Neurobiology of Stress Adaptation in the Mouse

Roles of Corticotropin-Releasing Factor and Urocortin 1

Layout	Arne Heijenga (www.stukjewebgebeuren.nl) & Anikó Kőrösi
Font	Palladius
Printer	Febodruk BV
ISBN	9039342229

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Neurobiologie van Stress-Adaptatie
in de Muis

Functies van Corticotropin-Releasing Factor en Urocortine 1

(met samenvattingen in het Nederlands en in het Hongaars)

PROEFSCHRIFT
ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op woensdag 26 april 2006 des middags te 12.45 uur

door

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geboren op 14 maart 1977, te Budapest, Hongarije

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This doctoral dissertation was produced with the financial support of:

PsychoGenics Inc., Tarrytown, NY, USA

Paranimfen: *M. Calle*
V.B.E. Jadin

And she's got safety pins and string holding it together

She can only wear her jacket in warm weather

Although the sleeves need to be shortened

The fact that the arms are falling off isn't important

PUSA, 2004

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Chapter 1

General Introduction

Adaptation to stress

Life requires organisms to adapt to changing environmental demands. The body's ability to adapt to external and internal factors that challenge the self-regulation of biological systems, or 'homeostasis' (Cannon, 1914), is essential for survival.

The stress concept, firstly outlined by Selye, refers to the pathophysiological state associated with specific physiological changes that is induced by diverse physiological and psychological stimuli (Selye, 1936). If exposure to a stressor persists or is intensified, the consequences for the animal may be severe, leading to disease or even death. A stressor can be defined as a change in the environment that is sensed by an organism, is aversive and potentially harmful to that organism, and elicits an acute and/or a chronic response (Ottenweller, 2000). Stress involves three events, input of a stimulus, the evaluation of this information, and a response output (Steckler, 2005). The emotional and/or physiological adaptation to any factor that interferes with homeostasis, is in general referred to as the stress response. Adaptation to stress is a complex phenomenon encompassing neuroendocrine, autonomic, physiological and behavioral changes, and for an appropriate adaptation to any challenge, a coordinated response by a number of regulatory, neural and endocrine mechanisms is required. The main systems employed by vertebrates to 'cope' with stress are the autonomic sympatho-adrenal medullar (SAM) axis and the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis (Chrousos and Gold, 1992). These systems are necessary to promote the 'FFF' ('fight-fright-flight') response, *i.e.*, non-specific, rapid autonomic and physiological changes necessary for mounting an acute stress response (*e.g.* an increase in heart rate, temperature, blood pressure, respiration rate, and arousal and increased attention to salient stimuli) and to inhibit non-essential vegetative behaviors, so that the organism can regain its homeostatic equilibrium. Individuals may considerably vary in their response to stressors, because both genes and environmental factors determine the way SAM- and HPA-axis shape the stress response. Failure of proper adaptation to the stressor will result in sustained over-activation of these stress systems and this may lead to the development of stress-related psychopathologies such as anxiety and depression.

Stress and the autonomic nervous system

The autonomic nervous system mainly controls heart muscle, smooth muscle and exocrine glands and mediates a variety of visceral reflexes. This system is a visceral sensory and motor system and permits rapid, integrated responses to changes in the environment. It has two major components: the sympathetic and the parasympathetic nervous system, which usually act in opposite ways. Generally, in response to stress the sympathetic system is activated and the parasympathetic part is suppressed. More precisely, the balance between the activities of the two systems promotes internal homeostasis ('le milieu intérieur'), in

the face of changing external conditions ('le milieu extérieur'; Claude Bernard 1865). The autonomic nervous system originates in the brain and through the spinal cord branches out to nearly every organ, blood vessel and gland. The nerve endings of the sympathetic system release adrenalin (Am.: epinephrine) and noradrenalin (Am.: norepinephrine).

Stress and the HPA-axis

The neuroendocrine stress response includes of activation of the HPA-axis. For vertebrates, appropriate functioning of this system is essential to survive. The HPA-axis is not only activated during stress but it also coordinates diurnal events such as food intake and sleep/wake (activity) cycle under normal, unstressed conditions. The diurnal activity of the HPA-axis results in a peak of glucocorticoid hormone secretion from the adrenals at the onset of the diurnal activity period. When the HPA-axis is activated during stress or around the activity peak, corticotropin-releasing factor (CRF) is produced in neurons of the paraventricular nucleus (PVN) of the hypothalamus and released into the portal-hypophyseal blood system. CRF stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the circulation. In the adrenal cortex, ACTH binds to its receptors to stimulate the synthesis and secretion of glucocorticoids (cortisol in humans, corticosterone in rodents) into the circulation. The role of glucocorticoids is to promote homeostatic adaptation to stress and this is achieved through catabolic actions that mobilize energy resources necessary for appropriate adaptive responses. Thus the integrity of the HPA-axis is critical, since homeostatic dysregulation may culminate in immunosuppression, neuroendocrine/autonomic dysfunction and tissue atrophy.

The HPA-axis functions as a closed-loop system involving tight negative-feedback control, which is mediated by the glucocorticoids exerting multiple regulatory actions on the brain and pituitary gland, including the HPA-axis itself. Autoregulation of the HPA-axis is essential for ensuring that the stress response is terminated, preventing excessive activations of peripheral organs and organ systems like the cardiovascular, gastrointestinal and immune system, and restoring normal internal homeostasis. Regulatory feedback occurs at several sites in the brain and pituitary gland and involves both rapid and delayed feedbacks, as shown in humans and rats (Keller-Wood and Dallman, 1984; Krishnan et al., 1991; Young et al., 1995). Rapid feedback occurs immediately following a rise in circulating glucocorticoids, whereas delayed feedback emerges 1-2 hours later and is dependent on glucocorticoid levels (de Kloet et al., 1998). Rapid feedback is exerted primarily via an inhibitory action of glucocorticoids at the hypothalamic level by decreasing mRNA expressions of CRF and of another peptide involved in the control of ACTH, vasopressin (Harbuz and Lightman, 1992). Delayed feedback is also manifest at the level of the pituitary adenohypophysis where glucocorticoids inhibit the mRNA expression of the ACTH precursor protein, pro-opiomelanocortin (POMC) (Harbuz and Lightman, 1992). In addition, glucocorticoids exert delayed negative feedback on the brain, at higher centers, such as the hippocampus (Ratka et al., 1989; Dallman et al., 2004).

The actions of the corticosteroids are mediated primarily through two types of specific nuclear receptor, viz. the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). These steroid receptors are located intracellularly in the cytoplasm and bind steroids that can freely diffuse across the plasma membrane. Once bound, the receptor-steroid complex translocates into the nucleus and interacts with palindromic hormone-responsive elements on the DNA. Thus, activated steroid receptors function as transcription factors and influence transcription of target genes, ultimately leading to changes in protein synthesis.

Therefore, adrenalin and noradrenalin (both acting within seconds), released in response to an autonomic stimulus and glucocorticoids (acting within minutes to hours) released as a neuroendocrine response to stress, account for a substantial part for what happens in an organism during stress.

The CRF peptide family

CRF is a 41-amino acid hypothalamic peptide characterized by Vale et al., (1981). It is the predominant hypothalamic neuropeptide regulating adrenal glucocorticoid release via pituitary ACTH release. The characterization of CRF was followed in 1995 by the identification of another mammalian CRF-related peptide involved in the stress response, urocortin 1 (Ucn1; Vaughan et al., 1995). The biological actions of CRF and Ucn1 are mediated via binding to two G protein-coupled receptors, type 1 and type 2 (CRF1 and CRF2), which occur throughout the central nervous system (CNS) and in various peripheral organs (Chalmers et al., 1996; Dieterich et al., 1997). CRF and Ucn1 activate both CRF1 and CRF2, with Ucn1 having higher affinity for CRF2 than CRF. In addition to CRF and Ucn1, more recent studies based on comparative genomic approaches have led to the identification of two other mammalian CRF/Ucn1-like peptides, urocortin 2 (also called 'stresscopin-related peptide'; Reyes et al., 2001) and urocortin 3 (also called 'stresscopin'; Lewis et al., 2001). Phylogenetic analysis and functional characterization studies have shown that these novel peptides represent a distinct evolutionary branch in the evolution of the CRF peptide family and have emerged as early as CRF and Ucn1 during vertebrate evolution. The sequences of both the CRF family ligands and the CRF receptors are highly conserved and can be traced back to invertebrates (Hsu et al., 2005). Presumably, the evolution of CRF/Ucn1 and Ucn2/Ucn3 peptides in 'modern' vertebrates has originated from two consecutive gene duplications of one CRF-like ancestral gene. Unlike CRF and Ucn1, Ucn2 and Ucn3 are specific CRF2 receptor agonists (Lewis et al., 2001, Reyes et al., 2001).

Presence and actions of CRF. CRF is involved in the neuroendocrine stress response and is further known as one of the key regulators of autonomic, behavioral and immunological responses to stress (Owens and Nemeroff, 1991; Koob, 1999; Koob and Heinrichs, 1999).

CRF immunoreactivity is heterogeneously distributed throughout the CNS, with similar distributions in rat and mouse (Merchenthaler et al., 1982; Swanson et al., 1983; Keegan et al., 1994; Arborelius et al., 1999). The main site of CRF production is the parvocellular part of the PVN, which projects to both the median eminence and the neocortex. While CRF released from the median eminence acts as a neurohormone on the pituitary ACTH-producing cells, in the neocortex it is assumed to have a neurotransmitter action, evoking behavioral stress responses (Swanson et al., 1983). Furthermore, CRF is present in the central nucleus of the amygdala and in the bed nucleus of the stria terminalis, modulating the neuroendocrine and autonomic stress response (Gray, 1993) via direct and indirect projections to the PVN and via projections to the brainstem. The presence of CRF in raphe nuclei, a site of serotonergic neurons, and in the locus ceruleus, where noradrenergic neurons are located, suggests a role of CRF in the modulation of emotions. Beside these production sites and projections in the brain there are numerous CRF fibers running in the spinal cord, indicating a role of CRF in the regulation of peripheral stress responses (Merchenthaler et al., 1983).

Ucn1 and the Edinger-Westphal system. The most prominent production site of Ucn1 in the brain is the Edinger-Westphal nucleus (EW). Central action of Ucn1 may account for some stress-related effects originally attributed to CRF (Spina et al., 1996; Skelton et al., 2000b). Moreover, various acute stressors activate EW-Ucn1 neurons (Weninger et al., 2000; Gaszner et al., 2004). Therefore, Ucn1-containing neurons in the EW are assumed to play a role in the regulation of stress adaptation. The presence of Ucn1 in the EW is a well-conserved phenomenon among vertebrates, including frog, rat, mice, sheep and human (Vaughan et al., 1995; Kozicz et al., 1998; 2002; Bittencourt et al., 1999; Calle et al., 2005; Ryabinin et al., 2005). Besides the EW, in rat, Ucn1 is present in the lateral superior olive and in the supraoptic nucleus in lateral hypothalamic area, and in several brainstem and spinal cord motoneuron nuclei (Kozicz et al., 1998; Bittencourt et al., 1999).

The EW is a dorsal midbrain, cholinergic parasympathetic nucleus with oculomotor functions. Nevertheless, it has recently been demonstrated that the Ucn1-producing neurons are not part of this preganglionic system as they are not cholinergic and, therefore, are unlikely to serve oculomotor functions (Cavani et al., 2003). Ucn1-immunoreactive fibers of possible EW-origin are observed in the lateral septum, substantia nigra, periaqueductal gray, dorsal raphe, spinal trigeminal nuclei, cerebellum and throughout the spinal cord. Since unlike CRF, Ucn1 is not a primary regulator of HPA activity, the involvement of EW-Ucn1 neurons in stress adaptation most probably occurs via the autonomic system. Neuroanatomical and neuropharmacological studies suggest that Ucn1 may be involved in many other processes besides stress adaptation, such as in regulation of food intake and energy expenditure, and in the control of motivation to consume alcohol (Bachtell et al., 2002a, 2003, 2004).

CRF receptors and their actions. CRF receptors belong to the super family of G-protein-coupled receptors that are characterized by the presence of a seven transmembrane domain. CRF receptor binding causes activation of adenylyl cyclase through a stimulatory G protein, resulting in an increased production of cAMP that subsequently binds to the regulatory subunit of PKA. The subunit then dissociates from the catalytic subunit, thereby activating it, which results in phosphorylation of a large variety of proteins (McKnight et al., 1988) including transcription factors (Gonzalez and Montminy, 1989). In addition to the cAMP pathway, other second messenger pathways involving MAP kinase, calcium ions and phospholipase C have been implicated in the actions of CRF (Rossant et al., 1999).

CRF1 mRNA is ubiquitously expressed in the CNS, for instance in the olfactory bulb, cerebral cortex, globus pallidus, red nucleus, pontine gray, substantia nigra and cerebellum (Potter et al., 1994; Van Pett et al., 2000). CRF2 mRNA is translated in two functional splice variants, $CRF2_{\alpha}$ and $CRF2_{\beta}$. The $CRF2_{\alpha}$ variant has been found in the CNS and shows a more restricted distribution than CRF1, *e.g.* in the lateral septal nuclei, ventromedial hypothalamic nuclei, amygdala, dorsal raphe and bed nucleus of the stria terminalis (Van Pett et al., 2000). The $CRF2_{\beta}$ variant is mainly present in non-neuronal structures, both centrally and peripherally, *e.g.* in the heart, skeletal muscle and blood vessels.

These heterogeneous distributions of CRF1 and CRF2 mRNAs suggest distinct functional roles of the two receptors. It has been proposed that CRF1 is particularly involved in cognition aspects including attention, executive functions, emotion and in sensory information processing and control of motor activity, whereas CRF2 would primarily influence processes necessary for survival, such as food intake and reproduction (Steckler and Holsboer, 1999).

The biological activities of CRF and Ucn1 are modulated by a secreted glycoprotein, the CRF-binding protein (Potter et al., 1992; Seasholtz et al., 2001). The binding of this protein to CRF and Ucn1 leads to the inhibition of CRF- and Ucn1- induced effects (Behan et al., 1995, 1996).

Aim of the thesis research

The brain controls the stress adaptation systems mainly in two ways, via the pituitary gland and the adrenal gland by the HPA-axis, and via the spinal cord by the sympathetic nervous system. The traditionally best-known peptide involved in stress adaptation is CRF. The discovery of Ucn1 acting, like CRF, through CRF receptors, is raising questions about how these two peptides and adaptation control systems interact. The present study aims to increase insight into mechanisms in the mouse brain and spinal cord that enable the coordinate functioning of these adaptation systems, with special attention to CRF, Ucn1 and their receptors CRF1 and CRF2.

Experimental approaches

In this section, some of the main techniques used in this thesis will be introduced.

Animals

In this thesis research, the mouse is the animal of choice, because it is an animal commonly used for the generation of genetically modified models and, consequently, its importance for experimental studies on the mechanisms of the stress response is steadily increasing (*e.g.* (Smith et al., 1998; Coste et al., 2000; Bale et al., 2002; Groenink et al., 2002; Imaki et al., 2003).

Experimental manipulation

The over-expression of the CRF gene basically occurs as follows. The CRF transgene element is composed of the complete coding sequence of rat CRF cDNA (0.6 kb fragment (Thompson et al., 1987), which was inserted at the XhoI restriction site into an 8.2 kb EcoRI genomic DNA fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence (Aigner et al., 1995). The Thy-1 regulatory sequences drives constitutive transgene expression in only postnatal and adult neurons (Morris and Grosveld, 1989; Vidal et al., 1990; Luthi et al., 1997). These procedures gave rise to two independent lines of CRF-over-expressing (CRF-OE) mice, viz. lines 2122 and 2123 (Dirks et al., 2001). Of these lines, as is shown in **Chapter 2,** line 2122 has proved to be a valuable tool for investigating the physiological and neurobiological effects of CRF overproduction.

In addition to the use of the CRF-OE mouse as a tool to study consequences of CRF-overproduction, in this thesis research some other experimental approaches have been used, such as exposure to acute and chronic homotypic stressors (*e.g.* exposure to ether), and chronically corticosterone treatment to mimic the chronically increased titer of glucocorticoids during stress.

Histological techniques

The expression of genes in the brain is regulated by multiple internal and external stimuli that can induce discrete and unique patterns of gene expression in different brain regions and even in a single neuron. These unique patterns of expression are involved in shaping the function of the brain and its ability to adapt and generate long-term responses to subsequent stimuli. Exposure to short- and long-term stressors produces clear and sometimes profound effects on neuronal function and behavior, and many of these effects occur at the level of gene transcription. To study the changes in gene transcription in

specific brain areas at the cellular level we have studied the expression of the transcription factor *Fos* by immunocytoochemistry and investigated the expression of the transcribed mRNA of various genes by *in situ* hybridization. Presence and possible co-existence of different peptides within the same neuron have been studied with single and double-labeling immunocytochemistry. The amount of a neuropeptide present in a neuron is an indication for neuropeptide storage, and changes in this amount is an indication for changed neuronal secretory activity.

The Fos method to identify neuronal activation

Immediate early genes are rapidly expressed upon cell stimulation, and predominantly encode transcription factors that modify the expression of target genes. Subsequently, target gene expression can alter the phenotype of the cell by shifting the affinity or number of receptors, or regulating the synthesis of certain enzymes and neurotransmitters. The immediate early gene c-fos is widely used as a functional anatomical mapping tool, for various reasons: its transcription level is low at basal condition, transcription can be experimentally induced by a wide range of stimuli, and the nuclear Fos protein product of the c-fos gene, can be easily detected and quantified using immunohistochemistry. In addition, the expression of Fos appears within in a short time frame (few hours) following a stimulus, which is suitable to combine microscopical studies with behavioral studies (Ceccatelli et al., 1989b; Morgan and Curran, 1991).

Outline of the thesis

This thesis focuses on the roles and regulation of CRF-like peptides and their receptors in the stress adaptation process.

In **Chapter 2** basic characteristics of the CRF-OE mouse model are described, with particular attention to the extent and the degree of central and peripheral CRF-over-expression, and some physiological aspects. In **Chapter 3** it is investigated whether CRF controls the expressions of CRF1 and CRF2 mRNAs in a brain site- and receptor-type specific manner, using the CRF-OE mice as a test model. **Chapter 4** provides support for the hypothesis that CRF and Ucn1 control peripheral aspects of the stress response via the spinal cord, by mapping and semi-quantitatively assessing the expressions of CRF1 and CRF2 mRNAs throughout the mouse spinal cord. **Chapters 5, 6** and **7** are dedicated to the regulation of the EW and its interaction with the CRF system. In **Chapter 5** it is studied whether EW-Ucn1 expression and the responsiveness of EW-Ucn1 expression to stressors are altered by chronic CRF over-expression. In **Chapter 6** the activation patterns of Fos and Ucn1 expression in the mouse EW are studied after a repeated, homotypic ether challenge. In **Chapter 7** it is investigated whether EW-Ucn1 expression is directly regulated by corticosterone by studying the possible co-existence of the glucocorticoid

receptor and Ucn1. Furthermore, the effects of 14 days corticosterone administration on Ucn1 neurons are described. Finally, in **Chapter 8**, the results of the thesis research are discussed with emphasis on their significance for an increased insight into fundamental mechanistic and clinical aspects of the mammalian stress adaptation response.

Chapter 2

Overexpression of corticotropin-releasing factor in transgenic mice and chronic stress-like autonomic and physiological alterations

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European Journal of Neuroscience (2002) 16:1751-1760

Abstract

To gain a greater insight into the relationship between hyperactivity of the corticotropin-releasing hormone (CRF) system and autonomic and physiological changes associated with chronic stress, we developed a transgenic mouse model of central CRF overproduction. The extent of central and peripheral CRF overexpression, and the amount of bioactive CRF in the hypothalamus were determined in two lines of CRF-overexpressing (CRF-OE) mice. Furthermore, 24 h patterns of body temperature, heart rate, and activity were assessed using radiotelemetry, as well as cumulative water and food consumption and body weight gain over a 7-day period. CRF-OE mice showed increased amounts of CRF peptide and mRNA only in the central nervous system. Despite the presence of the same CRF transgene in their genome, only in one of the two established lines of CRF-OE mice (line 2122, but not 2123) was overexpression of CRF associated with increased levels of bioactive CRF in the hypothalamus, increased body temperature and heart rate (predominantly during the light (inactive) phase of the diurnal cycle), decreased heart rate variability during the dark (active) phase, and increased food and water consumption, when compared with littermate wild type mice. Because line 2122 of the CRF transgenic mice showed chronic stress-like neuroendocrine and autonomic changes, these mice appear to represent a valid animal model for chronic stress and might be valuable in the research on the consequences of CRF excess in situations of chronic stress.

Introduction

Corticotropin-releasing hormone (CRF) plays a pivotal role in the response of an organism to various stressors. This neuropeptide, first described by Vale et al. (1981), coordinates neuroendocrine, autonomic, behavioural and immunological responses to stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Koob et al., 1993; Holsboer, 1999; Koob and Heinrichs, 1999). Chronically elevated levels of CRF are implicated in human stress-related and affective disorders, including major depression (see Mitchell, 1998; Arborelius et al., 1999).

To gain more insight into the relationship between hyperactivity of the CRF system and associated neuroendocrine, autonomic, physiological and behavioural changes, we have developed a transgenic mouse model of CRF overproduction. These CRF-OE mice are different from the CRF-OE mouse model earlier described by Stenzel-Poore et al. (1992), in that overexpression in our CRF-OE mice is under the control of the Thy-1 promotor, which drives constitutive transgene expression in neurons in postnatal and adult brain (*e.g.* Morris and Grosveld, 1989; Vidal et al., 1990; Moechars et al., 1996; Lüthi et al., 1997; Wiessner et al., 1999), resulting in central CRF overexpression only. Furthermore, there are differences in insertion sites in the genome, inherent to the random characteristics of the transgenesis technique.

