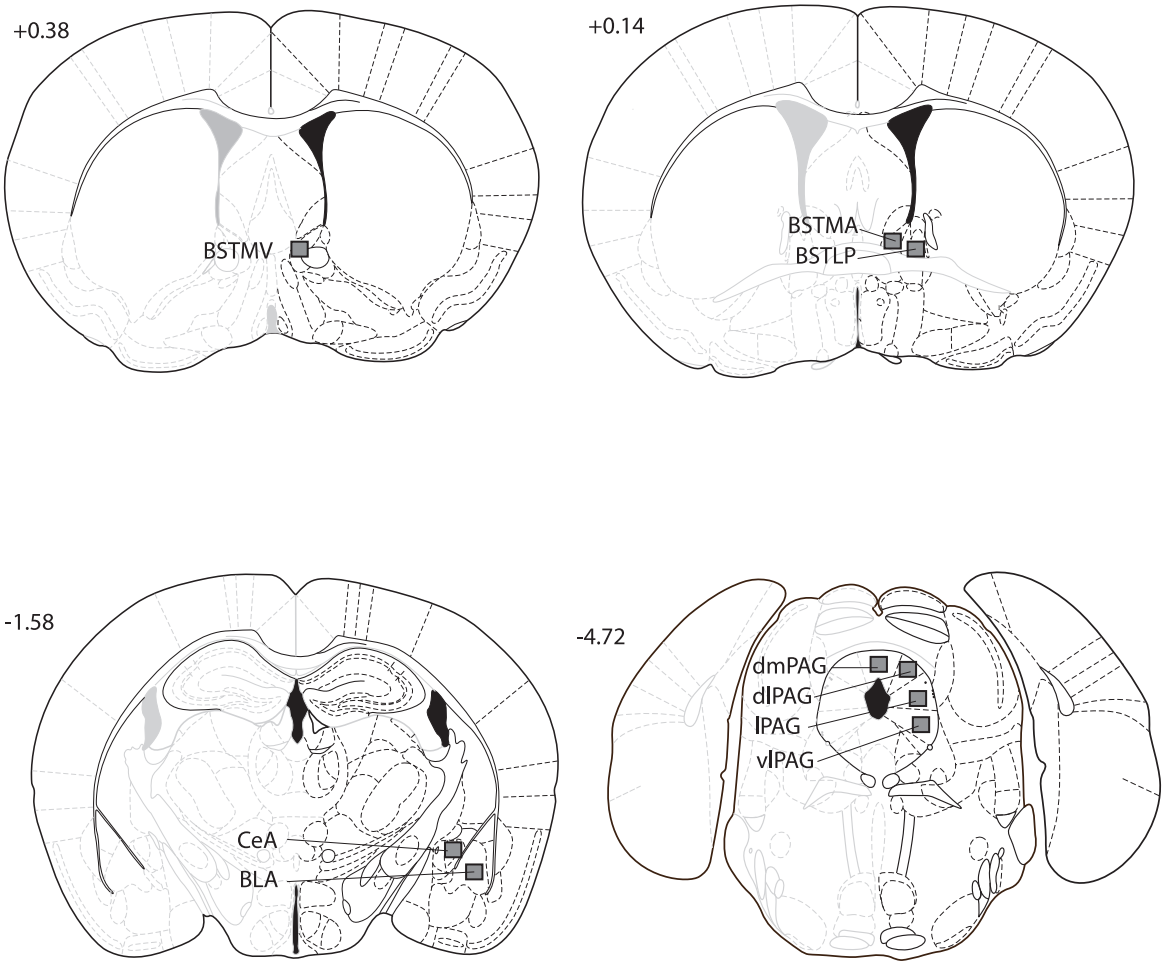
Appendix

List of abbreviations	
129P3	129P3/J
Amy	amygdala
ANOVA	analyses of variance
BALB/c	BALB/cJ
BLA	basolateral nucleus of the amygdala
BST	bed nucleus of the stria terminalis
BSTLP	lateral posterior part of the bed nucleus stria terminalis
BSTMA	medial anterior part of the bed nucleus stria terminalis
BSTMV	medial ventral part of the bed nucleus stria terminalis
CeA	central nucleus of the amygdala
CMS	chronic mild stress
CORT	corticosterone
CRFR1	corticotropin-releasing-factor receptor 1
DAB	diaminobenzidine tetrahydrochloride
DG	dentate gyrus
DIG	digoxigenin
dlPAG	dorsolateral part of the periaqueductal gray
DMH	dorsomedial hypothalamus
dmPAG	dorsomedial part of the periaqueductal gray
EPM	elevated plus maze
GABA	gamma aminobutyric acid
IEG	immediate early gene
IQR	interquartile range
lPAG	lateral part of the periaqueductal gray
LS	lateral septum
LSD	dorsal part of the lateral septum
LSI	intermedial part of the lateral septum
LSV	ventral part of the lateral septum
mGlu5	metabotropic glutamate receptor 5
mHB	modified hole board
MPEP	2-methyl-6-(phenylethynyl) pyridine
mRNA	messenger ribonucleic acid
OF	open field
ORT	object recognition test
PAG	periaqueductal gray
PreL	prelimbic cortex
PVN	paraventricular nucleus
RMA	repeated measures ANOVA
SEM	standard error of the mean