One of the hallmark signs of chronic stress in animals and humans is a consistent increased central drive of the hypothalamus-pituitary-adrenal (HPA) axis (Checkley, 1996), as shown for example by increased expression of CRF mRNA and/or vasopressin mRNA in whole hypothalamus, in particular in the hypothalamic paraventricular nucleus (see Imaki et al., 1991; Checkley, 1996). Conversely, after chronic intracerebroventricular (i.c.v.) infusion of CRF, rats show neuroendocrine features comparable to those in chronically stressed rats, including hypersecretion of HPA-axis hormones (Labeur et al., 1995; Linthorst et al., 1997). Studies examining the effects of chronic stress or chronic CRF hypersecretion on systems other than the HPA-axis are rare. With regard to the autonomic system, chronic CRF administration in rats induces alterations in body weight regulation, cardiovascular functioning and thermogenesis (*e.g.* Arase et al., 1988; Richard, 1993; Labeur et al., 1995; Buwalda et al., 1997; Linthorst et al., 1997). Chronic stress increases resting heart rate in rats (Bhatnagar et al., 1998; Grippo et al., 2002). Alterations in heart rate because of chronic stress have also been reported for human subjects (Brand et al., 2000; Cacioppo et al., 2000).

To investigate the effect of life-long CRF overexpression on parameters of the autonomic nervous system, in the present study 24h patterns in body temperature, heart rate and general activity in CRF-OE mice were determined by means of radiotelemetry. Radiotelemetry has the major advantage in that it allows a continuous and stress-free measurement of several parameters over a long period of time in undisturbed, freely moving animals. Furthermore, we examined the extent of central and peripheral CRF overexpression, the amount of bioactive CRF in the hypothalamus, as well as cumulative

water and food consumption and body weight gain over a 7-day period. If our CRF-OE mice could be regarded as an animal model for human chronic stress, than they should show elevated levels of CRF in the hypothalamus, with concomitant increases in body temperature and heart rate as has been demonstrated before in chronic stress.

Materials and Methods

Generation of transgenic mice

The generation of the CRF-OE mice is described extensively elsewhere (Dirks et al., 2002b). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (0.6 kb fragment; Thompson et al., 1987), which was inserted at the XhoI restriction site into a 8.2 kb EcoRI genomic DNA fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence (Aigner et al., 1995). The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons (Morris and Grosveld, 1989; Vidal et al., 1990; Moechars et al., 1996; Lüthi et al., 1997; Wiessner et al., 1999). The Thy1–CRF gene was prepared for microinjection by isolating a 9.0 kb NotI fragment containing the fusion gene, which was purified from a conventional agarose gel by electroelution. The fragment was microinjected into fertilized eggs (C57BL/6J), and the injected cells were transplanted to pseudopregnant foster mothers. To identify transgenic founder animals, tail DNA from offspring was screened by standard Southern dot-blot analysis using the 0.6 kb CRF cDNA fragment as probe. These procedures yielded three transgenic founder animals (one female and two males), which gave rise to three independent lines of transgenic animals. Subsequent breeding at the local breeding facilities (Central Laboratory Animal Institute, Utrecht, The Netherlands) consisted of matings between transgenic males and C57BL/6JIco females. Tail DNA from offspring, extracted with a High Pure polymerase chain reaction (PCR) Template Preparation Kit (Boehringer, Mannheim, Germany), was screened using PCR with transgene-specific primers. The forward-primers were specific for rat CRF and the reversed-primers originated from the Thy-1 promotor, thus excluding the possibility that the endogenous CRF and Thy-1 gene were amplified.

Animals

Male transgenic mice of two lines, CRF-OE$_{2122}$ and CRF-OE$_{2123}$, were used. Littermate wild type (WT) mice of both lines served as controls. Animals were housed at constant room temperature (21± 2 °C) and relative humidity (50–60%), in Macrolon-II cages (22×16×14cm) with EnviroDri (BMI, Helmond, The Netherlands) and a piece of PVC tubing as cage-enrichment. Standard rodent food pellets (Hope Farms, Woerden, The Netherlands) and water were available *ad libitum*. Mice were maintained on a 12 h light–dark cycle (lights on from 07.00h to 19.00h). All experiments were approved by the ethical committee on animal

experiments of the Faculties of Pharmacy, Biology and Chemistry of Utrecht University, The Netherlands.

Assessment of central and peripheral CRF expression

Separate cohorts of animals were used, one for the qualitative and quantitative assessment of central CRF expression (WT n=6; CRF-OE_{2122} n=6; CRF-OE_{2123} n=6; age 12-14 weeks), and one for the qualitative assessment of peripheral CRF expression (WT n=4; CRF-OE_{2122} n=3; CRF-OE_{2123} n=2; age 12–21 weeks).

Mice were deeply anaesthetized with Nembutal (60 mg/ml sodium pentobarbital, Sanofi Sante B.V., Maassluis, The Netherlands; 0.1 ml/mouse i.p.), transported to the perfusion room and then perfused transcardially with sterile saline followed by freshly prepared 4% paraformaldehyde in RNAse-free 0.1 M phosphate buffer (pH 7.4, approximately 150 ml/mouse). After perfusion, the brains, adrenal glands, testes and heart were removed and postfixed overnight in 4% paraformaldehyde at 4 °C, and subsequently stored in sterile 0.1 M PBS at 4 °C. Brains and peripheral organs were cut in 20 μm cryostat sections and mounted on gelatin-chrome-alum-coated slides.

The immunocytochemistry method used has been described extensively elsewhere (Veening et al., 1998). Briefly, sections were incubated overnight with rabbit anti-CRF (1 : 20,000) diluted in PBS containing 0.3% Triton X-100 and 0.1% bovine serum albumin (PBS-BT). Thereafter, sections were incubated in biotinylated donkey anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA; 1:1500) in PBS-BT for 90 min. Biotin was detected using the ABC-elite Kit (Vector Laboratories, Burlingame, CA, USA) 1 : 800 in PBS-BT, and the antibody-peroxidase complex was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining and nickel intensification. Finally, after rinsing, slides were dried, dehydrated, cleared in xylene and coverslipped with Entellan (Electron Microscopy Sciences, Fort Washington, PA, USA).

For the quantitative analysis of CRF-immunoreactivity, section planes were standardized according to the atlas of Franklin and Paxinos (1997). All subsequent indications of anterior-posterior (AP) planes are given in relation to Bregma and refer to this atlas. Because of the small size of the brain areas of interest, one section per animal was sufficient for reliable quantification. All structures were quantified bilaterally. The intensity of CRF-immunoreactivity was determined by measuring the average pixel value in the following brain areas, with corresponding AP plane and total size of quantified area: the parvocellular part of the hypothalamic paraventricular nucleus (PVH; AP −0.82 mm; 150 μm2); the bed nucleus of the stria terminalis, lateral division, dorsal part (BNST-LD; AP 0.26 mm; 200 μm2); and the central nucleus of the amygdala (CeA; AP −1.58 mm; 200 μm2). Intensity of CRF-immunoreactivity was quantified using an inverted light microscope (Zeiss Axiovert 135 TV, objective 10 ×), with the use of 0%, 2%, and 5% grey filters and a standardized light source. Images were taken through a monochrome Coolsnap digital camera (Roper Scientific, Tucson, AZ, USA), and the acquisition and measurements

were operated through the imaging software Metamorph (Universal Imaging Corporation, Downingtown, PA, USA) with standardized exposure time. The average pixel value has been measured in defined regions of 26 μm2, randomly positioned in the nucleus of interest (six times for the PVN and eight times for the CeA and BNST-LD).

For *in situ* hybridization, the procedure according to Jessell (http://cpmnet.columbia.edu/Department/neurobehav/jessell/insitu.html)was followed, with some minor modifications. The RNA probe was generated using rat CRF cDNA (kindly provided by Dr. W.W. Vale, The Clayton Foundation Laboratories of Peptide Biology, San Diego, CA, USA), and labelled with DIG-11-UTP using the labelling kit from Roche Molecular Biochemicals (F. Hoffmann La Roche, Basel, Switzerland). *In situ* hybridization incubation started with additional fixation in 0.1 M borax-buffered 4% paraformaldehyde (pH 9.5) at 4 °C for 10 min. The slides were then rinsed three times with 0.1 M PBS at room temperature, followed by a 10 min preincubation with proteinase K medium (0.1 M Tris/HCl, 0.05 M EDTA, 0.01 mg/ml proteinase K, pH 8.0) at 37 °C. After rinsing in distilled water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine (TEA) buffer (pH 8.0) at room temperature for 10 min, and rinsing in 2 × standard saline citrate (SSC) for 5 min. Hybridization medium, consisting of 50% deionized formamide, 0.3 M NaCl, 0.001 M EDTA, 1 × Denhardt's solution, 10% dextran sulphate, and 0.5 mg/ml tRNA, was placed together with the mRNA-DIG probe (100 ng/ml) in a water bath at 80 °C for 5 min and then on ice for at least 5 min. The 2 × SSC buffer was replaced by the hybridization solution (2 ml in each vial) for overnight incubation in a water bath at 60 °C. After incubation, slides were rinsed three times with 4 × SSC buffer at room temperature, followed by incubation in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, pH 8.0 and 0.01 mg/ml RNAse A that had been added just before the start of the incubation) for 30 min at 37 °C, and by stringent washing steps with decreasing concentrations of SSC (2 ×, 1 ×, 0.5 ×) with a final rinsing in 0.1 × SSC for 30 min at 58 C. The alkaline phosphatase method with NBT/BCIP as substrate was used for the detection of the DIG label. Briefly, after rinsing four times with buffer A (0.1 M Tris/HCl, 0.15 M NaCl, pH 7.5), sections were preincubated in buffer A containing 0.5% blocking agent (Roche Molecular Biochemicals) for 1 h, followed by a 3 h incubation at room temperature with sheep anti-DIG-AP (Roche Molecular Biochemicals; 1 : 5,000) in buffer A containing 0.5% blocking agent. Then, slides were rinsed four times for 5 min in buffer A, followed by 2 × 5 min in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M MgCl2, pH 9.5). After overnight incubation at room temperature in NBT/BCIP medium (10 ml buffer B, 2.4 mg levamisole, 175 μl NBT/BCIP mixture, Roche Mol. Biochem.) in a light-tight box, the reaction was stopped by placing the slides in buffer C (0.1 M Tris/HCl, 0.01 M EDTA, pH 8.0). After rinsing twice for 5 min, slides were dried overnight at 37 °C, rinsed in distilled water, dehydrated, cleared in xylene and coverslipped with Entellan.

In vitro CRF bioactivity assay in whole hypothalamus

Wild type (n = 9), CRF-OE_{2122} (n = 9) and CRF-OE_{2123} mice (n = 8), aged 15–18 weeks, were housed individually for 3 days in Macrolon-I cages (21 × 10 × 13 cm) with EnviroDri (BMI, Helmond, The Netherlands) as cage-enrichment. Then, animals were killed by decapitation, and brains were rapidly removed. Whole hypothalamus was dissected macroscopically, weighed, immediately frozen on dry ice in polypropylene vials, coated with 3% bovine serum albumin (BSA), and kept at − 80 °C until further use. For the extraction of tissue CRF, 1 ml ice-cold 1 N HCl containing 34 µl aprotinin (Trasylol; Bayer, Mijdrecht, The Netherlands) was added to whole hypothalamus. Tissues were microwaved for 10 s and subsequently disrupted by sonication on ice. Homogenates were centrifuged at 9000 g, for 10 min at 4 °C, after which the supernatant was transferred to BSA-coated polypropylene vials and freeze-dried in vacuum overnight using a SpeedVac VR1 centrifuge (Heto, Allerød, Denmark). For quantification of CRF biological activity, extracts were dissolved in 500 µl Dulbecco's medium without phenol red (Gibco BRL, Invitrogen BV, Breda, The Netherlands) and diluted by appropriate steps. L(tk) cells, stably transfected with a cAMP-responsive â-galactosidase gene (König et al., 1991; Liaw et al., 1994) and stably expressing the rat CRF2, were used for this bioassay (E. Ronken, personal communication). Briefly, cells were cultured in 96-well plates until confluency. Medium was aspirated and replaced by Dulbecco's medium without phenol red and kept at 37 °C with 95% O2 : 5% CO2. Cells were stimulated by adding CRF (Sigma, St. Louis, MO, USA) in known concentrations, or brain tissue extracts with known dilutions, for 3 h under culture conditions. After stimulation, cells were lysed in 0.1 µM phosphate buffer (pH 8) containing 2 mM $MgSO_4$ and 0.1 mM $MnCl_2$, and 0.5% (v/v) Triton X-100 (Sigma), containing 0.8 mg/ml o-nitrophenyl-β-galactopyranoside (Sigma). Spectrophotometric extinction was determined at 405 nm with an Anthos II spectrophotometer (Anthos Labtec Instruments, Salzburg, Austria) immediately and again after 60 min. The difference in optical density is a measure of enzymatic activity and therefore a measure for agonist activity at the CRF receptor. To confirm that obtained biological activity was derived exclusively from interaction with the cloned CRF2, parallel incubations were carried out in the presence of the CRF receptor antagonist astressin (kindly provided by Solvay Pharmaceuticals, Weesp, The Netherlands). The total amount of biologically active CRF in brain extracts was estimated by comparing the astressin-sensitive effects of the tissue extracts with that of known concentrations of CRF. The amount of biologically active CRF/mg tissue wet weight was calculated after adjustments for dilution factors and tissue weights.

Twenty-four hour patterns in body temperature, heart rate and activity, measured by radiotelemetry

Surgery. Wild type (n = 12), CRF-OE_{2122} (n = 9) and CRF-OE_{2123} (n = 13) mice were equipped with telemetric devices to study body temperature, heart rate and general activity in free-

ly moving animals. Surgery was performed as described previously (Bouwknecht et al., 2000). Briefly, a small wireless transmitter (type ETA-F20, Data Sciences International, St. Paul, MN, USA) was implanted in the abdominal cavity of each animal. Anaesthesia consisted of a mixture of Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone; Janssen Animal Health, Beerse, Belgium), Dormicum (5 mg/ml midazolam hydrochlorate; Roche Nederland B.V., Mijdrecht, The Netherlands), and sterile water (at 1 : 1 : 2, 10 ml/kg body weight s.c.) after pretreatment with the antibiotic Baytril (2.5% enrofloxacin, 0.025 ml/mouse s.c.; Bayer AG, Leverkusen, Germany). Two leads were positioned dorsal and ventral to the heart to allow measurement of the biopotential across the heart. At the beginning of surgery, mice were 13–18-weeks old. Following surgery, animals were housed individually and were allowed to recover from anaesthesia and surgery for 2 weeks before telemetric measurements started. They were weighed daily in order to follow the recovery process and if necessary were given additional injections with saline (0.5 ml/mouse s.c.). After 5–7 days postsurgery mice are fully recovered from anaesthesia and surgery, as evidenced by return of normal circadian rhythms and return of heart rate and blood pressure to presurgery baselines (Butz and Davisson, 2001).

Data acquisition. The telemetry system consisted of transmitters, implanted in the abdomen of the mice, and receivers (type RLA1020, Data Sciences International), positioned under each home cage. Data were transmitted from the receiver to a computer and translated into body temperature (°C), heart rate (beats per minute), and activity (counts/min) values (software under OS/2 Warp Connect: Dataquest ART., Data Sciences International). All three parameters were sampled at 1 min intervals.

Procedure. To assess 24 h rhythms of the CRF-OE mice and WT mice, body temperature, heart rate, and activity were measured on six consecutive days. Animals were only mildly disturbed by the experimenter for a short period of time (for 5–10 min between 09.30 h and 09.45 h) on three of these days to check their health and water and food availability, and perform data sampling without any handling. During the rest of the light–dark cycle animals were left undisturbed.

On day 0 and day 7, on which the transmitters were switched on and off, respectively, the animals, food racks including food pellets, and water bottles were weighed to assess body weight gain and food and water intake.

Data analyses

Results of the quantitative analysis of CRF-immunoreactivity and *in vitro* CRF bioassay were analysed by one-way analysis of variance (ANOVA) on genotype, followed by a Bonferroni *post hoc* test.

For technical reasons, data from two animals regarding telemetry, food and water intake, and body weight were excluded from the analyses (one WT and one CRF-OE_{2122}).

Because preliminary statistical analyses had shown that the effect of disturbance by the experimenter on day 3 was similar in all groups (statistics not shown), body temperature, heart rate and activity data across all days were reduced to average values per hour per animal. These data were analysed by repeated measures ANOVA with time as a within-subject factor (24 levels) and genotype as a between-subject factor (three levels) followed by the Bonferroni *post hoc* test when appropriate. Heart rate variability, a parameter for autonomic control of heart rate (Friedman and Thayer, 1998a,b), was computed for the light and dark phase separately as the mean standard deviation of hourly heart rate values (Stiedl and Spiess, 1997; Pattij et al., 2002), and analysed by repeated measures ANOVA with phase as a within-subject factor (two levels) and genotype as a between-subject factor (three levels) and by Bonferroni *post hoc* test when appropriate.

Differences in body weight at day 0 and day 7 of the telemetric recording were analysed by repeated measures ANOVA with day as a within-subject factor (two levels) and genotype as a between-subject factor (three levels) and by Bonferroni *post hoc* tests when appropriate. Weight gain, food intake and water intake were analysed by oneway ANOVA on genotype, followed by a Bonferroni *post hoc* test.

In all repeated measures ANOVAs, the Greenhouse–Geisser correction factor (ε) was used to adjust the degrees of freedom in case of violation of the sphericity assumption (Vasey and Thayer, 1987). The level of significance was set at $P < 0.05$.

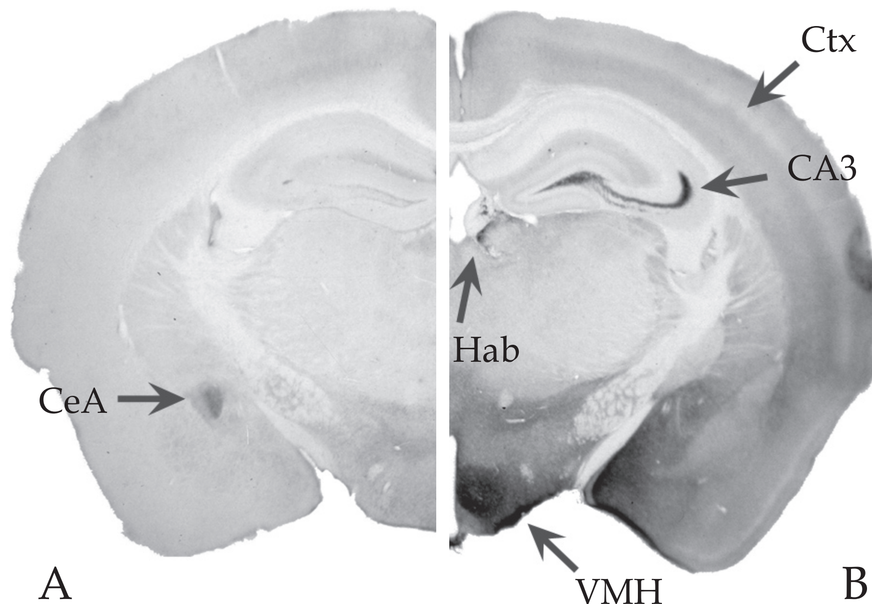

Figure 1. Representative overview (at mid-hypothalamic level) of CRF-immunoreactivity in the brain of a WT (A) and CRF-OE_{2122} mouse (B). Arrows indicate brain areas in which differences in CRF-IR between WT and CRF-OE_{2122} mice are evident. CeA, central nucleus of the amygdala; CA3, CA3 region of hippocampus; Ctx, cortex; Hab, habenula; VMH, ventromedial nucleus of the hypothalamus.

Results

Central and peripheral CRF overexpression

Increased amounts of CRF peptide and CRF mRNA were observed throughout the CNS in transgenic mice of both lines, as illustrated for WT and CRF-OE_{2122} mice in Figure 1. In CRF-OE_{2122} mice the expression was more extensive and the amounts of immunoreactive CRF appeared higher than in CRF-OE_{2123} mice. In both lines, overexpression of CRF was not uniform as it occurred in some brain regions whereas other regions seemed virtually unaffected.

Quantitative data corroborate the observed differences in amounts of CRF peptide. In the PVN and CeA, CRF-immunoreactivity was increased in CRF-OE_{2122} mice compared with WT and CRF-OE_{2123} mice (Table 1; $F_{2,16} = 8.63$, $P < 0.01$ and $F_{2,16} = 15.83$, $P < 0.001$, respectively). Increased amounts of CRF were also present in the BNST of CRF-OE_{2122} mice compared with WT mice (Table 1; $F_{2,23} = 4.55$, $P < 0.05$).

In CRF-OE_{2122} mice, occasionally, granular expression of CRF was observed in the adrenal medulla, identical to the observations in WT and CRF-OE_{2123} mice (Fig. 2A,B). Furthermore, CRF-immunoreactive fibres, originating from the spinal cord, with numerous varicosities were evident in the adrenal medulla of CRF-OE_{2122} mice (Fig. 2D). These immunostained fibers were not observed in WT mice (Fig. 2C), whereas in the CRF-OE_{2123} mice no more than an occasional fibre or varicosity could be detected (not shown).