Brain areas

Schematic drawings of the investigated brain areas





Schematic drawings were adapted from the mouse brain atlas by Franklin and Paxinos (1997).
BLA: basolateral amygdala; BSTLP; lateral posterior part of the bed nucleus terminalis; BSTMA medial anterior part of the bed nucleus terminalis; BSTMV: medial ventral part of the bed nucleus terminalis; CeA: central nucleus of the amygdala; dlPAG: dorsolateral part of the periaqueductal gray; DMH: dorsomedial hypothalamus; dmPAG: dorsomedial part of the periaqueductal gray; lPAG: lateral part of the periaqueductal gray; LSD: dorsal part of the lateral septum; LSI: intermedial part of the lateral septum; LSV: ventral part of the lateral septum; PreL: prelimbic cortex; PVN: paraventricular nucleus; vlPAG: ventrolateral part of the periaqueductal gray.

Chapter

11

Nederlandse samenvatting

Nederlandse samenvatting

Angst is een biologische zinvolle reactie op potentiële dreiging en helpt mens en dier te overleven. Echter angststoornissen in mensen komen vrij veel voor en hoewel er wel enkele risicofactoren bekend zijn, blijven de achterliggende neurobiologische oorzaken onduidelijk. Om deze mechanismen te onderzoeken wordt vaak gebruik gemaakt van diermodellen. Niettemin meten veelgebruikte diermodellen voor angst normale adaptieve angst en kunnen dus geen uitspraken gedaan worden over pathologische angst.
Daarbij rijst de vraag of dieren überhaupt pathologisch angstig kunnen zijn. Een gepast diermodel voor pathologische angst zou niet al.leen van waarde zijn voor de studie van humane angststoornissen, maar zou dus ook van betekenis kunnen zijn voor dierenwelzijn.
In dit proefschrift staat de vraag centraal of gedragsmatige habituatie aan een testomgeving gebruikt kan worden om pathologische angst bij muizen te identificeren. Wanneer een dier niet kan wennen na herhaalde blootstelling aan een testomgeving en dus een gebrek aan habituatie laat zien, zou dit indicatief kunnen zijn voor pathologische angst. Naast gedrag, werden ook stresshormonen gemeten en c-Fos, een eiwit indicator voor neurale activiteit werd onderzocht in hersengebieden betrokken bij angst.
In hoofdstuk 3 hebben we twee inteelt muistammen, waarvan bekend is dat ze verschillen in initieel angst gedrag, herhaaldelijk getest in de modified hole board test getest (mHB). BALB/c muizen die initieel erg angstig bleken, lieten een duidelijk habituatie patroon zien, wat zich uitte in een verminderd vermijdingsgedrag van het aversieve gedeelte van de mHB en een verhoging van locomotorisch- en exploratief gedrag over de tijd. In tegenstelling lieten 129P3 muizen, die initieel lager angstiger waren, juist een verhoging van hun angstgedrag over de tijd zien, terwijl locomotie en exploratie op hetzelfde niveau bleven. De 129P3 muis liet dus duidelijk een gebrek aan habituatie zien. Hieruit trokken we de conclusie dat herhaaldelijk testen een indicatie kan geven over adaptief en niet adaptief angst gerelateerd gedrag. In aanvulling op de gedragsmetingen hebben we gekeken naar activiteit in hersengebieden betrokken bij angst. De neurale marker c-Fos komt tot expressie na blootstelling aan verscheidene stimuli, zoals een nieuwe omgeving. Hiermee kan gekeken worden welke hersengebieden actief zijn. BALB/c muizen lieten een hogere c-Fos expressie in de prelimbische cortex en laterale septum zien, twee gebieden betrokken bij de integratie van emotionele en cognitieve processen. In combinatie met het non-adaptieve gedragsprofiel, suggereerden deze stamverschillen dat 129P3 muizen een probleem hebben met de integratie van cognitieve en emotionele processen, waardoor ze te weinig cognitieve controle over hun angst hebben.
In hoofdstuk 4 hebben we gekeken of chronische milde stress het gebrek aan habituatie in 129P3 muizen kon beïnvloeden.