Table 1. CRF-immunoreactivity in brain areas of WT and CRF-OE mice (optical density).

	n	**PVN**	**BNST**	**CeA**
WT	6	3.27 ± 4.44	33.53 ± 5.69	21.44 ± 5.46
CRF-OE_{2122}	6	28.90 ± 3.83*+	50.16 ± 3.40*	50.46 ± 2.55*+
CRF-OE_{2123}	6	9.65 ± 1.60	47.13 ± 3.01	34.21 ± 2.74

*P < 0.05 *vs.* WT, +P < 0.05 *vs.* CRF-OE_{2123}.

Table 2. CRF bioactivity in whole hypothalamus of WT and CRF-OE mice (in vitro bioassay on tissue extracts).

	n	CRF (ng/mg ± SEM)
WT	9	2.8 ± 1.4
CRF-OE_{2122}	9	10.5 ± 2.1*+
CRF-OE_{2123}	8	4.0 ± 1.6

*P < 0.05 *vs.* WT; +P < 0.05 *vs.* CRF-OE_{2123}.

Table 3. Body weight and cumulative food and water intake in wildtype and CRF-OE mice over 7-day period.

	WT (n = 11)	CRF-OE$_{2122}$ (n = 8)	CRF-OE$_{2123}$ (n = 13)
Body weight day 0 (BW, g)	32.8 ± 0.6	30.0 ± 0.7*+	32.5 ± 0.6
Body weight day 7 (g)	33.2 ± 0.5	30.6 ± 0.8*+	33.0 ± 0.6
Weight gain (g)	0.3 ± 0.14	0.6 ± 0.20	0.5 ± 0.20
Food intake (g)	30.0 ± 0.7	33.9 ± 1.1*	31.7 ± 0.9
Food intake (g/g BW)	0.9 ± 0.02	1.1 ± 0.04*+	1.0 ± 0.02
Water intake (g)	28.8 ± 1.0	40.8 ± 2.0*	34.9 ± 2.9
Water intake (g/g BW)	1.0 ± 0.03	1.3 ± 0.08*+	1.1 ± 0.08

BW, body weight; g, gram; *$P < 0.05$ *vs*. WT; +$P < 0.05$ *vs*. CRF-OE$_{2123}$.

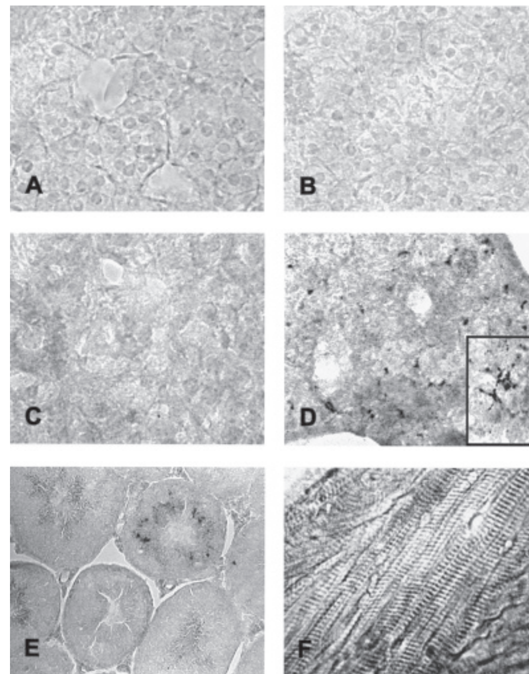

Figure 2. CRF-immunoreactivity and CRF mRNA distribution in peripheral organs of WT and CRF-OE mice. (A) Distribution of CRF mRNA expression in the adrenal medulla of a WT mouse. (B) Distribution of CRF mRNA in the adrenal medulla of a CRF-OE$_{2122}$ mouse. Changes in the (non)occurrence of CRF mRNA were not observed. (C) Distribution of CRF-immunoreactivity in the adrenal medulla of a WT mouse. (D) Distribution of CRF-immunoreactivity in the adrenal medulla of a CRF-OE$_{2122}$ mouse, showing the occurrence of CRF-immunoreactive fibres with varicosities, magnified in the insert. (E) Occasional expression of CRF mRNA in the testis of a WT mouse, not changed in the CRF-OE$_{2122}$ mouse. (F) Heart muscle of a CRF-OE$_{2122}$ mouse not expressing CRF mRNA.

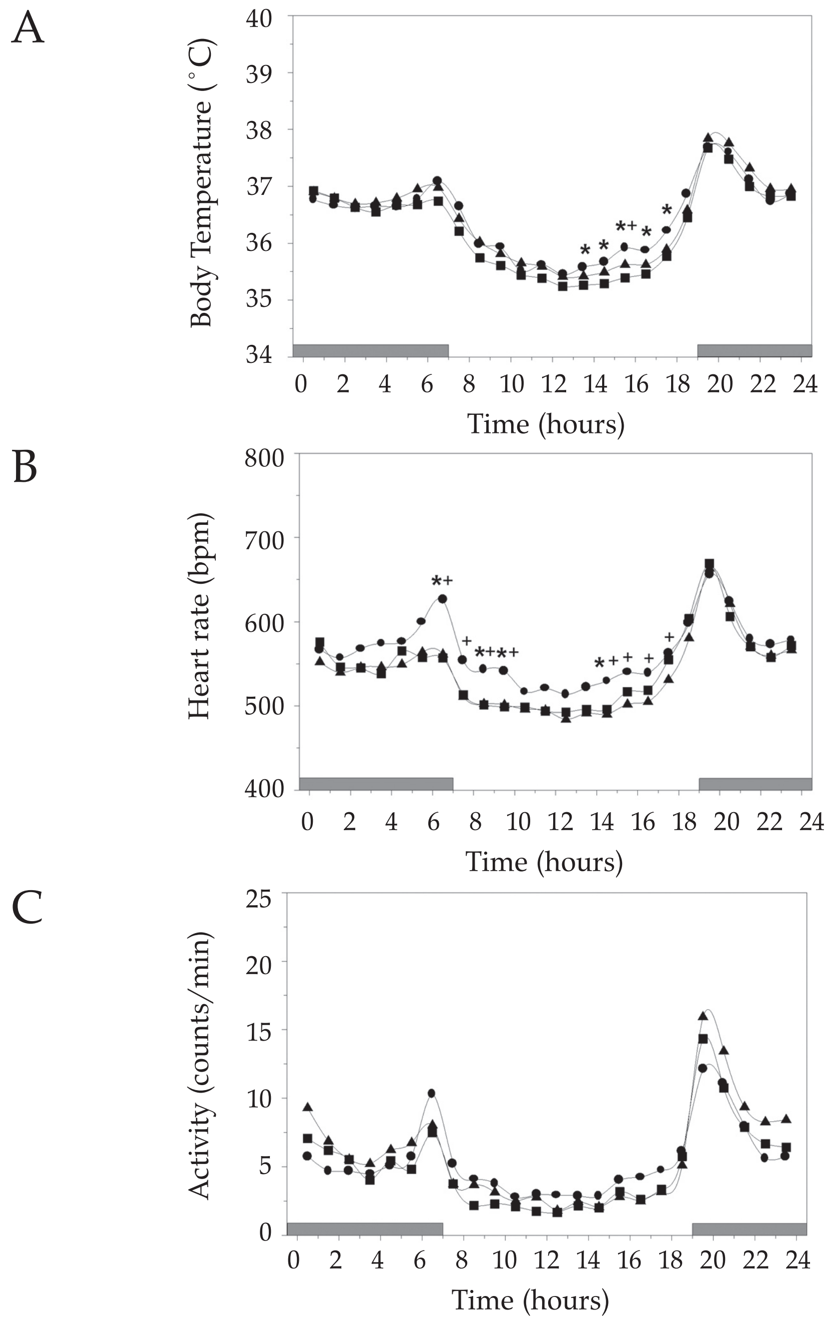

Figure 3. Twenty-four hour patterns of body temperature in degrees Celsius (A), heart rate in beats per min (b.p.m.) (B), and locomotor activity in counts per min (C) in WT (■, n = 11), CRF-OE_{2122} (●, n = 8) and CRF-OE_{2123} (▲, n = 13) mice. For details, see Materials and Methods. The shaded areas indicate the dark periods. Data points represent means over 60 min *$P < 0.05$ *vs*. WT; + $P < 0.05$ *vs*. CRF-OE_{2123}.

In the testis, moderate CRF expression was observed in WT and CRF-OE mice of both lines, without obvious differences between genotypes (Fig. 2E). No CRF expression was seen in the heart muscle with *in situ* hybridization in WT, CRF-OE_{2122}, or CRF-OE_{2123} mice (Fig. 2F).

In vitro CRF bioactivity assay in whole hypothalamus

The amount of bioactive CRF equivalent in tissue extracts of whole hypothalamus differed between groups (Table 2; $F_{2,23} = 5.64$, $P < 0.05$). The Bonferroni *post hoc* test revealed that in CRF-OE_{2122} mice the levels of bioactive CRF in the hypothalamus were substantially increased compared with both WT and CRF-OE_{2123} mice. Wild type and CRF-OE_{2123} mice did not differ from each other.

Twenty-four hour patterns in body temperature, heart rate and activity measured by radiotelemetry

As shown in Figure 3A-C, all mice revealed a distinct diurnal rhythm of body temperature, heart rate, and locomotor activity. Body temperature (Fig. 3A) showed a rapid decrease after lights were switched on. It was lowest halfway through the light phase of the diurnal cycle and started to rise in the late afternoon, reaching its maximum level approximately around the time when lights were switched off. In general, heart rate (Fig. 3B) and locomotor activity (Fig. 3C) followed similar diurnal patterns, with the exception of a period of increased activity 30–60 min before lights were switched on.

Wild type and transgenic mice had different body temperatures over time as indicated by a significant time–genotype interaction ($F_{46,667} = 2.07$, $P < 0.05$, $\varepsilon = 0.23$), without an overall effect of genotype ($F_{1,29} = 2.55$, n.s.). *Post hoc* tests revealed that CRF-OE_{2122} mice had increased body temperatures compared to WT and CRF-OE_{2123} mice, predominantly in the second half of the light period (Fig. 3A). Also heart rate varied over 24 h dependent on genotype (genotype $F_{1,29} = 2.92$, n.s.; time × genotype $F_{46,667} = 2.21$, $P < 0.01$, $\varepsilon = 0.37$). As shown in Figure 3B, CRF-OE_{2122} mice, but not WT and CRF-OE_{2123} mice, showed a marked increase in heart rate about 1 h before lights were switched on. Furthermore, heart rate of CRF-OE_{2122} mice was increased at a number of time points during the light phase of the diurnal rhythm, compared to WT and CRF-OE_{2123} animals. In the dark (active) phase, heart rate variability was reduced in CRF-OE_{2122} mice compared with WT and CRF-OE_{2123} mice (WT 44.66 ± 2.82, CRF-OE_{2122} 31.67 ± 2.74; CRF-OE_{2123} 42.38 ± 1.92; $F_{2,29} = 6.65$, $P < 0.005$) despite similar heart rates. In the light phase, during which heart rate was significantly increased in CRF-OE_{2122} mice, heart rate variability did not differ between genotypes, although statistical significance was nearly reached (WT 28.25 ± 2.05, CRF-OE_{2122} 33.33 ± 1.61; CRF-OE_{2123} 25.48 ± 2.37; $F_{2,29} = 2.99$, $P = 0.066$). Activity levels did not differ between groups (Fig. 3C; genotype $F_{2,29} = 1.14$, n.s.; time × genotype $F_{46,667} = 1.79$, n.s., $\varepsilon = 0.18$).

Body weight gain, cumulative food and water intake over 7-day period

Before surgery, the animals had similar body weights (WT 27.5 ± 0.6 g; CRF-OE_{2122} 26.2 ± 1.5 g; CRF-OE_{2123} 28.6 ± 0.7 g; $F_{2,33}$ = 1.80 n.s.). However, body weight was lower in CRF-OE_{2122} mice compared with WT and CRF-OE_{2123} mice at the start and at the end of the telemetric sampling (Table 3; $F_{2,29}$ = 4.81, $P < 0.05$). Weight gain during this 7-day period was similar in all groups (Table 3; $F_{2,29}$ = 0.49 n.s.). Furthermore, during the 7 days of measurements, the cumulative food and water intakes were higher in CRF-OE_{2122} mice (Table 3; food intake $F_{2,29}$ = 4.18, $P < 0.05$; water intake $F_{2,29}$ = 5.95, $P < 0.01$). Analysis of food and water consumption normalized to body weight on day 7, yielded similar results (Table 3; food intake/g body weight: $F_{2,29}$ = 17.42, $P < 0.001$; water intake/g body weight $F_{2,29}$ = 11.32, $P < 0.001$).

Discussion

In the present study, we only observed alterations in autonomic and physiological parameters in one of the two established lines of CRF-OE mice. Despite the presence of the same CRF transgene in their genome, the level of overexpression of CRF in the CNS was different between the two transgenic lines. Furthermore, *in vitro* CRF bioassay studies indicated that CRF concentrations were increased markedly in tissue extracts from whole hypothalamus of CRF-OE_{2122}, but not of CRF-OE_{2123} mice. These changes in hypothalamic CRF content were accompanied by increased plasma corticosterone levels and adrenal gland hypertrophy, whereas basal plasma adrenocorticotrophic hormone (ACTH) concentrations were not increased in CRF-OE_{2122} mice (Groenink et al., 2002). This suggests that the adrenal cortex of CRF-OE_{2122} mice is hyperresponsive to ACTH, which would be in accordance with the increase in CRF fibres in the adrenal medulla of CRF-OE_{2122} mice observed in the present study. It is very likely that these fibres originate from preganglionic neurons in the spinal cord (Bagdy et al., 1990), and reach the medulla via the splanchnic nerves (Pomerantz et al., 1996; Li and McDonald, 1997). Splanchnic nerve stimulation could increase the adrenal sensitivity to ACTH (Jasper and Engeland, 1997), possibly via an intra-adrenal CRF mechanism (Andreis et al., 1991; Van Oers et al., 1992). This suggests that the sympathetic system could be involved in the alterations found at the adrenal glands of CRF-OE_{2122} mice. Because no alterations in CRF expression were observed in other peripheral organs of CRF-OE mice, the increased number of CRF fibres in the adrenal medulla is most likely an indirect result of central CRF overexpression.

Absence of elevated basal ACTH secretion upon prolonged CRF hypersecretion as observed in CRF-OE_{2122} mice suggests adaptations at the pituitary level, such as CRF1 downregulation (Hauger et al., 1990), or alterations in pro-opiomelanocortin content, which is also regulated by CRF (Levin et al., 1989). Interestingly, after footshock stress, the levels of plasma corticosterone in CRF-OE_{2122} mice did not differ from WT mice (Groenink et al., 2002), whereas CRF-OE_{2122} mice showed blunted Fos-immunoreactivity

in the paraventricular nucleus of the hypothalamus in reaction to footshock stress (Dirks, 2001). Activation of the HPA-axis superimposed on existing CRF- and corticosterone-hypersecretion could be explained by a shift from CRF to vasopressin controlled pituitary-adrenal activation, which is also seen in animal models of chronic stress (Hauger and Aguilera, 1993; Scott and Dinan, 1998).

Only CRF-OE_{2122} mice clearly exhibited significant changes in body temperature and heart rate patterns as well as food and water intake, although CRF-OE_{2123} mice did not differ from WT mice. Chronic stress in man and rodents, is characterized by increased central drive of the HPA-axis, with increased CRF content of the hypothalamus, disrupted negative feedback by glucocorticoids and hypertrophy of the adrenal glands (see Imaki et al., 1991; Checkley, 1996) as well as by increased cardiovascular functioning and thermogenesis (Rothwell, 1990; Labeur et al., 1995; Buwalda et al., 1997; Linthorst et al., 1997; Brand et al., 2000). Thus, only transgenic mice of line 2122 display chronic stress-like increases in hypothalamic CRF content and neuroendocrine and autonomic changes. Therefore, we propose that CRF-OE_{2122}, but not CRF-OE_{2123}, mice may represent a suitable animal model for studying chronic hyperactivity of the CRF system in chronic stress, and that there is a relationship between central CRF-overproduction and autonomic and physiological changes associated with chronic stress.

Stenzel-Poore et al. (1992) were the first to describe the effects of CRF-overproduction in transgenic mice. Our CRF-overexpression model appears to be a valuable addition to the mouse model described by Stenzel-Poore et al. (1992) because only in our CRF-OE mice: (i) overexpression is limited to the CNS because of the promotor used; (ii) the selection of the founder animals was not biased towards an overactivity of the HPA-axis; and (iii), only centrally mediated effects of CRF are measured. The transgene Stenzel-Poore mouse exhibits elevated HPA-axis activity, and develops a Cushing's syndrome-like phenotype, consisting of excess fat accumulation, muscle atrophy, thin skin and alopecia from a very early age (Stenzel-Poore et al., 1992). The CRF-OE_{2122} mice, however, have no Cushing-like phenotype, despite elevated plasma corticosterone levels. Only after 6 months does increased fat deposition and hair loss become apparent. Furthermore, the CRF-OE mice show more anxiety related behaviours (Stenzel-Poore et al., 1994; Heinrichs et al., 1997) and impaired learning (Heinrichs et al., 1996). However, aspects of autonomic nervous system functioning in these CRF-OE mice have not been studied.

In the present study it has been shown that, compared to WT and CRF-OE_{2123} mice, body temperature in CRF-OE_{2122} mice is increased, predominantly during the second half of the light (inactive) phase. This result is consistent with the pronounced elevation of core body temperature demonstrated in rats after acute (Rothwell, 1990; Diamant and de Wied, 1991; Morimoto et al., 1993; Buwalda et al., 1998; Heinrichs et al., 2001), and chronic CRF administration for 7 or 10 days (Buwalda et al., 1997; Linthorst et al., 1997). The time point of the elevation in our study, that is in the second half of the light (inactive) phase, is not consistent with other reports indicating effects of CRF in both the light and dark phase of the diurnal cycle (Buwalda et al., 1997; Linthorst et al., 1997). The effects of

CRF on body temperature are most likely to be the result of CRF-induced activation of the sympathetic nervous system, including brown adipose tissue involved in thermogenesis (see Brown et al., 1982b; Richard, 1993). Increased catecholamine release might mediate the increased body temperature in the light phase in CRF-OE_{2122} mice.

Cardiovascular functioning is increased in CRF-OE_{2122} mice, as demonstrated by increased heart rate in the light phase. These results correspond well with reports showing that CRF administered acutely i.c.v. increases heart rate (Fisher et al., 1982; Diamant and de Wied, 1991; Korte et al., 1993; Morimoto et al., 1993; Richter and Mulvany, 1995; Buwalda et al., 1998; Nijsen et al., 2000; Heinrichs et al., 2001). To our knowledge, publications showing effects of chronic CRF administration on cardiovascular functioning in rodents are not available. Chronic stress increases resting heart rate in rodents measured during the light phase (Bhatnagar et al., 1998; Grippo et al., 2002). Studies in humans indicate that subjects suffering from high, chronic stress also have a higher baseline heart rate (Brand et al., 2000; Cacioppo et al., 2000). Taken together, these findings correspond with our demonstration of an increased heart rate in CRF-OE mice. Furthermore, in humans a link has been proposed between low heart rate variability and risk for anxiety disorders (*e.g.* Friedman and Thayer, 1998a,b), or major depressive disorder (see Nemeroff et al., 1998), both associated with elevated CRF levels. The finding of decreased heart rate variability in CRF-OE_{2122} mice in the dark (active) phase, when heart rates of CRF-OE and WT mice are similar, fits nicely with these ideas and favours our hypothesis that the CRF-OE_{2122} mice represents an animal model for chronic stress.

Increased general activity, as measured by radiotelemetry, does not provide an explanation for the observed heart rate values, because activity levels in CRF-OE_{2122} mice did not differ from WT or from CRF-OE_{2123} mice. In rats, acutely administered CRF induces a marked hyperactivity in a familiar environment (Sutton et al., 1982; Diamant and de Wied, 1991; Morimoto et al., 1993; Nijsen et al., 2000; Heinrichs et al., 2001). After chronic treatment, CRF increases activity only during the first 3 days of a 7-day regimen (Linthorst et al., 1997), whereas in another study no overall effect of CRF was measured on motor activity (Buwalda et al., 1997), a result corresponding well with our findings. It should be noted that the activity as measured by radiotelemetry is highly variable and only reflects gross activity and horizontal displacements. Thus, it cannot be excluded that with more refined measuring techniques, subtle changes in activity level, such as during eating, drinking and grooming, can be detected in our mouse model.

Our results demonstrate that food and water consumption is increased in CRF-OE_{2122} mice. However, decreased food intake is a well-documented effect of acutely or chronically infused CRF in rodents (*e.g.* Morley and Levine, 1982; Arase et al., 1988; Hotta et al., 1991; Richard, 1993; Buwalda et al., 1997; Linthorst et al., 1997; Pellymounter et al., 2000; Heinrichs et al., 2001). Besides direct effects on food intake, it has also been suggested that CRF blunts energy storage by reducing energy intake and augmenting energy expenditure (Richard et al., 2000). Conversely, energy demand would be increased by CRF-induced increases in locomotor activity (Morley and Levine, 1982; Sutton et al.,

1982) or by CRF-induced activation of the sympathetic nervous system and metabolism (Brown et al., 1982a; Arase et al., 1988), including activation of intrascapular brown adipose tissue involved in thermogenesis (Arase et al., 1988). Because our CRF-OE_{2122} mice did not display altered activity levels, increased thermogenesis and heart rate might be responsible for the increased energy intake, *i.e.* food and concomitant water consumption. Despite their lower body weight at the start of the telemetric measurement, body weight gain is the same for all groups in the present study. These results seem to suggest that CRF-OE_{2122} mice have adjusted their energy balance, *i.e.* the difference between energy intake and energy expenditure (Richard et al., 2000), in such a way that their caloric needs are met and that their increased energy demand because of CRF-induced increases in heart rate and body temperature are fulfilled.

Because CRF-OE mice are overproducing CRF throughout postnatal development, numerous neurochemical and developmental changes could have occurred to compensate for the increased levels of this neuropeptide. Thus, compensatory adaptations in other neurotransmitter systems involved in thermogenesis, cardiovascular functioning, activity, and feeding cannot be ruled out. Furthermore, regulation and adaptation to the CRF-induced changes, as often seen in rats, might be compromised or even absent in CRF-OE mice. Lastly, given the slight discrepancies in 24 h patterns of body temperature, heart rate, activity and food consumption between the present findings in CRF-OE mice and aforementioned effects of acute and chronic CRF administration in rats, species differences in CRF effects must also be considered.

In conclusion, chronic hyperactivity of the CRF system, as mimicked in line 2122 of transgenic mice overexpressing CRF, is associated with chronic-stress like neurochemical, autonomic and physiological changes. Therefore, CRF-OE_{2122} mice appear to represent a valid animal model for chronic stress and might be valuable in the research on chronic stress and the consequences of CRF excess in situations of chronic stress.

Acknowledgements

We dedicate this manuscript to the memory of Paul Toorop, who initiated this project on CRF-OE mice. We thank Solvay Pharmaceuticals, Weesp, The Netherlands, and in particular Guus van Scharrenburg for the initiation of and their financial support to the generation and maintenance of the lines of CRF-OE mice. We thank Herman van der Putten for his kind gift of the Thy-1 expression cassette and F. Tilders for the kind gift of the CRF antiserum. We also thank Marlies Lutje Schipholt and Ronald Oosting for screening tail DNA and Hira Binesari and Evert-Jan de Rond for excellent technical assistance.

Chapter 3

Distribution and expression of CRF receptor 1 and 2 mRNAs in the CRF over-expressing mouse brain

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Brain Research (2006) 1072:46-54

Abstract

Corticotropin-releasing factor (CRF) acts through CRF 1 and CRF 2 receptors (CRF1, CRF2). To test the hypothesis that CRF controls the expression of these receptors in a brain site- and receptor-type specific manner, we studied CRF1 mRNA and CRF2 mRNA expressions in mice with central CRF over-expression (CRF-OE) and using in situ hybridization. CRF1 and CRF2 mRNAs appear to be differentially distributed across the brain. The brain structures expressing the receptors are the same in wild-type (WT) and in CRF-OE mice. We therefore conclude that chronically elevated CRF does not induce or inhibit expression of these receptors in structures that normally do not or do, respectively, show these receptors. However, from counting cell body profiles positive for CRF1 and CRF2 mRNAs, clear differences appear in receptor expression between CRF-OE and WT mice, in a brain structure-specific fashion. Whereas some structures do not differ, CRF-OE mice exhibit remarkably lower numbers of CRF1 mRNA-positive profiles in the subthalamic nucleus (-38.6%), globus pallidus (-31.5%), dorsal part of the lateral septum (-23.5%), substantia nigra (-22,8%), primary somatosensory cortex (-18.9%) and principal sensory nucleus V (-18.4%). Furthermore, a higher number of CRF2 mRNA-positive profiles is observed in the dorsal raphe nucleus (+ 32.2%). These data strongly indicate that central CRF over-expression in the mouse brain is associated with down-regulation of CRF1 mRNA and up-regulation of CRF2 mRNA in a brain structure-specific way. On the basis of these results and the fact that CRF-OE mice reveal a number of physiological and autonomic symptoms that may be related to chronic stress, we suggest that CRF1 in the basal nuclei may be involved in disturbed information processing and that CRF2 in the dorsal raphe nucleus may play a role in mediating stress-induced release of serotonin by CRF.

Introduction

Corticotropin-releasing factor (CRF) is a main regulator of neuroendocrine, autonomic and behavioral responses, and is particularly involved in the control of adaptation to stressful conditions (Vale et al., 1981; Fisher, 1989; Holsboer et al., 1995; Nemeroff, 1996). It has a widespread distribution in the mammalian brain, in both hypothalamic and extrahypothalamic areas (Swanson et al., 1983; Valentino et al., 1992), where it acts by binding to two receptors, CRF receptor 1 (CRF1) and CRF receptor 2 (CRF2) (Chalmers et al., 1995). CRF1 is ubiquitously expressed, *e.g.* in the olfactory bulb, cerebral cortex, globus pallidus (GP), red nucleus (RN), pontine gray (Pn), substantia nigra (SN), sensory and motor trigeminal nuclei, and cerebellum (for complete distribution, see Van Pett et al., 2000) and is assumed to be primarily involved in sensory information processing and control of motor activity (Potter et al., 1994; Van Pett et al., 2000). CRF2 has a more restricted distribution, *e.g.* in the lateral septal nuclei (LS), ventromedial hypothalamic nuclei (VMH), amygdala, dorsal raphe nucleus (DR) and the bed nucleus of the stria terminalis (BNST) (Van Pett et al., 2000). This clearly differential distribution of CRF1 and CRF2 implicates that these receptors exert different functions that are controlled by different mechanisms. Although these control mechanisms are largely unknown, especially work on rat has indicated that they may involve stressor-specific up- and down-regulations of CRF-receptor expression. In rat, acute stress results in increased CRF1 mRNA expression in the hypothalamic paraventricular nucleus (PVN) and the supraoptic nucleus (SON) (Luo et al., 1994; Makino et al., 1995a, 1997; Rivest et al., 1995; Bonaz and Rivest, 1998; Imaki et al., 2001), whereas after chronic stress CRF1 mRNA is decreased in the frontal cortex and increased in the hippocampus and in the PVN, but less strongly in the latter than after acute stress (Makino et al., 1995a; Iredale et al., 1996; Bonaz and Rivest, 1998; Brunson et al., 2002).

There are indications that CRF controls CRF1 and CRF2 plasticity, either directly or by controlling the action of other factors such as corticosteroids, and that the nature of this regulation may differ among CRF1 and CRF2 receptors and among brain structures (Luo et al., 1994; Brunson et al., 2002). For example, in rat, exogenous CRF can stimulate transcription of CRF1 in the PVN (Imaki et al., 1996; Mansi et al., 1996) and CRF administration to juvenile rats increases CRF1 mRNA in the frontal cortex and hippocampus but not CRF2 expression in the basomedial amygdala (BLA) and VMH (Brunson et al., 2002). As to corticosteroids, chronic corticosterone administration or adrenalectomy results in decreased CRF1 mRNA in the rat PVN (Makino et al., 1995a, 1997) whereas extrahypothalamic regions such as the amygdala and the BNST are unaffected by these treatments (Makino et al., 1995a). On the other hand, in the VMH CRF2 mRNA expression increases by acute and chronic corticosterone treatment but is reduced after adrenalectomy, whereas it remains unaffected in the PVN (Makino et al., 1997, 1998).

Although these data suggest that CRF1 and CRF2 receptors are regulated in a receptor-specific and brain-specific way, the involvement of CRF and other stress-regulatory

factors in controlling CRF receptor expressions needs further elucidation, because up till now data have been derived from studies involving a variety of stressors (*e.g.* chronic, acute, environmental, pharmacological) hampering generalized conclusions, and have been mainly concerned with restricted parts of the rat brain. In the present study we focus on the primary role of CRF in the regulation of CRF1 and CRF2 mRNA expressions throughout the mouse brain. For this purpose we have used a mouse model of central CRF over-expression (CRF-OE mouse) (Dirks et al., 2002a,b; Groenink et al., 2002). Central over-expression of CRF in this animal is associated with a chronically increased level of bioactive CRF in the hypothalamus, and increased CRF-immunoreactivity in the PVN and in extrahypothalamic areas such as the BNST and the central nucleus of the amygdala (CeA) (Dirks et al., 2002a). In these animals, CRF is expressed in a large number of additional brain structures, such as the thalamus and the basal nuclei, which do not contain any CRF in wild-type (WT) mice (Swanson et al., 1983). Furthermore, the CRF-OE mouse exhibits down-regulation of the CRF-related peptide, urocortin 1 (Ucn1) in the Edinger-Westphal nucleus (EW), which is a factor assumed to play an important role in stress adaptation responses (Skelton et al., 2000b; Kozicz et al., 2004). This animal model shows hypercortisolism, adrenal gland hypertrophy and dexamethasone nonsuppression (Groenink et al., 2002), aberrant autonomic activities such as increased thermogenesis and accelerated cardiovascular activity (Dirks et al., 2002a), and impaired sensory information processing associated with reduced behavioral reactivity to environmental stimuli (Dirks et al., 2002b) and reduced locomotor activity (Groenink et al., 2003).

This study aims to test the hypothesis that CRF plays a role in controlling CRF1 and CRF2 mRNA expressions in the mouse, in a receptor- and brain area-specific fashion. To this end we have used a quantitative *in situ* hybridization approach to compare CRF receptor mRNA expression patterns in WT with those in the CRF-OE mice brain. By relating our results to what is known about the aberrant physiological and behavioral characteristics of the CRF-OE mouse, in the Discussion some suggestions will be made as to the physiological and pathophysiological aspects of CRF1 and CRF2 receptor regulations, in distinct brain areas.

Experimental Procedure

Animals

CRF-OE mice were generated as previously described (Dirks et al., 2002a). Briefly, the CRF transgene was composed of the complete coding sequence of rat CRF cDNA (0.6 kb fragment (Thompson et al., 1987), which was inserted at the XhoI restriction site into an 8.2 kb EcoRI genomic DNA fragment encompassing the murine Thy-1.2 gene, including regulatory regions and polyadenylation signal sequence (Aigner et al., 1995). The Thy-1 regulatory sequences drive constitutive transgene expression in postnatal and adult neurons (Morris and Grosveld, 1989; Vidal et al., 1990; Luthi et al., 1997). The Thy1-CRF

gene was prepared for microinjection by isolating a 9.0 kb NotI fragment containing the fusion gene, which was purified from a conventional agarose gel by electroelution. The fragment was microinjected into fertilized eggs (C57BL/6J), and the injected cells were transplanted into pseudopregnant foster mothers. To identify transgenic founder animals, tail DNA from offspring was screened by standard Southern dot-blot analysis using the 0.6 kb CRF cDNA fragment as a probe. These procedures yielded three transgenic founder animals, which gave rise to three independent lines of transgenic animals, one of which (CRF-OE 2122 line) was bred further at the local breeding facilities (Central Laboratory Animal Institute, Utrecht University, The Netherlands) and used for the present study. Breeding consisted of mating between transgenic male and C57BL/6J female mice. Tail DNA from offspring, extracted with the High Pure PCR Template Preparation Kit (Boehringer, Mannheim, Germany), was screened using PCR with transgene-specific primers. The forward-primers were specific for rat CRF and the reverse-primers originated from the Thy-1 promoter, thus excluding the possibility that the endogenous CRF and Thy-1 genes were amplified.

Adult male transgenic CRF-OE mice (n=7) were used in all experiments. Adult male littermate WT mice served as controls (n=7). Mice were housed 2-3 per cage, had free access to food and water and were adapted to housing conditions 14 days prior to perfusion for histology. All procedures were approved by the ethical committee for animal experimentation of the Faculties of Pharmaceutical Sciences, Biology and Chemistry, Utrecht University (Dec-GNK-FSB), according to the Dutch law for animal experimentation and the Declaration of Helsinki.

Tissue processing

Animals were deeply anesthetized with nembutal (60 mg/ml sodium pentobarbital, Sanofi Santé B.V., Maassluis, The Netherlands; 0.1 ml/mouse intraperitoneally) and perfused transcardially with sterile saline followed by freshly prepared 4% paraformaldehyde in RNAse-free 0.1 M sodium phosphate buffer (pH 7.4; 100 ml/mouse). After perfusion, animals were decapitated, and brains were removed and postfixed in 4% paraformaldehyde for 16 h at 4 °C, and subsequently stored in autoclaved 0.1 M sodium phosphate-buffered saline (PBS) at 4 °C, till use. Prior to cutting, tissues were transferred to 30% sucrose in 0.1 M PBS for 16 h at 20 °C and then frozen with dry ice. Of each brain 30-μm thick coronal sections with 150-μm intervals, were saved in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C, until histological processing.

In situ hybridization

In situ hybridization for CRF1 and CRF2a mRNAs was carried out with the free-floating section method according to Jessell (http://cpmcnet.columbia.edu/dept/neurobeh/jessell/insitu.html), with minor modifications, as follows. Antisense and sense (control)

RNA probes were generated using rat CRF1 and CRF2a cDNAs (kindly provided by Dr W.W. Vale, San Diego, CA, USA) and labeled with DIG-11-UTP using a labeling kit from Roche Molecular Biochemicals (Basel, Switzerland). *In situ* hybridization steps were carried out at room temperature (20 °C) unless stated otherwise. First, sections were fixed in 0.1 M borax-buffered 4% paraformaldehyde (pH 9.5), at 4 °C for 30 min. Then sections were rinsed four times for 7 min with 0.1 M PBS, followed by preincubation in proteinase K medium (0.1 M Tris/HCl, 0.05 M EDTA, 0.1 mg proteinase K), for 10 min at 37 °C. After rinsing in autoclaved MQ water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by rinsing in 2 times concentrated (2X) standard saline citrate buffer (SSC; pH 7.0) for 5 min. Hybridization mixture (50% deionized formamide, 0.3 M NaCl, 0.001 M EDTA, Denhardt's solution, 10% dextran sulphate) together with 0.5 mg/ml tRNA and the mRNA-digoxigenin (DIG) probe (ca. 2.5 ng/ml), were placed into a water bath at 80 °C for 5 min, and then on ice for another 5 min. Sections were incubated in hybridization solution for 16 h at 60°C, rinsed four times for 7 min with 4X SSC, incubated for 30 min at 37 °C in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, 0.01 mg/ml RNAse A; pH 8.0) that had been added just before the start of incubation, and stringently washed in steps with decreasing SSC concentrations (2X, 1X, 0.5X, 0.1X), for 30 min at 58 °C. The alkaline phosphatase method with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) as substrate was used for the detection of the DIG label. Briefly, after rinsing four times for 5 min with buffer A (0.1 M Tris/HCl, 0.15 M NaCl; pH 7.5) sections were preincubated in buffer A containing 0.5% blocking agent (Roche Molecular Biochemicals) for 60 min, followed by 3 h incubation with sheep anti-DIG-AP (Roche Molecular Biochemicals; 1:5,000) in buffer A containing 0.5% blocking agent. Subsequently, sections were rinsed four times for 5 min in buffer A, followed by two times of 5 min rinsing in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M MgCl2; pH 9.5). After 16 h incubation in NBT/BCIP medium (10 ml buffer B, 2.4 mg levamisole, 175 ml NBT/BCIP mixture; Roche Molecular Biochemicals) in a light-tight box, the reaction was stopped by placing the sections in buffer C (0.1 M Tris/HCl, 0.01 M EDTA; pH 8.0). After rinsing twice for 5 min, sections were mounted on gelatin-coated glasses, dried for 16 h at 37 °C, rinsed in distilled water, dehydrated, cleared in xylene, and coverslipped with Entellan. Sections of WT and CRF-OE mice brains were processed for *in situ* hybridization in parallel for each CRF receptor mRNA.

Quantitative analyses

To obtain quantitative information about the presence of receptor (CRF1 and CRF2) mRNAs in various brain structures, the numbers of neuronal cell body profiles in *in situ* hybridized sections were counted by direct microscopic examination, by an observer who was neuroanatomically experienced but unaware of the scientific aim of the study and of the key of the encoded slides. Counts were carried out using a Zeiss Axiovert 35

M microscope (Carl Zeiss AG, Germany) and Neurolucida software (MicroBrightfield, Williston, VT, USA). All cells that were visible, *i.e.*, contained receptor mRNA, were counted. Moreover, only cells with a clearly visible nucleus were counted. Per receptor type and experimental group (WT, CRF-OE) brains of 7 animals were studied. Per brain, cell body profiles with a positive hybridization signal for a receptor mRNA were counted in the two most central sections present of a brain structure, according to the co-ordinates given in the atlas of Paxinos and Franklin (2001). The brain structures and their co-ordinates are given in Tables 1 and 2. Per structure, the counts of the two sections were averaged, and the average value obtained for each mouse was used to calculate group means. The area of measurement included the whole brain structure, except for the somatosensory cortex (layers I-IV) and the CA1, CA3 and dentate gyrus of the hippocampus, which were analyzed in a central square (0.040 mm^2 for the somatosensory cortex, 0.013 mm^2 for each hippocampal region). Data were statistically analyzed with Student's unpaired t-test (α = 5%).

Results

General remarks and observations

In both WT and CRF-OE mice, hybridization signals were seen throughout the brain, in various brain areas as identified on the basis of the co-ordinates in the mouse brain atlas of Paxinos and Franklin (2001). For CRF1 mRNA the major expressing areas were the cerebral cortex, hippocampus, dorsal part of the lateral septal nucleus, medial septal nucleus, olfactory bulb, globus pallidus, red nucleus, principal sensory nucleus V, pontine gray, cerebellum, subthalamic nucleus, substantia nigra pars compacta and pedunculopontine nucleus. For CRF2 mRNA major expression was in the medial amygdala, the intermediate part of the lateral septal nucleus, the posterodorsal region of the bed nucleus of the stria terminalis, the ventromedial nucleus of the hypothalamus and in the dorsal and median raphe. No other elements in the brain, like glial cells, or blood vessels, were found positive. No appreciable background staining was noticeable, and also with the sense probes (control) no hybridization signal was seen. The hybridization signal was always located in the neuronal perikarya (Fig. 1A), with variable staining intensity (Fig. 1B).

We assessed both receptor mRNA expressions by counting the numbers of stained (hybridization-positive) cell body profiles of sectioned neurons. Since it was not the aim of our study to quantify the amount of mRNA in an absolute sense but only to test for a relative difference in expressions between the two, equally treated, mouse genotypes, we have chosen for the relatively simple method of profile counting. The results confirm that this method provides the requested answers with adequate reproducibility. CRF1 and CRF2 receptor mRNAs show different distribution patterns, which, however, do not differ between WT and CRF-OE mice. On the other hand, for some brain structures the quantification demonstrates, clear differences between the two genotypes whereas

other structures did not differ between WT and CRF-OE mice. Below we will describe the expressions of both receptor types in detail, for a number of brain areas selected on the basis of technical criteria (well-delineation of the structure, ease of identification of individual perikarya).

CRF1 mRNA distribution in WT and CRF-OE mouse brain

Looking at the average number of neuronal cell bodies with a positive hybridization signal in Table 1, it is remarkable that all brain areas showed a lower number of CRF1 mRNA-positive cell bodies in the CRF-OE mouse brain, but studying these data into more detail by means of statistical analysis, a more differential picture appears. No difference was found

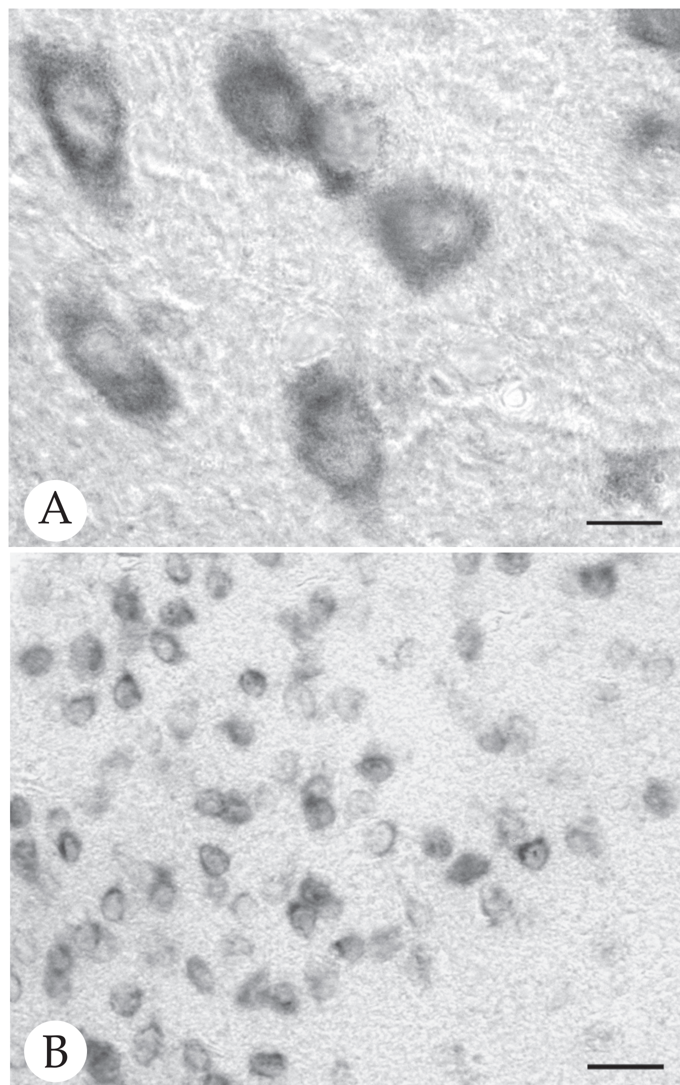

Figure 1. *In situ* hybridization of CRF2 mRNA in perikarya in the lateral septum of a WT mouse (a). Detail, showing neurons with different staining intensities (b). Scale bar a: 250 μm, b: 100 μm.

in the numbers of cell bodies between WT and CRF-OE mice in the three subdivisions of the hippocampus (CA1, CA3, DG), the medial septal nucleus, the pedunculopontine nucleus, the Pn (Fig. 2A,B), the RN and the interposed cerebellar nucleus. However, in the CRF-OE mice markedly lower numbers of CRF1 mRNA-positive cell bodies were observed in the subthalamic nucleus (STh) (-38.6%) (Fig. 2C,D) and in the GP (-31.5%) (Fig. 2 E,F), whereas moreover, lower numbers were also observed in the dorsal part of the LS (-23.5%), the SN (-22,8%), the principal sensory V nucleus (-18.9%) and the somatosensory cortex (-18.4%). Detailed data are given in Table 1.