Het is bekend dat chronische stress variërende effecten kan hebben op angstgedrag. Na een periode van veertien dagen milde stress, lieten 129P3 muizen niet al.leen het bekende gebrek aan habituatie zien, maar waren naast het angstgedrag ook andere gedragsdimensies betrokken. Interessant was dat het initieel angst niveau onveranderd bleef na een periode van chronische milde stress wat nog eens duidelijk maakt dat adaptieve capaciteit niet gemeten kan worden na eenmalig testen. Hogere c-Fos expressie werd gevonden in de bed nucleus stria terminalis en de periaqueductale grijs van gestreste dieren. Deze eerste is een belangrijk tussenstation in de stress response en de tweede speelt een rol bij de passieve emotionele coping reactie. Deze verschillen suggereren dat 129P3 muizen gevoeliger zijn voor stress wat uiteindelijk leidde to een versterking van het gebrek aan habituatie.
In hoofdstuk 5 hebben we gekeken of de vrouwelijke individuen van de twee muizen stammen een zelfde gedragprofiel laten zien als hun mannelijke tegenhangers. Vrouwelijke BALB/c muizen hadden een duidelijk eenzelfde habituatie patroon, terwijl vrouwelijke 129P3 muizen een gebrek aan habituatie lieten zien. Interessant was dat vrouwelijke 129P3 muizen een sterker gebrek aan habituatie hadden dan mannelijke individu's, wat zich uitte in lagere locomotie op gedragsniveau en een lagere c-Fos expressie op hersenenniveau (prelimbische cortex en hippocampus). Het lijkt er dus op dat vrouwelijke 129P3 muizen minder in staat zijn te habitueren dan hun mannelijke soortgenoten. Interessant gegeven is dat in de humane situatie indicaties bestaan dat vrouwen gevoeliger zijn voor de ontwikkeling van angststoornissen.
In hoofdstuk 6 hebben we de hypothese getest of het gebrek aan habituatie primair veroorzaakt wordt door een hoog angst niveau, omdat habituatie bijvoorbeeld ook door cognitieve vaardigheden beïnvloed kan worden. Daartoe hebben we de mannelijke muizen voor het testen behandeld met twee anxiolytica (angst reducerend), diazepam en MPEP en vervolgens hebben we gekeken naar habituatie in de twee angsttesten (open field, elevated plus maze) en een cognitief object recognitie test. Diazepam, een klassieke benzodiazepine werkte vooral angst reducerend in BALB/c muizen, terwijl het een sterk sedatief effect had in 129P3 muizen. In tegenstelling, MPEP, een metabotrope glutamaat 5 (mGlu5) antagonist oefende voornamelijk een angst verlagende werking uit in 129P3 muizen en verbeterde habituatie in deze stam, terwijl MPEP in BALB/c muizen vrijwel geen effect had. En verhoging van c-Fos expressie in de prelimbische cortex en een verlaging van c-Fos expressie in de emotie regulerende amygdala na MPEP behandeling in 129P3 muizen, suggereerde een rol van mGlu5 receptoren in het non-adaptieve fenotype. Daarnaast verbeterde MPEP object recognitie in 129P3 muizen. Dit leidde tot de hypothese dat het non-adaptieve fenotype van 129P3 muizen tot stand komt door een

verslechterde neuro-transmissie tussen de prelimbische cortex en emotionele hersengebieden, veroorzaakt door glutamaat transmissie. Deze hypothese werd deels onderzocht in hoofdstuk 7, waarin we de regulatie van mGlu5 receptoren hebben gemeten in de prelimbische cortex en amygdala in mannelijke naïeve en geteste dieren.
Omdat glutamaat de belangrijkste exciterende neurotransmitter in het brein is en vele processen beïnvloed, achtte we glutamaat niet als enige speler in het in het non-adaptieve fenotype.
Een van belangrijkste neuromodulators betrokken bij angst is de corticotropin-releasing factor (CRF). CRF en zijn receptoren, in het bijzonder receptor 1 (CRFR1) spelen een belangrijke rol bij de angst response. In vitro resultaten suggereren dat CRF peptiden glutamaat transmissie kunnen beïnvloeden onder normale en stressvolle omstandigheden. In hoofdstuk 7 beschrijven we de eerste voorlopige resultaten van mRNA expressie in de prelimbische cortex en amygdala in BALB/c en 129P3 muizen onder naïeve condities en na herhaaldelijk testen in de mHB. Uit deze resultaten blijkt dat een verlaging van CRFR1 mRNA expressie in de prelimbische cortex in verband kan worden gebracht met habituatie. In mGlu5 mRNA expressie vonden we geen verschillen, echter de rol van mGlu5R in habituatie kunnen we op dit moment nog niet uitsluiten en meerdere hersengebieden moeten nog onderzocht worden.
De resultaten van alle experimenten in het licht van de centrale onderzoeksvraag worden besproken in hoofdstuk 8. Uit de resultaten blijkt dat 129P3 muizen een non-adaptief gedragsprofiel laten zien, wat waarschijnlijk wordt veroorzaakt door een beperkte integratie van cognitieve informatie met de emotionele perceptie.
Een verlaging van CRFR1 mRNA expressie in de prelimbische cortex wijst erop dat CRFR1 een rol speelt in dit specifieke fenotype.
De vraag of muizen pathologische angst kunnen vertonen, lijkt hierop positief beantwoord. De 129P3 muizen zijn daarom een potentieel translationeel diermodel voor pathologische angst. Echter, voordat ze als diermodel gebruikt kunnen worden, moeten ze aan een drietal criteria voldoen. De eerste daarvan is dat symptomen overeen moeten komen met de humane symptomen van pathologische angst. De karakteristiek van 129P3 muizen is hun gebrek aan habituatie, een eigenschap wat ook in humane angststoornissen wordt gevonden. In de humane situatie blijkt dat zeer angstige mensen vaak geen onderscheid kunnen maken tussen wat veilig is en wat niet. Daarmee lijken 129P3 muizen aan dit eerste criterium te voldoen met betrekking tot het gebrek aan habituatie.
Niettemin moet het fenotype op andere eigenschappen worden onderzocht, zoals hun cognitieve vermogens. Het tweede criterium betreft dat een diermodel voor angst gevoelig is voor klinisch gevalideerde anxiolytica. In onze studies hebben we laten zien dat de 129P3 muis gevoelig is voor angst reducerende drugs, en daarmee voldoen aan dit criterium.

Ook hier geldt, dat meer onderzoek nodig is, met name de effecten van langdurige behandeling moeten onderzocht worden. Het derde en laatste criterium betreft het onderliggende mechanisme. Echter aan dit criterium wordt vaak niet voldaan, omdat het onderzoek naar psychiatrische stoornissen in diermodellen juist vaak wordt gedaan om onderliggende mechanismen te ontrafelen. Wij kwamen tot de hypothese dat het fenotype van 129P3 muizen veroorzaakt wordt door een verstoorde integratie van cognitieve en emotionele processen, waarin voornamelijk de verminderde cognitieve controle door de prelimbische cortex een rol speelt. Interessant gegeven is dat sommige theorieën betreft de oorzaak van humane angststoornissen gebaseerd zijn op een primaire cognitieve deficiëntie. Toch blijft het de vraag of modulatie van cognitieve processen het habituatie profiel kunnen beïnvloeden.
In hoofdstuk 8 wordt verder besproken wat het gevolg is van de 129P3 fenotype op hun welzijn. Wanneer angst de normale levenstandaard beïnvloedt kan ook welzijn in het gedrang raken. Terwijl BALB/c muizen zich na een initiële hoge angstresponse prima lijken aan te passen is dit niet het geval voor 129P3 muizen. De vraag rijst dan ook of het welzijn van 129P3 muizen gegarandeerd kan worden onder de standaard huisvestings en experimentele condities die wij in de studies hebben toegepast. Er is echter weinig bekend hoe de adaptieve capaciteit in laboratorium dieren bevorderd kan worden. Toekomstig onderzoek zal zich dan ook moeten wijden aan de vraag hoe het emotionele fenotype van 129P3 muizen tot stand komt en in welke mate de genetische achtergrond of omgevingsfactoren een rol spelen.