CRF2 mRNA distribution in WT and CRF-OE mouse brain

Our quantitative analysis shows that no differences exist between WT and CRF-OE mice in the number of CRF2 mRNA-positive neurons in the medial nucleus of the amygdala, the intermediate part of the LS (Fig. 3 A,B), the posterodorsal region of the BNST, the VMH and the median raphe nucleus. However, the number of CRF2 mRNA-positive neurons in the DR (Fig. 3 C,D) in the CRF-OE mouse brain is clearly higher (+ 32.2%) than that in the WT. Detailed data are given in Table 2.

Table 1. Number of neurons in brain structures with their Bregma co-ordinates ref. (Paxinos and Franklin, 2001), expressing corticotropin-releasing factor receptor 1 (CRF1) mRNA in WT and CRF-OE mouse brains (n=7).

Brain area	Bregma (mm)	WT	CRF-OE	t-test
Medial septal nucleus	0.62	101.9 ± 9.2	87.4 ± 9.6	ns
Primary somatosensory cortex	0.86	82.0 ± 3.0	66.9 ± 7.0	P = 0.04
Lateral septum (dorsal part)	0.86	400.3 ± 16.6	306.1 ± 39.5	P = 0.02
Globus pallidus	-0.82	360.0 ± 40.4	246. 6 ± 46.8	P = 0.04
CA1 (hippocampus)	-1.82	47.5 ± 2.5	42.8 ± 2.3	ns
CA3 (hippocampus)	-1.82	48.7 ± 2.9	40.3 ± 3.9	ns
Dentate gyrus (hippocampus)	-1.82	38.7 ± 2.4	38.2 ± 3.9	ns
Subthalamic nucleus	-2.06	234.4 ± 25.0	143.9 ±19.8	P = 0.008
Substantia nigra compacta	-2.92	553.8 ± 31.8	427.4 ± 52.7	P = 0.03
Red nucleus	-3.80	266.8 ± 21.4	249.3 ± 27.1	ns
Pontine gray	-4.16	1750.6 ± 105.8	1618.0 ± 146.7	ns
Pedunculopontine nucleus	-4.48	126.5 ± 9.0	105.6 ± 14.7	ns
Principal sensory nucleus V	-4.96	674.0 ± 29.9	546.6 ± 64.0	P = 0.04
Interposed cerebellar nucleus	-6.12	422.9 ± 33.2	337.6 ± 40.8	ns

ns, statistically not significant.

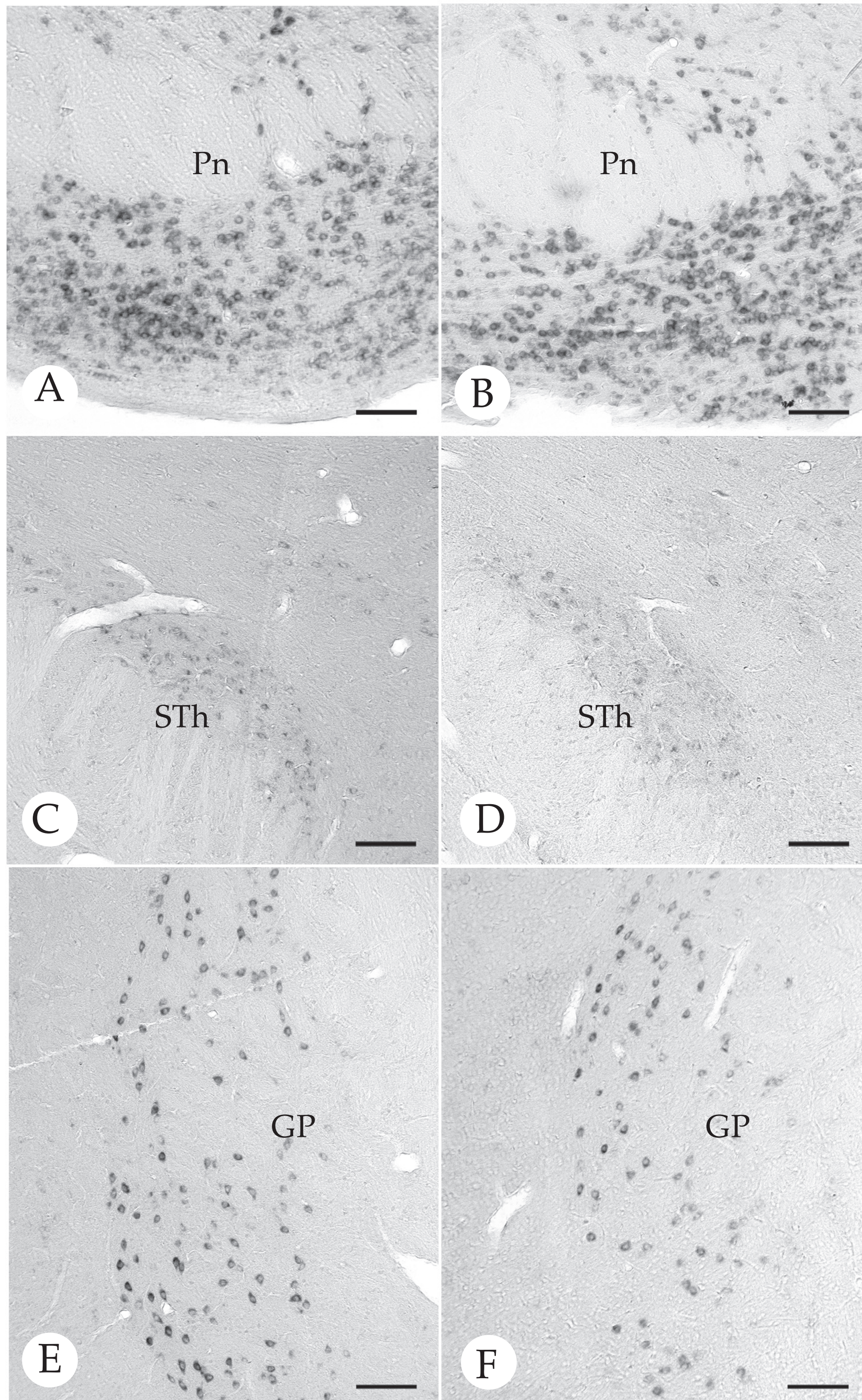

Figure 2. Comparison of *in situ* hybridization of CRF1 mRNA in three brain structures, between WT (left panels) and CRF-OE (right panels) mice. The number of positive neurons in the pontine gray does not differ (a,b), but in the subthalamic nucleus and globus pallidus it is much lower in CRF-OE (e,f) than in WT (c,d) mice. GP: globus pallidus; Pn: pontine gray; STh: subthalamic nucleus. Scale bar: 50 μm.

Discussion

General considerations

In the present study we investigated the possible involvement of CRF in the regulation of CRF1 and CRF2 receptors in the mouse brain. This has been done by testing the hypothesis that CRF controls, differentially as to receptor type and brain structure, the expressions of CRF1 mRNA and CRF2 mRNA, in a mouse strain with chronic CRF over-expression in the central nervous system (Dirks et al., 2002a,b; Groenink et al., 2002). In addition, as this CRF-OE mouse shows a number of physiological and behavioral aberrations from WT mice, our data provide a basis for the further elucidation of the functional significance of these two CRF receptor types and the brain areas that express them. These two aspects of our studies will be discussed below.

CRF over-expression leads to differential expression of CRF1 and CRF2 receptors

Our immunocytochemical study show strong hybridization signals with probes for CRF1 mRNA and CRF2 mRNA in various brain structures of WT and CRF-OE mice. The absence of background reaction, and of any positive signal with control sense-probes indicates that the signals specifically reveal the presence of CRF1 and CRF2 mRNAs in neuronal cell bodies. The anatomical analysis of the WT brain demonstrates that the two receptor mRNAs have non-overlapping patterns, and that these patterns are essentially the same

Table 2. Number of neurons in brain structures with their Bregma co-ordinates according to Paxinos and Franklin (2001), expressing corticotropin-releasing factor receptor 2 (CRF2) mRNA in WT and CRF-OE mouse brains (n=7).

Brain area	Bregma (mm)	WT	CRF-OE	t-test
Lateral septal nucleus (intermediate part)	0.86	784.8 ± 59.7	744.2 ± 65.9	ns
Bed nucleus of the stria terminalis (posterodorsal part)	-0.46	187.8 ± 42.0	223.7 ± 30.2	ns
Medial nucleus of the amygdala	-1.22	110.1 ± 7.0	98.7 ± 7.4	ns
Ventromedial nucleus of the hypothalamus	-1.22	96.0 ± 8.9	100.0 ± 7.5	ns
Dorsal raphe	-4.60	73.9 ± 3.0	97.7 ± 5.4	P = 0.001
Median raphe	-4.60	49.6 ± 7.5	53.8 ± 5.0	ns

ns, statistically not significant.

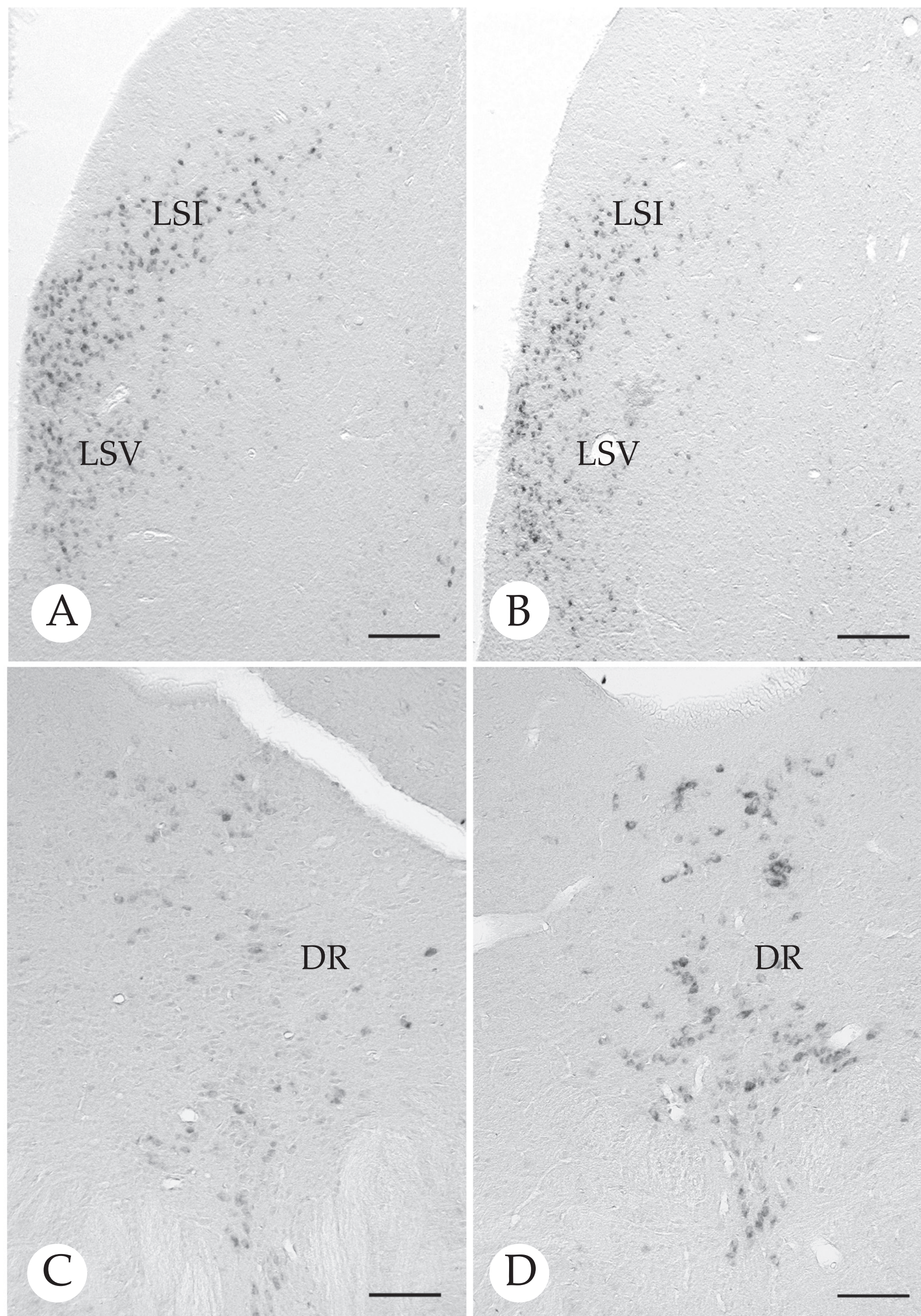

Figure 3. Comparison of *in situ* hybridization of CRF2 mRNA in two brain structures, between WT (left panels) and CRF-OE (right panels) mice. The number of positive neurons in the lateral septum does not differ (a,b), but in the dorsal raphe nucleus it is much higher in CRF-OE (d) than in WT (c) mice. DR: dorsal raphe nucleus; LSI: intermediate part of lateral septum; LSV: ventral part of lateral septum. Scale bar: 50 μm.

as described previously for the brain of the same mouse C57BL/6 strain (Van Pett et al., 2000). The distinctly different distributions of the two mRNAs suggest that the CRF1 and CRF2 receptors have different functions.

The same anatomical distributions of the two receptor mRNAs as occur in WT mice, were found in all CRF-OE mice. Therefore, we conclude that chronically elevated CRF does not induce CRF receptor expression in brain areas that normally (in WT) do not express these receptors and, similarly, does not inhibit these expressions in areas that normally do express these receptors. In our CRF-OE mice, CRF is expressed in brain structures that do not express CRF in normal mice (Dirks et al., 2002a). Apparently, these 'novel CRF-expressing areas' are not associated with 'novel CRF receptor-expressing areas' in the CRF-OE mouse brain. Assuming that these novel areas release CRF, it remains to be established if this CRF acts on targets also present in WT mice or on targets present in the mutant only.

As to the lack of a visible effect of CRF over-expression on CRF receptor expression in the PVN, this result is in line with the observation that restraint stress-induced CRF mRNA expression in the mouse PVN was not associated with CRF1 expression in this nucleus (Imaki et al., 2003). In rat, in contrast, the same stressor as well as exogenous CRF administration induces CRF1 mRNA in the PVN (Imaki et al., 1996; 2003; Mansi et al., 1996). Therefore, our data support the notion that CRF-dependent regulatory mechanisms controlling CRF receptor expression in the PVN differ between mouse and rat.

Although our finding that the distribution patterns of CRF1 mRNA and CRF2 mRNA do not differ between WT and CRF-OE mice might suggest that CRF excess does not affect the expression of the receptors, the detailed quantitation of the numbers of neuronal cell body profile positive with the respective receptor mRNA probes shows a different picture. Our analyses clearly demonstrate that the degree of expression of both receptor mRNAs is substantially different when individual brain structures are considered. In a number of structures no differences in the number of receptor mRNA-expressing neurons were seen. However, the fact that compared to WT mice, CRF-OE mice exhibit a lower number of CRF1 mRNA-expressing neurons in the dorsal part of the LS, the GP, SN pars compacta, STh, primary somatosensory cortex and principal sensory nucleus V whereas a higher number of CRF2 mRNA-expressing neurons were found in the DR, strongly indicates that a chronically elevated CRF level is associated with down-regulation of CRF1 mRNA and up-regulation of CRF2 mRNA, in a brain area-specific way. This conclusion confirms and extends the assumption of Brunson et al. (2002) in rat that CRF may regulate CRF1 and CRF2 in a differential manner.

Possible regulatory mechanisms of CRF1 and CRF2 receptor expression

The question emerges as to the precise relationship between CRF over-expression and changed CRF1 and CRF2 expressions. Obviously, the primary cause may be an elevated release and binding of CRF to these receptors, but the effect of CRF may be more indirect,

e.g. via CRF-induced release of other factors known to be involved in stress-related neuronal activities, such as glucocorticoids and other CRF-related peptides. This idea receives support from studies on CRF receptor mRNA expression in rat, where chronic unpredictable stress decreases CRF1 mRNA in the frontal cortex and increases CRF1 mRNA in the hippocampus (Iredale et al., 1996), chronic corticosterone administration decreases CRF1 mRNA in the PVN and the BLA (Makino et al., 1995a, 1997) and increases CRF2 mRNA in the VMH (Makino et al., 1997, 1998), and subchronic exposure to $GABA_A$-benzodiazepines receptor agonist decreases CRF1 mRNA in the BLA but increases CRF2 mRNA in the LS (Skelton et al., 2000a). These data reveal that not only CRF but also other stress-related factors may control CRF1 and CRF2 expression, but also indicate that the same factors can have opposite effects on different brain structures and can regulate the two receptor types in opposite ways. The present study is the first to show that such opposite regulations, either by CRF and/or by such other factors, can occur in the mouse brain. In this respect it is noteworthy that the CRF-OE mouse shows an elevated basal plasma corticosterone level (Groenink et al., 2002) and down-regulation of Ucn1 in the EW (Kozicz et al., 2004). Ucn 1 is a CRF-related peptide, which also acts through CRF1 and CRF2, and has been implicated in the regulation of the stress response and more specifically in the control of CRF-regulated adaptive processes (Skelton et al., 2000b; Latchman, 2002; Oki and Sasano, 2004). Below we will look in some detail how CRF and other stress-regulating factors might control some of the brain structures in CRF-OE that express CRF receptor mRNA differently from WT mice.

As to the dorsal part of the LS, the down-regulation of CRF1 mRNA may be under the control of CRF-producing neurons in the hypothalamus, which shows a strong upregulation of CRF in CRF-OE mice (Dirks et al., 2002a). Possibly, the projection from this nucleus to the LS is involved in the control of various types of behavior including stress-related anxiety (Sakanaka et al., 1988). Similarly, the down-regulation of CRF1 mRNA observed in the SN might be caused by CRF-neurons located in the CeA and the BNST, which also show a clear up-regulation of CRF in CRF-OE mice (Dirks et al., 2002a). The latter two brain areas innervate dopaminergic neurons in the substantia nigra, and therefore are likely to modulate dopaminergic activity in the striatum (Gray, 1993). Furthermore, an increased expression of CRF2 mRNA in the DR may not (only) be caused by over-expressed CRF but (also) by decreased expression of Ucn1 (Kozicz et al., 2004). In CRF-OE mice, such a decrease has been reported in the EW, which has major projections to the DR nucleus (Chung et al., 1987; Vaughan et al., 1995; Bittencourt et al., 1999; Bittencourt and Sawchenko, 2000; Weitemier et al., 2005). The EW Ucn1 acts through both CRF1 and CRF2 receptors, but has a strong preference for CRF2 (Potter et al., 1994; Turnbull and Rivier, 1997; Reul and Holsboer, 2002).

Possible functional significance of changed receptor expressions

Clearly, the control of physiological and cognitive functions does not only involve changes in CRF release but also plastic changes in the expressions of CRF1 and CRF2. This makes the CRF-OE mouse a suitable model to obtain information about the role of CRF receptors in these physiological and cognitive functions, namely in relating brain site-specific changes in CRF receptor content to specific physiological or cognitive aberrations. Below we will apply this approach to a number of physiologically rather well-defined brain structures.

Among the brain areas with a lower number of CRF1 mRNA-positive neurons, the strongest effect of CRF over-expression was observed in the STh, the GP and the SN, all belonging to the basal nuclei system. These ganglia are considered as important nodes in cortico-subcortical networks involved in the processing of information in motor, cognitive and limbic domains (Tisch et al., 2004). Indeed, CRF-OE mice reveal reduced and non-habituating startle reactivity as well as impairment of prepulse inhibition (Dirks et al., 2002b), symptoms of impaired information processing. Therefore, the current data suggest that CRF1 in basal nuclei may be important for adequate information processing.

The only difference in CRF2 expression observed in CRF-OE mice when compared to WT is a remarkably higher number of mRNA-positive cell body profiles in the DR nucleus that also exhibits the vast majority of serotoninergic neurons in mammals (Dahlstrom and Fuxe, 1964; Abrams et al., 2004). The prominent participation of serotonin is generally acknowledged in stress-induced arousal as occurs during anxiety and depression (for reviews, see *e.g.* Graeff et al., 1996, 1997; Carrasco and Van de Kar, 2003). Microinjection of CRF into the dorsal raphe nucleus alters raphe neuronal activity *in vivo* (Kirby et al., 2000) and *in vitro* (Kirby et al., 2000; Lowry et al., 2000; Price et al., 2002), and the tone of the raphe-serotonin system is regulated in a dynamic manner through CRFR2 activation (Pernar et al., 2004). Therefore, our result of an increased expression of DR CRF2 in CRF-OE mice may contribute to the identification of the neuronal networks involved in stress-related diseases such as anxiety and major depression.