Chapter

12

Dankwoord

Dankwoord

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Liefs
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Chapter

About the author and list of publications

About the author

Amber Rasika Salomons was born on the 2nd of December 1981 in Colombo, Sri Lanka. She was adopted by a Dutch family and moved to Emmeloord, the Netherlands at the age of 9 weeks. After having completed her secondary education (VWO) at the Emelwerda College in Emmeloord in 2000, she started her study Biology at the University of Groningen in Groningen, The Netherlands. During her studies she did her first internship at the Department of Animal Physiology under the supervision of Dr. Gertjan van Dijk where she did research on the interaction of the central nervous cholinergic system and the melanocortin system in the regulation of energy balance in rats. In her second research project she studied the behavioural and neurological effects of the metabotropic glutamate receptor 2/3 antagonists in a rat model of schizophrenia under the supervision of Dr. Gabor Imre at the Department of Psychiatry at the University Medical Center Groningen. After a small project at the Department of Behavioral Biology under Dr. Simon Verhulst studying vocalizations in jackdaws she graduated in August 2005 and started as educational assistant at the Department of Animal Physiology, University of Groningen. In April 2006 she started her PhD research at the Department of Animals in Science and Society at the Faculty of Veterinary Medicine at the Utrecht University, The Netherlands under the supervision of Prof dr. Frauke Ohl and Dr. Saskia Arndt of which the results are presented and discussed in this thesis.
At the moment she is still appointed as junior researcher within the same department.

List of publications

AR Salomons, G Bronkers, S Kirchhoff, SS Arndt and F Ohl (2010) Behavioural habituation to novelty and brain area specific immediate early gene expression in female mice of two inbred strains. Behavioural Brain Research 215; 95-101

AR Salomons, TA Kortleve, NR Reinders, S Kirchhoff, SS Arndt and F Ohl (2010) Susceptibility of a potential animal model for pathological anxiety to chronic mild stress. Behavioural Brain Research 209; 241-249

AR Salomons, JAKR van Luijk, NR Reinders, S Kirchhoff, SS Arndt and F Ohl (2010) Identifying emotional adaptation: behavioural habituation to novelty and immediate early gene expression in two inbred mouse strains. Genes Brain and Behavior 9; 1-10

AR Salomons, SS Arndt and F Ohl (2009) Anxiety in relation to animal welfare and environment. Scandinavian Journal of Laboratory Animal Science 36; 37-45

SS Arndt, MC Laarakker, HA van Lith, FJ van der Staay, E Gieling, AR Salomons, JG van't Klooster and F Ohl (2009) Individual housing of mice--impact on behaviour and stress responses. Physiology and Behavior 97; 385-393

Imre G, Salomons A, Jongsma M, Fokkema DS, Den Boer JA, Ter Horst GJ (2006). Effects of the mGluR2/3 agonist LY379268 on ketamine-evoked behaviours and neurochemical changes in the dentate gyrus of the rat. Pharmacology Biochemistry and Behavior 84;:392-9

List of abstracts

AR Salomons, SS Arndt and F Ohl. Identification of pathological anxiety in rodents (2006). Veterinary Science day, Zeist

AR Salomons, SS Arndt and F Ohl (2007). Repeated exposure to a novel environment; evaluating the adaptive capacity in the BALB/c and 129P3/J mouse strain, Rudolf Magnus Institute, Summerschool, Zeist 2006 and the European Brain and Behaviour Society, Trieste

AR Salomons, SS Arndt and F Ohl (2007). Behavioural habituation to a novel environment as indicator for anxiety in BALB/c and 129/J mice. Dutch Endo-Neuro-Psycho meeting, Doorwerth

AR Salomons JG van't Klooster, SS Arndt and F Ohl (2007). Habituation after successive trials in the same test-set-up is dependent on strain and light conditions. Biotechnische dagen, Noordwijk

AR Salomons, SS Arndt and F Ohl (2008). Habituation as indicator for anxiety phenotype in inbred mouse strains. Stress and Behaviour conference, St. Petersburg

AR Salomons, SS Arndt and F.Ohl (2009). Identifying emotional adaptation on inbred mice. Euron workshop Drugs and the brain, an update in psychopharmacology, Braga

AR Salomons, N Espitia Pinzon, NR Reinders, S Kirchhoff, SS Arndt and F Ohl (2009). Characterisation of non-adaptive anxiety in two inbred mouse strains 41st European Brain and Behaviour Society Meeting. Rhodes Island

AR Salomons, N Espitia Pinzon, NR Reinders, S Kirchhoff, SS Arndt and F Ohl (2009). Characterisation of non-adaptive anxiety in male mice. Dutch Endo-Neuro-Psycho Meeting, Doorwerth

AR Salomons, G Bronkers, SS Arndt and F Ohl (2010). Emotional adaptation characterized by behavioral habituation to novelty and immediate early gene expression in male and female mice. Federation of European Neuroscience Society, Amsterdam and Neuroscience and Cognition Research Day, Utrecht