Chapter 4

Distribution of CRF receptor 1 and 2 mRNAs in the mouse spinal cord

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Submitted to Journal of Comparative Neurology

Abstract

Corticotropin-releasing factor (CRF) and urocortin 1 (Ucn1) are assumed to play important roles in stress adaptation responses. Various stressors elicit rapid activation of the autonomic nervous system via extrahypothalamic CRF present in the brain and in the spinal cord, where CRF acts as a neurotransmitter, and via Ucn1-projections from the Edinger-Westphal nucleus to the spinal cord. The CRF receptor 1 (CRF1) binds CRF and Ucn1 with similar high affinity but the CRF receptor 2 (CRF2) binds Ucn1 with much higher affinity than CRF. Both CRF receptors and both ligands are present in distinct brain areas, suggesting site- and receptor-specific actions of CRF and Ucn1. In analogy we hypothesize that CRF and Ucn1 in the spinal cord, control by similar actions peripheral components of the stress response. Here we provide evidence to support this hypothesis, by mapping and semi-quantitatively assessing the expression of CRF1 and CRF2 mRNAs in the four parts (cervical, thoracic, lumbar and sacral) of the mouse spinal cord, using non-radioactive in situ hybridization. We show the presence of both mRNAs throughout the spinal cord, in all laminae of Rexed, with exception of the superficial laminae in the dorsal horn. The two CRF receptor mRNAs reveal different distributions, CRF2 having a wider occurrence (laminae III-X) than CRF1 (laminae III-VIII). Comparing the sites of expression of the CRF receptor mRNAs with the presence of CRF and Ucn1, strongest coexistence appears in lamina VII and in the intermediolateral column (IML). Moreover, CRF2 mRNA predominates in lamina IX where it coexists with Ucn1, and there is a predominance of CRF2 mRNA in lamina X where it coexists with both ligands. In view of the lamina-specific coexistences of the two CRF receptor mRNAs with their ligands, we finally make some suggestions as to their functional roles in peripheral adaptation to stress. In particular both CRF receptors may play a role in the modulation of stress induced analgesia whereas CRF2 may mediate visceral nociceptive information.

Introduction

Corticotropin-releasing factor (CRF; Vale et al., 1981) in the hypothalamic paraventricular nucleus plays a critical role in activating the hypothalamo-pituitary-adrenal (HPA) stress axis. In addition, various stressors elicit rapid alterations in autonomic nervous system activity via CRF contained in various extrahypothalamic sites in the central nervous system including the spinal cord (Merchenthaler et al., 1982, 1983; Schipper et al., 1983; Swanson et al., 1983; Puder and Papka, 2001). Extrahypothalamic CRF may act as neurotransmitter (Brown et al., 1982a, 1986; De Souza, 1995; Koob and Heinrichs, 1999) and mediate a wide variety of peripheral components of the stress response (Fisher, 1989; Koob and Heinrichs, 1999) such as cardiovascular activity, gastric acid secretion, gastro-intestinal motility (Brown et al., 1982a, 1986; Owens and Nemeroff, 1991), stress-induced analgesia (Lariviere and Melzack, 2000) and visceral pain perception (Song and Takemori, 1990; Nijsen et al., 2005). These responses appear to be mediated primarily by CRF produced in and acting on various brain centers such as the cerebral cortex, the limbic system, the medulla and the pons (Owens and Nemeroff, 1991). However, the inhibitory effects of intrathecal administration of CRF on gastric acid secretion (Bell and De Souza, 1988) and on perception of visceral pain in rat (Song and Takemori, 1990; Nijsen et al., 2005) suggest that also the spinal cord may be an important site of control by CRF of autonomic stress responses. This idea is supported by the immunocytochemical demonstration of extensive CRF fibers throughout the rat spinal cord, in the various laminae of Rexed (Rexed, 1952), *e.g.* in the marginal zone (lamina I), deeper regions of the dorsal horn (laminae V-VII) and around the central canal (lamina X). Moreover, CRF-immunoreactive fibers occur in the intermediolateral column (IML) of the thoracic and lumbar parts of the spinal cord (Merchenthaler et al., 1983).

Another member of the CRF peptide family, urocortin 1 (Ucn1) (Vaughan et al., 1995) is also assumed to play important roles in stress adaptation responses (Skelton et al., 2000b; Gaszner et al., 2004; Kozicz et al., 2004; Korosi et al., 2005). Ucn1 is mainly expressed in the Edinger-Westphal nucleus (Chung et al., 1987; Vaughan et al., 1995; Bittencourt et al., 1999; Skelton et al., 2000b; Latchman, 2002; Oki and Sasano, 2004) and projects to the lateral septal nucleus, dorsal raphe nucleus and spinal cord. The extensive ramifications of Ucn1 fibers in the spinal cord (Bittencourt et al., 1999) may be crucial in controlling thermogenesis (Parkes et al., 2001; De Fanti and Martinez, 2002) and stress-induced immunosuppression (Okamoto et al., 1998), possibly via the sympathetic nervous system. In all spinal cord segments, Ucn1 fibers occur in the intermediate gray (lamina VII), the central gray (lamina X) and the IML. Furthermore, a moderate number of Ucn1-immunoreactive fibers are present in lamina I and in the ventral horn, some of them being closely apposed to motoneurons (Bittencourt et al., 1999).

In the brain, CRF and Ucn1 bind to two types of G protein-coupled membrane receptor (Potter et al., 1994), viz. the CRF receptor 1 (CRF1) and the CRF receptor 2 (CRF2). CRF1 binds CRF and Ucn1 with similar high affinity (Vaughan et al., 1995; Chalmers et al.,

1996) but CRF2 binds Ucn1 with about 40 times higher affinity than CRF (Lovenberg et al., 1995b; Vaughan et al., 1995). CRF and Ucn1, as well as CRF1 and CRF2, are present in distinct brain areas. This differential occurrence suggests that each peptide plays its own role in orchestrating behavioral, neuroendocrine and autonomic responses to stress, by acting in specific brain structures on either CRF1 or CRF2 (Turnbull and Rivier, 1997; Reul and Holsboer, 2002). In analogy, one would expect that in the spinal cord the same site- and receptor-specific actions of CRF and Ucn1 are involved in controlling peripheral components of the stress response. However, evidence supporting this latter hypothesis is lacking, as information with regard to the possible presence of CRF receptors in the spinal cord is scarce. Receptor autoradiography studies have demonstrated that in rat strong CRF binding takes place in the superficial layers of the dorsal horn (laminae I and II) and moderate binding in the ventral horn of lumbar segments (Skofitsch et al., 1985; Bell and De Souza, 1988; De Souza, 1995). Furthermore, CRF2 mRNA has been shown in the rat spinal cord by RT-PCR (Million et al., 2006). Spinal cord CRF receptors have been implicated in mediating colonic motor responses to centrally injected CRF and to stressors (Maillot et al., 2003) and, furthermore, in mediating sensory transmission of visceral noxious stimuli (Lariviere and Melzack, 2000).

Overviewing these data, it can be concluded that only in rat there is some circumstantial evidence for a role of CRF and CRF2 in the spinal cord in the regulation of peripheral stress responses. However, evidence is lacking to support the hypothesis that in addition to CRF, Ucn1 would also be involved in this regulation and, moreover, that each of these peptides would act on different CRF receptors in different regions of the spinal cord. Therefore, the present study aims to map and semi-quantitatively assess by *in situ* hybridization the distributions of CRF1 and CRF2 mRNAs in the four parts of the mouse spinal cord, with particular attention to their presence in the different laminae of Rexed. The functional significance of the results will be discussed in relation to the presence of CRF- and Ucn1-containing fibres and their possible targets. The mouse was chosen for this study because of its increasing importance for experimental studies on the mechanisms of the stress response (*e.g.* Smith et al., 1998; Coste et al., 2000; Bale et al., 2002; Groenink et al., 2002; Imaki et al., 2003; Korosi et al., 2005) and because of our recent experience in demonstrating CRF1 and CRF2 mRNAs in the mouse brain at the cellular level using non-radioactive *in situ* hybridization (Korosi et al., 2006).

Materials and Methods

Animals

Three, twelve weeks-old, male C57BL/6J mice were housed in standard plastic cages in a temperature- and humidity-controlled environment, and maintained on a 12/12-h light/dark cycle. They had permanently free access to food and water. All procedures had been approved by the ethical committee on animal experimentation of the Departments of

Pharmacy, Biology and Chemistry of Utrecht University, according to the Dutch law for animal experimentation and the Declaration of Helsinki.

Tissue processing

Each mouse was deeply anesthetized by intraperitoneal injection of 0.1 ml 6% sodium pentobarbital (nembutal; Sanofi Santé B.V., Maassluis, The Netherlands) and transcardially perfused with sterile saline (2 min) followed by freshly prepared 4% paraformaldehyde in RNAse-free 0.1 M sodium phosphate buffer (pH 7.4; 100 ml/mouse; 15 min). After perfusion, spinal cords were dissected and postfixed in 4% paraformaldehyde, for 16 h at 4°C, and stored in autoclaved 0.1 M sodium phosphate-buffered saline (PBS) at 4°C. Prior to cutting, tissues were transferred to 30% sucrose in 0.1 M PBS, for 16 h at 20°C, and frozen in dry ice. Each spinal cord was cut in 30-µm thick coronal sections at intervals of 210 µm, which were stored in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20°C, until processing.

In situ hybridization

In situ hybridization of CRF1 and CRF2α mRNAs was carried out with the free-floating section method according to Jessell (http://cpmcnet.columbia.edu/dept/neurobeh/jessell/insitu.html), with minor modifications, as follows. Antisense and sense (control) RNA probes were generated using rat CRF1 and CRF2α cDNAs (kindly provided by Dr W.W. Vale, San Diego, CA, USA) and labeled with DIG-11-UTP using a labeling kit from Roche Molecular Biochemicals (Basel, Switzerland). *In situ* hybridization steps were carried out at room temperature (20 °C) unless stated otherwise. First, sections were fixed in 0.1 M borax-buffered 4% paraformaldehyde (pH 9.5), at 4 °C for 30 min. Then they were rinsed four times for 7 min with 0.1 M PBS, and preincubated in proteinase K medium (0.1 M Tris/HCl, 0.05 M EDTA, 0.1 mg proteinase K), for 10 min at 37 °C. After rinsing in autoclaved MQ water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by rinsing in 2 times concentrated (2X) standard saline citrate buffer (SSC; pH 7.0), for 5 min. Hybridization mixture (50% deionized formamide, 0.3 M NaCl, 0.001 M EDTA, Denhardt's solution, 10% dextran sulphate) together with 0.5 mg/ml tRNA and the mRNA-digoxigenin (DIG) probe (ca. 2.5 ng/ml), were placed into a water bath, for 5 min at 80 °C, and then on ice for another 5 min. Sections were incubated in hybridization solution, for 16 h at 60°C, rinsed four times for 7 min with 4X SSC, incubated for 30 min at 37 °C in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, 0.01 mg/ml RNAse A; pH 8.0) that had been added just before the start of incubation, and stringently washed with decreasing SSC concentrations (2X, 1X, 0.5X, 0.1X), for 30 min at 58 °C. The alkaline phosphatase (AP) method with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) as substrate was used to detect DIG label. Briefly, after rinsing

four times for 5 min with buffer A (0.1 M Tris/HCl, 0.15 M NaCl; pH 7.5) sections were preincubated in buffer A containing 0.5% blocking agent (Roche Molecular Biochemicals), for 1 h, followed by 3 h incubation with sheep anti-DIG-AP (Roche Molecular Biochemicals; 1:5,000) in buffer A containing 0.5% blocking agent. Subsequently, sections were rinsed four times for 5 min in buffer A, followed by two times of 5 min rinsing in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M $MgCl_2$; pH 9.5). After 16 h incubation in NBT/BCIP medium (10 ml buffer B, 2.4 mg levamisole, 175 µl NBT/BCIP mixture; Roche Molecular Biochemicals) in a light-tight box, reaction was stopped by rinsing twice for 5 min in buffer C (0.1 M Tris/HCl, 0.01 M EDTA; pH 8.0). Then, sections were mounted on gelatin-coated glasses, dried for 16 h at 37 °C, rinsed in distilled water, dehydrated, cleared in xylene, and coverslipped with Entellan.

Microscopical analysis

Different parts of the spinal cord were identified on the basis of Sidman et al. (1971) whereas the laminae of Rexed were determined according to the description by Molander and Grant (1995) for the rat, which equally applies to that of the mouse (Li and Clark, 2001). For each receptor mRNA in a given lamina, the number of labeled neurons and the strength of the hybridization signal in the perikarya were assessed by direct microscopical analysis and translated into a semi-quantitative scale of receptor density, viz. as ‘absent or very rare’ (-), ‘low’ (+), ‘moderate’ (++) or ‘high’ (+++) (Fig. 1). This method does not allow to compare directly the amount of CRF1 mRNA with that of CRF2 mRNA, as the efficiencies of the hybridizations of the two mRNAs are unknown and may differ from each other, but it provides detailed information on the relative distribution of an mRNA within the four parts and the various laminae of the spinal cord.

Results

General remarks

The distributions of CRF1 mRNA and CRF2 mRNA were examined in coronal sections in all segments of the four parts (cervical, thoracic, lumbar and sacral) of the spinal cord (Figs. 2-5). The three mice studied gave essentially the same results. Both mRNAs were seen throughout the whole length of the spinal cord in neurons. Non-neuronal elements were never found to be positive. With the sense probes (control), no hybridization signals are visible. Hybridization signals are always in perikarya, with variable staining intensity. Although CRF1 and CRF2 show partly overlapping distributions, they exhibit clear differences in specific areas of the spinal cord (Table 1). The distributions of the two CRF receptor mRNAs will be described separately, with special reference to the ten laminae of Rexed and the four parts of the spinal cord. The distributions of both CRF receptor mRNAs appeared to be essentially the same in all segments within a given part of the

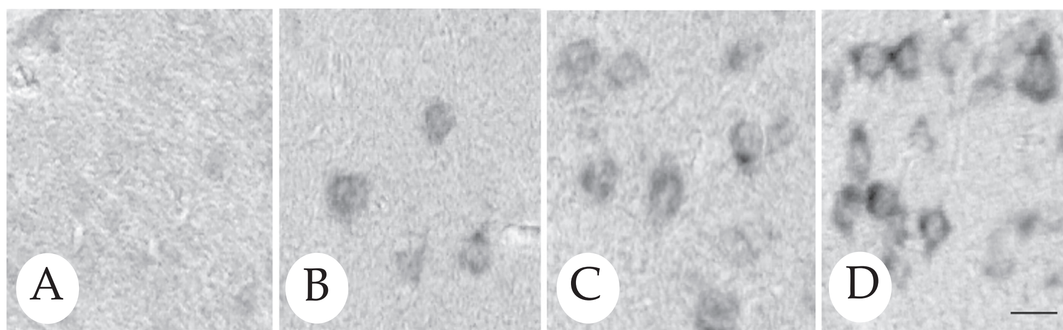

Figure 1. A-D: Photomicrographs showing examples of different densities of neuronal perikarya labeled for CRF1 mRNA. CRF2 mRNA neuronal densities showed similar differences in density (not shown). (A) Complete lack or isolated positively labeled cells; (B) low density; (C) moderate density; (D) high density. Scale bar = 20 µm.

spinal cord. (Representative illustrations are given in Figs 2 and 3.)

CRF1 mRNA distribution

In the cervical part (Fig. 3A), CRF1 mRNA is present in neuronal perikarya in laminae III-VIII, with lowest expression in lamina III (Fig. 4A) and most labeled neurons present in laminae VII and VIII (Fig. 4B). In the thoracic part (Fig. 3B), labeled neurons were seen in all laminae except in lamina IX. The lowest number of positive neurons and weakest signal was observed in the superficial laminae of the dorsal horn (I-IV) and the highest number of labeled neurons in laminae V and VII and in the IML (Fig. 4C). The lumbar part (Fig. 3C) reveals the highest number of labeled neurons in laminae VII and VIII and slightly lower numbers in laminae III-VI. No evidence was found for labeled neurons in the most superficial laminae in this part of the cord (Fig. 4D). In the sacral part (Fig. 3D) a few positive neurons occur in the dorsal horn (laminae III-V) but strong signals occur in laminae VII and VIII. There are no labeled neurons in lamina X (Fig. 4E) but some CRF1 mRNA-positive neurons are present in lamina IX. These data are summarized in Table 1 and in Fig. 2.

CRF2 mRNA distribution

In the cervical part (Fig. 3E), CRF2 mRNA-containing neurons occur in laminae IV-X (Fig. 5A) with strongest signals in laminae VII-IX, (Fig. 5B). As to the thoracic part (Fig. 3F), in all laminae hybridizations signals were observed with similar high strength, except for the two most superficial laminae I and II in the dorsal horn, where labeling intensity is less strong, and for lamina III, which reveals an only moderate signal. Furthermore, the IML shows strong hybridizations. In the lumbar part (Fig. 3G) labeled neurons are present in all laminae, with low densities in laminae I and II. In laminae III-VIII (Fig. 5C) and around the central canal (lamina X) (Fig. 5D) neurons reveal a particularly strong labeling,

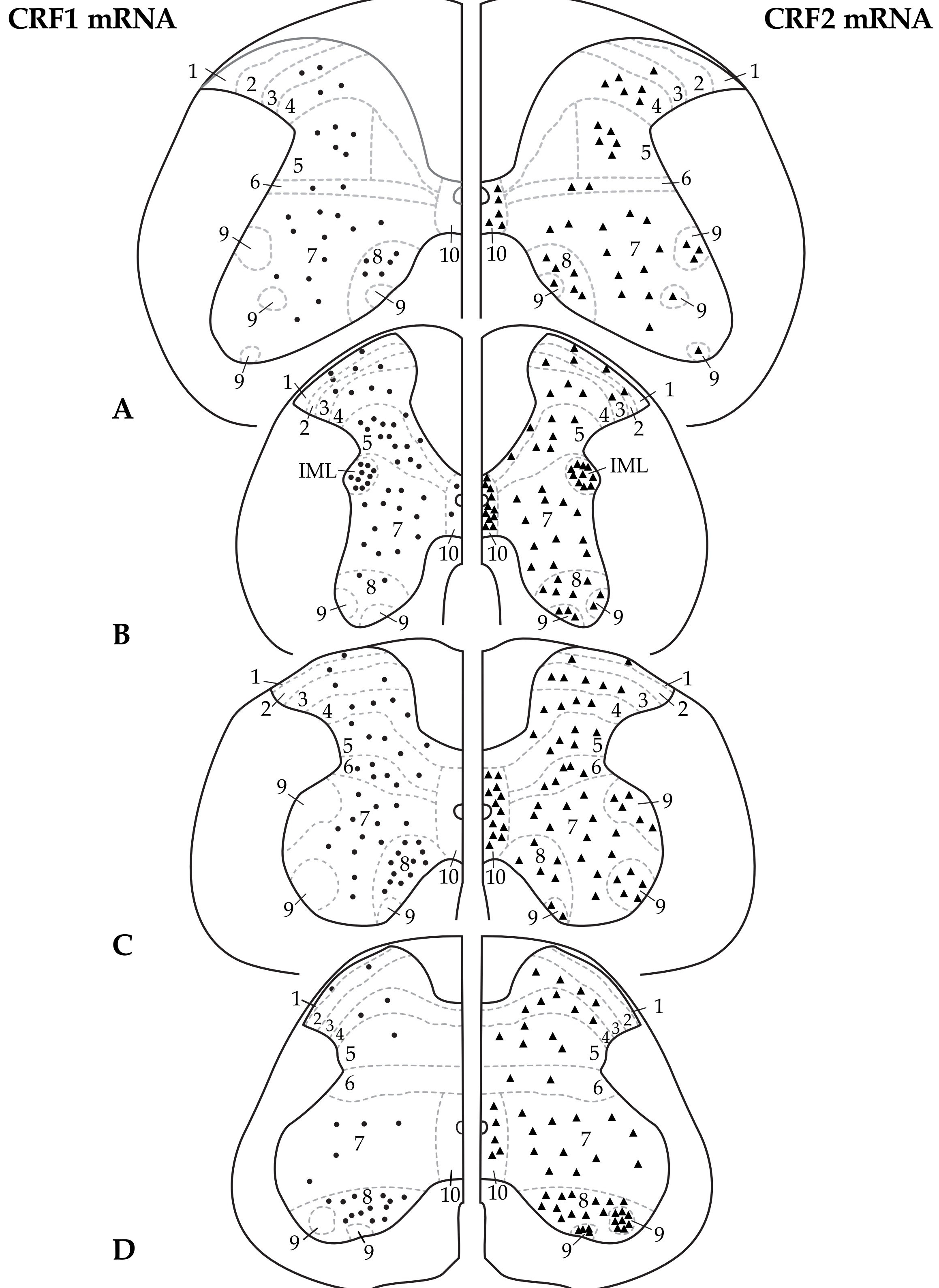

Figure 2. A-D: Distribution of the CRF1 and CRF2 mRNAs in the laminae of Rexed (1-9) in the mouse spinal cord. Schematic drawing of coronal sections showing the distribution of cells expressing CRF1 mRNA (●) and CRF2 mRNA (▲) in a representative section of (A) the cervical part (segment C7), (B) thoracic part (segment T3), (C) lumbar part (segment L5), and (D) sacral part (segment S1).

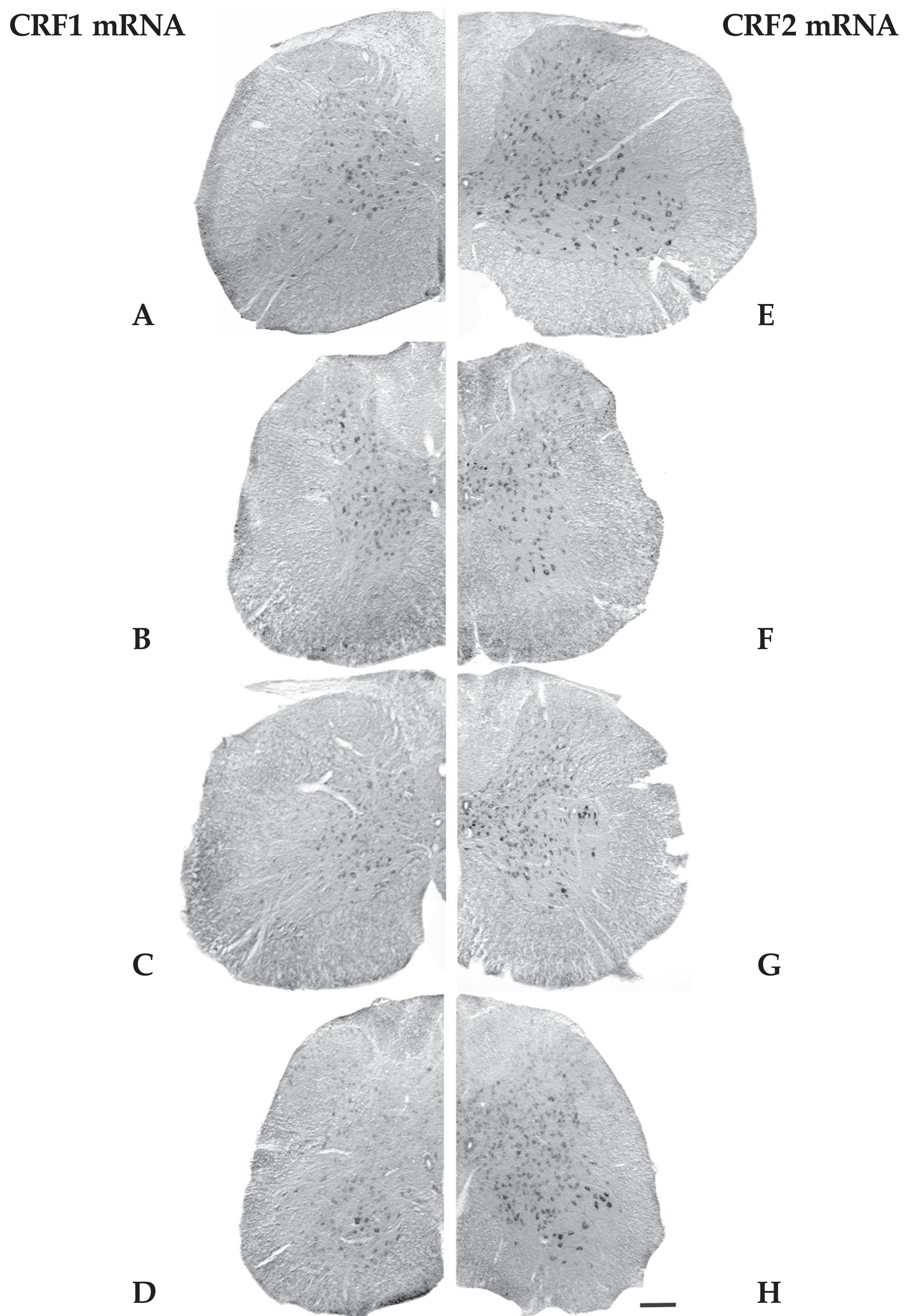

Figure 3. A-H: Distribution of the CRF1 and CRF2 mRNAs in the mouse spinal cord, showing the distribution of (A-D) cells expressing CRF1 and (E-H) cells expressing CRF2 mRNAs, in the cervical (A,E; segments C7,C5, respectively), thoracic (B,F; segment T3), lumbar (C,G; segment L5) and sacral part (D,H; Segment S1). Scale bar = 100 μm.

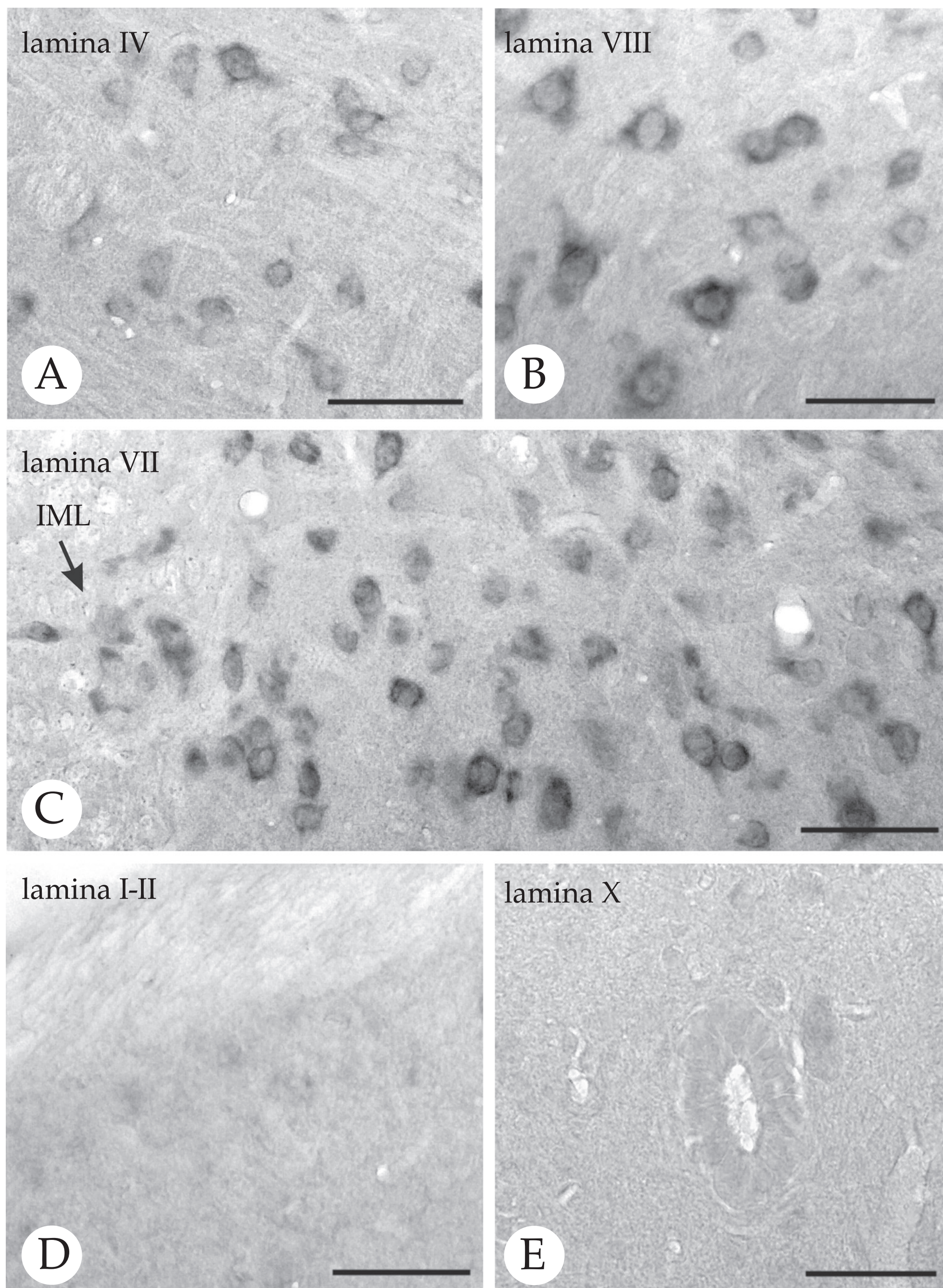

Figure 4. A-E: CRF1 mRNA expression in coronal sections of mouse spinal cord, in (A) lamina IV and (B) lamina VIII both in the cervical part, and (C) in the intermediate gray (lamina VII) and the IML in the thoracic part. No evidence for CRF1 mRNA-positive neurons was observed in (D) laminae I-II of the lumbar part and in (E) lamina X of the sacral part. Scale bar = 50 μm.

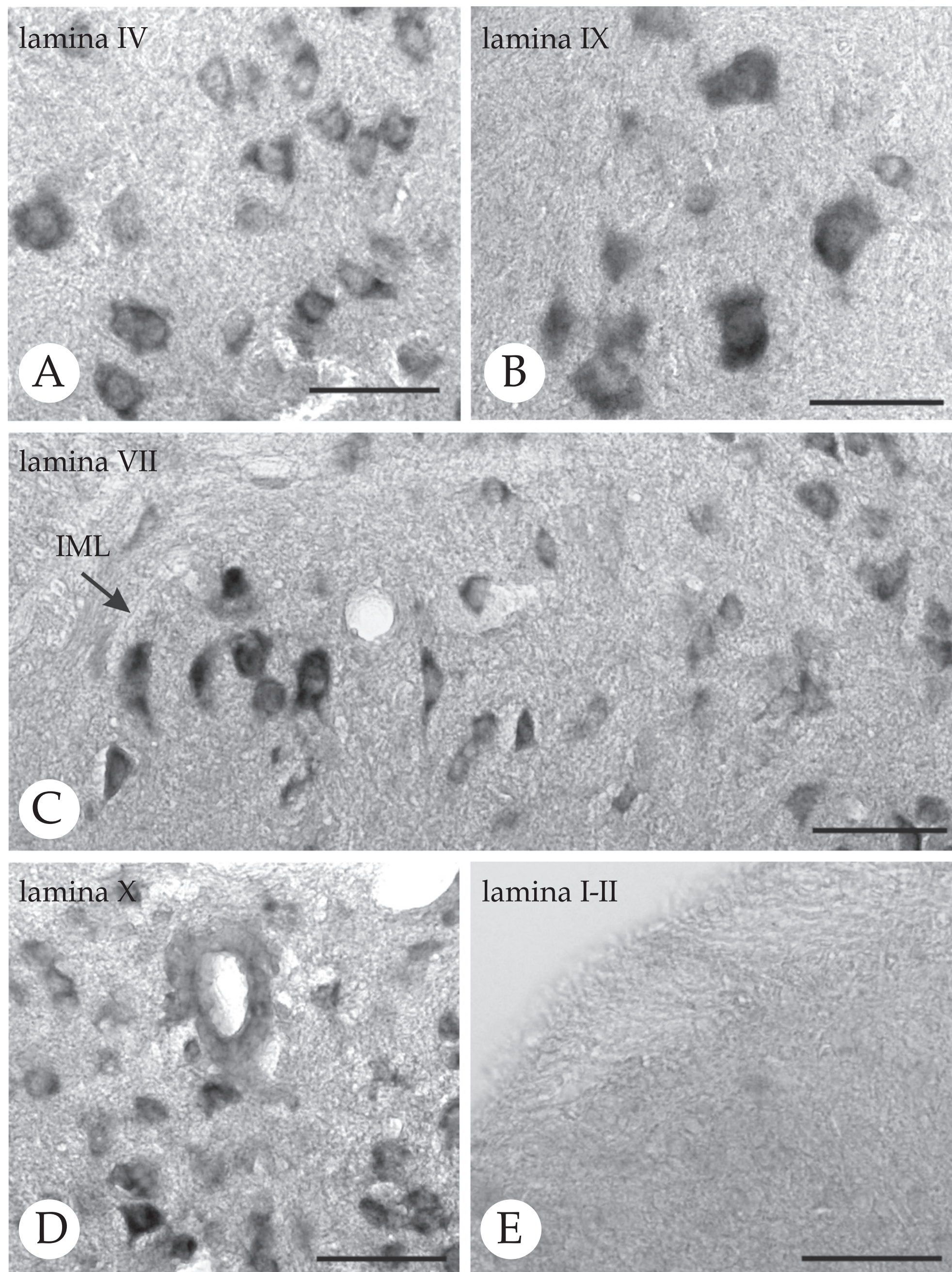

Figure 5. A-E: CRF2 mRNA expression in coronal sections of mouse spinal cord in (A) lamina IV and (B) lamina IX both in the cervical part, and (C) in the intermediate gray (lamina VII) and the IML and in (D) lamina X both in the lumbar part. No evidence for CRF2 mRNA-positive neurons was observed in (E) laminae I-II of the sacral part. Scale bar = 50 μm.

Table 1. Distribution of the CRF1 and CRF2 mRNA in the mouse spinal cord with semi-quantitative ratings of the density of positively labeled cells with in the laminae of Rexed, in four parts of the spinal cord: (-) absence or rare positively labeled cells, (+) low density, (++) moderate density, (+++) high density. The two most right columns give similar ratings for the amount of, respectively, CRF- and Ucn1-immunoreactivity (ir) present in the rat spinal cord, according to the literature.

Part of spinal cord	Lamina of Rexed	CRF1 mRNA	CRF2 mRNA	CRF-ir (rat) (Merchenthaler et al.,1982)	Ucn1-ir (rat) (Bittencourt et al.,1999)
Cervical					
dorsal horn	I-II	-	-	+	+
	III	+	-		
	IV	++	++		
	V	++	+	+	
	VI	+	+	+	
Intermediate zone	VII	+++	+++	++	+++
ventral horn	VIII	++	+++		
	IX	-	+++		+
around central canal	X	-	++	++	++
Thoracic					
dorsal horn	I-II	+	+	+	+
	III	++	+		
	IV	++	++		
	V	+++	++	+	
(only T1)	VI	+	++	+	
	IML	+++	+++	+++	+++
Intermediate zone	VII	+++	+++	++	+++
ventral horn	VIII	++	++		
	IX	-	++		+
around central canal	X	+	+++	++	++
Lumbar					
dorsal horn	I-II	-	-	+	+
	III	+	++		
	IV	++	++		
	V	++	++	+	
	VI	++	++	+	
(only from L1 to L3)	IML	++	+++	+++	+++
Intermediate zone	VII	+++	+++	++	+++
ventral horn	VIII	+++	++		
	IX	-	+++		+
around central canal	X	-	+++	++	++
Sacral					
dorsal horn	I-II	-	-	+	+
	III	-	++		
	IV	+	++		
	V	+	++	+	
(only S1)	VI	-	+	+	
Intermediate zone	VII	++	+++	++	+++
ventral horn	VIII	+++	+++		
	IX	+	+++		+
around central canal	X	-	++	++	++

which is stronger than the labeling seen in any other part and lamina of the cord. In the sacral part (Fig. 3H), except for laminae I and II (Fig. 5E), all laminae show substantial hybridization,with strongest labeling in lamina IX. For a summary of these data, see Table 1 and Fig. 2.

Discussion

General considerations

Up to now, only fragmentary evidence was available for the presence of CRF receptors and CRF2 mRNA in the spinal cord. The present study provides the first description of the distribution of mRNAs of CRF1 and CRF2 in the mouse spinal cord at the neuronal level. The distributions will be discussed, and compared with the distributions of the ligands of these receptors, CRF and Ucn1, in rat. Finally, some functional implications of our results will be considered.

Differences in CRF1 mRNA and CRF2 mRNA distributions

With our non-radioactive *in situ* hybridization approach to demonstrate CRF receptors (Korosi et al., 2006) we here show hybridization signals with CRF1 and CRF2 mRNA probes, in neuronal perikarya in all four parts of the mouse spinal cord. The absence of background staining and of staining with sense probes indicates that the signals specifically reveal the presence of CRF1 and CRF2 mRNAs. This is the first report on the presence of CRF1 mRNA in the mammalian spinal cord and the first detailed description of the distribution of CRF1 mRNA and CRF2 mRNA in the mouse spinal cord. We reveal the presence of these mRNAs in all four parts and in all laminae of Rexed, with the exception of superficial laminae in the dorsal horn. Moreover, our data support our hypothesis that the distributions of the two receptor mRNAs are not identical, as appears from the following observations. CRF2 mRNA has a wider distribution than CRF1 mRNA, occurring throughout the spinal cord in all laminae, with the exception of lamina I-II, where it lacks in the cervical, lumbar and sacral parts and of laminae III, where it lacks in the cervical part. In contrast, CRF1 mRNA is almost completely restricted to laminae III-VIII, with the exception of the thoracic part, where it occurs in laminae I-II and X and of the sacral part, where it lacks in lamina VI.

Obviously, the demonstration of receptor mRNA does not directly prove the presence of receptor protein. However, it is likely that our demonstration of receptor mRNAs is indicative of the presence of CRF1 and CRF2 receptors in the same laminae. First of all, perikarya in the spinal cord can be assumed to receive synaptic input particularly on their dendrites, soma and/or proximal axon (Grant and Koerber, 2004). Therefore, most receptor protein is transported and inserted into the neuronal plasma membrane inside the lamina that contains the mRNA. An exception might hold for the superficial layers

of the spinal cord that are known to contain the dendrites of the so-called 'antenna-like neurons' situated in laminae IV (Grant and Koerber, 2004). As we did not find CRF receptor mRNA in the superficial laminae (I-II) of the cervical, lumbar and sacral parts, it may be that the binding of CRF in these laminae, as described before (Skofitsch et al., 1985; Bell and De Souza, 1988; De Souza, 1995) is due to CRF receptors that have been produced in the perikarya of antenna-like neurons situated in laminae IV. Nevertheless, our finding of a predominance of CRF2 mRNA in laminae IX matches with electrophysiological evidence for the presence of CRF receptors in motoneurons of the ventral horn (Bell and De Souza, 1988). Also, the fact that experimentally applied CRF and Ucn1 increases the number of cfos-positive cells in the IML and in laminae I-VII and X (Maillot et al., 2003), sites we show to contain CRF receptor mRNAs, supports the assumption that CRF receptors generally occur in the same laminae as their mRNAs.

CRF1 mRNA and CRF2 mRNA distributions in relation to CRF and Ucn1 occurrence

On the basis of our assumption that a receptor mRNA and its receptor protein mainly occur within the same lamina, it is of interest to compare the sites of the expression of the respective mRNAs with the decriptions of the presence in the spinal cord of their receptor ligands CRF (Merchenthaler et al., 1983; Schipper et al., 1983; Swanson et al., 1983; Puder and Papka, 2001) and Ucn1 (Bittencourt et al., 1999). These descriptions refer to the rat spinal cord, but in view of the similar distributions of CRF and Ucn1 in the brain of rat and mouse (Keegan et al., 1994; Weitemier et al., 2005) they likely hold for the mouse spinal cord as well.

When the total spinal cord is considered we can conclude that CRF, Ucn1 and both CRF receptors occur together in every part. Such coexistence, however, is hardly useful to generate a hypothesis as to the possible functional relation between a specific ligand and a given receptor. For that purpose, we have to take a closer look at the level of the individual laminae, taking both strength of mRNA expression and strength of ligand immunostaining into account. Then two situations are of interest: (1) 'matching': one or both ligands match with one or with both receptor mRNAs, and (2) 'mismatching': one or more receptor mRNAs but no ligands are present. Below, these situations will be considered into some detail.

(1) Matching. As Table 1 shows, the strongest coexistence of the two receptor mRNAs with both ligands occurs in lamina VII of all four parts of the spinal cord, and in the IML. Furthermore, all parts show coexistence of CRF with CRF1 mRNA and with CRF2 mRNA in lamina V and VI, and of Ucn1 with CRF2 mRNA in lamina IX. Some coexistence is restricted to more restricted regions, such as the coexistence of both receptor mRNAs and both ligands in lamina I-II and X of the thoracic part, and the coexistence of CRF and Ucn1 with only CRF2 mRNA in lamina X of the cervical, lumbar and sacral part. Clearly, these data are only correlative and do not provide conclusive proof that the ligands are

actually released and bind to these receptors, but it should be noted that, for instance, the exclusive presence of Ucn1 and CRF2 mRNA in lamina IX is in line with the high affinity of the CRF2 receptor for Ucn1 (Vaughan et al., 1995).

(2) Mismatching. In some laminae where we show the presence of CRF receptor mRNAs, no CRF or Ucn1 seems to occur (Merchenthaler et al., 1983; Schipper et al., 1983; Swanson et al., 1983; Bittencourt et al., 1999; Puder and Papka, 2001). This holds for lamina III, IV, VIII and IX in case of CRF and for laminae III-VI and VIII with regard to Ucn1. Assuming that the mRNAs found in these layers are indicative of the local presence of their receptors, it may be that other ligands than CRF and Ucn1 act on these receptors. Candidate ligands are two other members of the CRF-family, viz. Ucn2 and Ucn3. Indeed, both in the mouse and the rat brain the distribution of Ucn2 mRNA and in the mouse brain the distribution of Ucn3 mRNA reveals a high degree of overlap with the distribution of CRF2 mRNA, and the involvement of these two peptides in autonomic functions has been suggested (Lewis et al., 2001; Reyes et al., 2001). Moreover, Ucn2 mRNA expression has been shown in the mouse and rat spinal cord (Reyes et al., 2001) .

Functional considerations

On the basis of our mapping and semi-quantitative assessment of the presence and strength of expression of the two CRF receptor mRNAs, and in view of their presumed lamina-specific coexistence with CRF- and Ucn1-containing fibers, proposals may be made as to the functional significance of CRF1 and CRF2 receptors in the mouse spinal cord.

CRFR mRNAs are located throughout the spinal gray matter. Consequently, it may be expected that the CRF receptors and their ligands influence both sensory and motor systems involved in autonomic processes. For example, the predominance of CRF1 and CRF2 mRNAs in lamina VII and in the IML, both containing spinal preganglionic autonomic neurons (Grant and Koerber, 2004), indicates that CRF1 and CRF2 may play a role in mediating sympathetic actions of CRF and Ucn1. Furthermore, the abundance of CRF-immunoreactive and Ucn1-immunoreactive terminals in lamina VII and in the IML (Merchenthaler et al., 1983; Bittencourt et al., 1999) supports the idea that autonomic actions of CRF and Ucn1 are mediated by these spinal cord CRF receptors. Obviously, such activities could be crucial contributions of the spinal mechanism by which CRF and Ucn1 control peripheral stress responses.

It is assumed that CRF controls nociception via CRF receptors in the spinal cord, because intrathecally administered CRF has an analgesic effect that can be antagonized by intrathecal administration of the CRF receptor antagonist, α-helical CRF (Song and Takemori, 1991; Larivière and Melzack, 2000; Millan, 2002). Our data support and extend this idea, by revealing the presence of CRF1 and CRF2 mRNAs in lamina V, which is known to receive nociceptive inputs (Song and Takemori, 1991).

We demonstrate a predominance of CRF2 mRNA in lamina X, which has been suggested to be involved in the control of temperature and visceral nociception (Wang et al., 1999; Grant and Koerber, 2004). There is evidence that CRF and Ucn1 are involved in modulating visceral pain sensitivity (Song and Takemori, 1991; Martinez et al., 2004; Nijsen et al., 2005) and that this modulation takes place via CRF2 (Nijsen et al., 2005; Million et al., 2006). Therefore, the coexistence of CRF- and Ucn1-immunoreactive nerve terminals with CRF2 mRNA in lamina X suggests that the modulation of CRF and Ucn1 of visceral pain perception proceeds through CRF2 in this lamina.

Finally, the abundance of CRF2 mRNA in lamina IX, which contains motoneurons that innervate skeletal muscle (Grant and Koerber, 2004), may reflect an involvement of CRF2 and Ucn1 in the modulation of somatic muscle activity. Especially the coexistence of Ucn1 fibers and CRF2 mRNA in the large motoneurons of the ventral horn (lamina IX), strongly supports the notion that Ucn1 is involved in modulating motor activities via CRF2.

Chapter 5

Urocortin expression in the Edinger-Westphal nucleus is down-regulated in transgenic mice over-expressing neuronal corticotropin-releasing factor

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Neuroscience (2004) 123: 589-594

Abstract

In recent years a large body of evidence has emerged linking chronic stress to increased vulnerability for depression and anxiety disorders. As corticotropin-releasing factor (CRF) is hypersecreted under these psychological conditions, we used our CRF-overexpressing (CRF-OE) mouse line to study underlying brain mechanisms possibly causing these disorders. Urocortin1 (Ucn1), a recently discovered member of the CRF peptide family may play a role in the pathophysiology of stress-induced disorders. Stressors recruit Ucn1-immunoreactive neurons in the Edinger-Westphal nucleus (EW), which is the major site of Ucn1 expression. Furthermore, EW Ucn1 mRNA levels are upregulated in CRF-deficient mice. Based on these findings, we hypothesized the down-regulation of EW Ucn1 in CRF-OE mice and consequently, altered responsiveness to stressful stimuli. Our results support this hypothesis as we found weaker immunohistochemical labelling with anti-Ucn1 and a six times weaker Ucn1 mRNA signal in EW in CRF-OE mice. Moreover, EW Ucn1-expressing neurons mounted a response to acute challenge in CRF-OE mice too. From these results it is concluded that the CRF and EW Ucn1 neuronal systems work in concert in response to acute challenges, but are inversely regulated in their activities during chronic hyperactivity of the hypothalamo-pituitary-adrenal axis.

Introduction

A number of studies have shown that the hypothalamo-pituitary-adrenal (HPA) axis is hyperactive during major depression (Plotsky et al., 1998) and anxiety (Stenzel-Poore et al., 1994; Fossey et al., 1996). Patients suffering from such disorders have strongly elevated CRF levels in the cerebrospinal fluid (Arato et al., 1989). Moreover, *post mortem* studies have revealed an increase in CRF and CRF mRNA in the hypothalamic paraventricular nucleus (Raadsheer et al., 1994). These data indicate that under these psychological conditions CRF is hypersecreted.

Recently, urocortin peptides were identified that are structurally related to CRF, viz. urocortin (Ucn1) (Vaughan et al., 1995), Ucn 2, also known as stresscopin-related peptide (Hsu et al., 2001; Reyes et al., 2001) and Ucn 3, also named stresscopin (Hsu et al., 2001; Lewis et al., 2001). Whereas the functions of CRF are relatively well-known, the physiological significance of urocortins is largely elusive, albeit that central administration of Ucn1 induces a variety of physiological responses (for review, see Skelton et al., 2000b) and behavioural changes as to locomotion and anxiety (Jones et al., 1998; Sajdyk et al., 1999).

The most dominant site of Ucn1-immunoreactivity in the rat brain is the Edinger-Westphal nucleus (EW) (Kozicz et al., 1998; Bittencourt et al., 1999). This compact, cholinergic brain structure is involved in oculomotor adaptation (Westphal, 1887) but the presence of a stress-related neuropeptide and the complexity of ascending and descending connections between this nucleus and non-oculomotor brain areas (Klooster et al., 1993) suggest that the EW is involved in the control of other physiological functions as well. Urocortin mRNA expression in the EW as revealed by *in situ* hybridisation is upregulated 3 hours after restraint stress (Weninger et al., 2000). In concert with this finding, we recently reported increased immediate early gene c-fos expression in the rat EW Ucn1 neurons in response to acute formalin-induced stress (Kozicz et al., 2001b), suggesting a role for EW urocortin in the modulation of the stress response. Interestingly, EW Ucn1 mRNA levels are clearly upregulated in CRF-deficient mice (Weninger et al., 2000) and an inverse relationship between CRF- and Ucn1-containing neuronal systems has been postulated (Weninger et al., 1999; Skelton et al., 2000a).

Based on these findings, we hypothesized the down-regulation of EW Ucn1 in CRF-OE mice and consequently, altered responsiveness to stressful stimuli. To test this, we used our transgenic mouse line, which overexpresses CRF (CRF-OE) exclusively in neuronal tissues (Dirks et al., 2002a) and forms an established experimental model to study the neurobiological, physiological and behavioural changes as occur in human major depression and anxiety (for review, see Groenink et al., 2003). We provide evidence indeed that EW Ucn1 mRNA is substantially downregulated in CRF-OE mice, and that EW urocortinergic neurons can be activated by acute ether stress in a condition of chronically increased levels of neuronal CRF.

Experimental procedures

Animals

Eight weeks old male mice (CRF-OE and wild type littermates-WT) were housed in standard plastic cages, in a temperature- and humidity-controlled environment, and maintained on a 12/12-h light/dark cycle (lights on 06:00). The generation of CRF-OE transgenic mice has been described by Dirks et al. (2002a). In these studies we used the GG-2122 line of CRF-OE mice and their littermates as controls. All procedures were conducted in accordance with the Declaration of Helsinki and the Dutch law for animal welfare. All efforts were made to minimize the number of animals used and their suffering.

Acute ether stress

CRF-OE mice and WT littermates (n=4/experimental group) were kept in a glass container (diameter 20 cm; height 20 cm), saturated with ether vapour, until they collapsed and frequency of breathing slowed down, which took about 1 min. Control mice were handled in the same way, except that they were not challenged. The survival time was 2 h after the acute ether challenge.

Fixation

Animals were deeply anaesthetized with sodium pentobarbital (100 mg/kg body weight, Sanofi Sante, Maassluis, The Netherlands). After opening the chest cavity they were transcardially perfused with 20 ml of 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) followed by 100 ml of 4% ice-cold paraformaldehyde solution.

In situ hybridization

Midbrains from control and stressed CRF-OE mice and WT littermates (n=4) were individually marked and sectioned using a Leica VT 1000S vibratome (Leica, Rijswijk, The Netherlands). Coronal sections (25 µm) were collected in autoclaved 0.1 M PBS, and processed in the same vials allowing reliable comparison of hybridisation signal. *In situ* hybridisation was carried out using antisense and sense (control; no hybridisation signal was seen upon using the sense probe) cRNA probes transcribed from a linearized 550 bp Ucn1 cDNA (generous gift from Dr. P.E. Sawchenko, Salk Institute, La Jolla, CA, USA) and labelled with digoxigenin-UTP. Sections were pre-incubated with 1µg /ml proteinase K, for 10 min at 37 °C. Probes were applied at a probe concentration of 0.2 ng/ml, for 16 h at 20 °C, in a solution containing 25 ml 50% formamide, 3 ml 5 M NaCl, 10 µl 0.5 M EDTA (pH 8.0), 1 ml 50x Denhardt's solution, 10 ml 50% dextransulfate and 1.6 ml milliQ water.

Then, sections were treated with 25 μg/ml ribonuclease A, for 30 min at 37 °C, rinsed in 2x, 1x and 0.5x SSC solutions, and pre-incubated in 0.5% blocking agent, for 30 min. Next, they were incubated with sheep-anti-DIG-AP (1:5,000) for 3 h at 20 °C. After a rinse in buffer A (10 ml 1 M Tris, 3 ml 5 M NaCl and 87 ml milliQ, autoclaved at pH 7.5) and in buffer B (10 ml 1 M Tris, 3 ml 5 M NaCl, 5 ml 1 M $MgCl_2$ and 82 ml milliQ, autoclaved at pH 9.5) sections were incubated in NBT/BCIP medium, for 16 h at 20 °C.

Immunohistochemistry

For DAB immunohistochemistry, 25 μm thick coronal sections from midbrains of CRF-OE (n=6) and WT littermates (n=6) were cut on a Vibratome and rinsed in a solution of 0.1% Triton X-100 (Sigma Chemical, Zwijndrecht, The Netherlands) in PBS for 10 min to enhance antigen penetration. To block non-specific binding sites, sections were placed for 1 h into 5% normal goat serum (Vector ABC Elite Kit; PK-6101; Vector Laboratories, Burlingame, CA, USA). The polyclonal (rabbit) anti c-Fos antiserum (#sc 52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:800, for 48 h at 4 °C, followed by 4x15 min washes in PBS, and incubation in the secondary anti-rabbit IgG (1:200; Vector Labs) for 1 h at 20 °C. After 3x15 min washes in PBS, sections were incubated in ABC reagent (1:50) (Vector Labs) for 1 h at 20 °C, and in 10 mg 3-3'-diaminobenzidine (D 5637; Sigma Chemical) in 50 ml Tris buffer (pH 7.6), for 10 min. Sections of challenged and control animals were processed at the same time and under the same conditions.

For double immunofluorescence labelling, sections were washed for 4x15 min in PBS, incubated in 0.5% Triton X-100 solution for 30 min, and in 5% normal donkey serum (Jackson Immunoresearch Laboratories, West Grove, PA, USA) in PBS for 1 h. Then they were incubated in a mixture of polyclonal (goat) anti-Fos (1:50) and polyclonal rabbit anti-urocortin (1:30,000; PBL#5779, generous gift from Dr W.W. Vale, Salk Institute, La Jolla, CA, USA) for 48 h at 4 °C. After 3x15 min PBS washes, a secondary antiserum cocktail (Cy^2-conjugated anti-goat IgG; 1:50, and Cy^3 conjugated anti-rabbit IgG; 1:100) was applied for 3 h at 20 °C.

The high specificity of the Ucn1 antiserum has previously been reported (Bittencourt et al., 1999). Furthermore, when primary antisera against Fos and Ucn1 were omitted or replaced by non-immune goat or rabbit sera at dilutions of the primary antisera, no immunoreactions were observed.

Counting of labeled neuronal structures

Simple cell counting by direct light microscopic examination assessed differences in the relative amounts of Fos- and Ucn1-expressing cells. Counts of DAB-stained nuclei (Fos) and DIG-labelled neurons (Ucn1) were made at the midlevel of the EW (Bregma: -3.2 to -3.6 mm) in regularly spaced series of 10 sections per animal. Similarly, Cy^2-labeled Fos

nuclei and Cy[3]-stained Ucn1 neurons were quantified for colocalization counts. Since the formula of Floderus (1944) yielded a correction factor of nearly 1 (0.965), no mathematical correction of counts for section thickness was applied.

Image analysis

Images of *in situ* hybridisation material were taken with a Leica DC 500 digital camera mounted on a Leica DMRBE microscope (Leica Microsystems, Heerbrugg, Switzerland). Semi-quantitative image analysis was performed using Scion Image software (version 3.0b; NIH, Bethesda, MR, USA). To quantify Ucn1 mRNA levels, the specific signal density (SSD) was determined relative to neutral background density present in the same section. Four representative sections of the EW per animal were used to produce a single value for each WT, stressed WT, CRF-OE and stressed CRF-OE mice. Statistical analyses were carried out on average values of each experimental group (n=4), and expressed as means ± standard error of the mean (SEM). A random selection procedure was maintained throughout the study. Data were tested by a one-way ANOVA (α=5%), using Statistica (StatSoft, Tulsa, OK, USA), after appropriate transformation of data if needed to fulfil the criteria of normality and homogeneity of variance.

Digital images, using software supplied with the Leica DC 500 digital camera mounted on a Leica DMRBE microscope were taken at a resolution of 1200x1600 pixels, imported into Adobe Photoshop 7.0, and digitally processed (histogram levels, brightness, contrast and sharpness were adjusted).

Results

Presence of Ucn1 and Ucn1 mRNA in CRF-OE vs. WT mice

Ucn1 *in situ* hybridisation revealed numerous, strongly positive Ucn1-expressing neurons in the EW in WT mice (Fig. 1A). In contrast, in CRF-OE mice, we observed a dramatic down-regulation of the Ucn1 signal in this brain area, as neurons were less positive than in WT mice (Fig. 1A,B). Similarly, much weaker Ucn1-immunoreactivity was seen in EW neurons in CRF-OE than in WT mice, indicating a decreased amount of Ucn1 peptide in this nucleus (Fig. 1E,F). We also found a strong difference in the number of neurons expressing Ucn1. In WT mice the mean number of cells per section positive for Ucn1 mRNA was 31±3 whereas in CRF-OE mice only 19±2 neurons per section were labelled ($P<0.01$; $F_{3,22}$:91.08; Fig. 2). Moreover, semiquantitative image analysis demonstrated clearly lower levels (about six times) of Ucn1 mRNA in EW neurons in CRF-OE mice (specific signal density-SSD=11.8±2.0) than in WT mice (SSD=73.8±2.7; $P<0.01$; $F_{3,22}$:116.92) (Fig. 3).

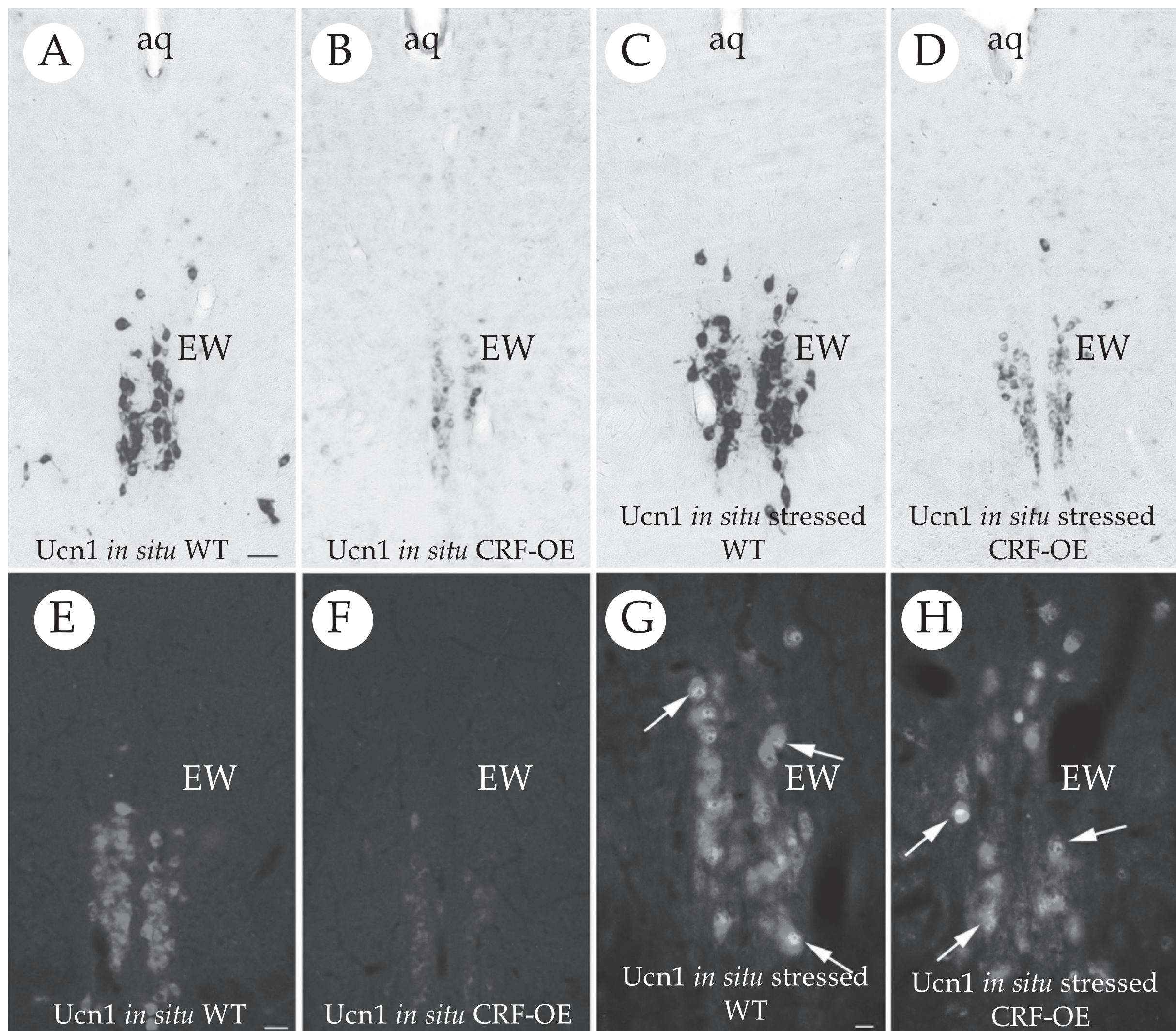

Figure 1. Representative bright field photomicrographs showing expression patterns of Ucn1 mRNA in the EW nucleus in stressed and non-stressed CRF-OE and WT mice (upper images). Ucn1 mRNA expression in (a) WT non-stressed, (b) CRF-OE non-stressed, (c) challenged WT and (d) stressed CRF-OE mice. Scale bar: 50 µm. Fluorescent images showing Ucn1-immunoreactivity (lower left two pictures) and colocalization of Fos and Ucn1-immunoreactivities (lower right two photomicrographs). Ucn1-immunofluorescence labelling in the EW reveals that CRF-OE mice (f) exhibit weaker labelling than WT mice (e). Scale bar: 50 µm. Double immunofluorescence labelling shows colocalization (arrows) of Ucn1 and Fos-immunoreactivities in stressed WT (g) and stressed CRF-OE (h) mice. Scale bar: 25 µm. aq (cerebral aqueduct); IHC (immunohistochemistry); PG (periaqueductal grey).

Effects of ether stress

Quantitative Fos-imaging revealed that in non-challenged WT and CRF-OE mice only occasional activation of EW neurons had taken place (1±1 nucleus per section), whereas acute ether stress had strongly activated EW neurons in WT mice (26±3 nuclei per section). Similarly, in challenged CRF-OE mice the number of Fos-immunoreactive nuclei was much higher (18 ± 2; $P<0.01$) than in non-challenged CRF-OE mice (1±1). However, the

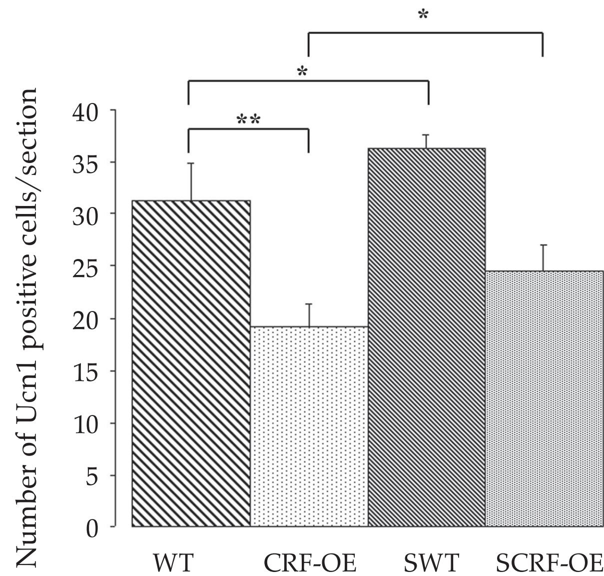

Figure 2. Ucn1 mRNA-expressing perikarya in EW in non-stressed WT and CRF-OE mice *vs.* stressed WT (SWT) and stressed CRF-OE (SCRF-OE) mice. Vertical bars represent the means ± SEM (n=4 mice/group). *P<0.05; ** P<0.01.

mean number of Fos-positive nuclei in stressed CRF-OE mice (18±2) was clearly smaller (P<0.01) than in stressed WT ones (26±3).

We also found a strong increase in the number of EW Ucn1-expressing cells in stressed *vs.* non-stressed CRF-OE mice. The mean number of Ucn1-positive cells was substantially increased (P<0.05; $F_{3,22}$:13.28), viz. from 19±2 (CRF-OE) to 24±2 (stressed CRF-OE) (Fig.

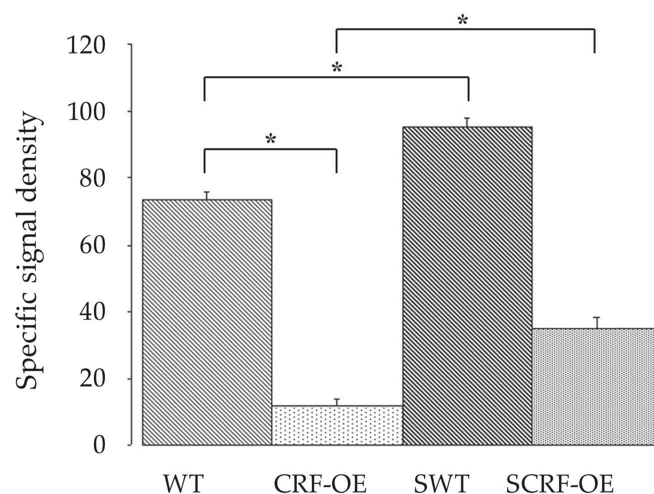

Figure 3. Specific Ucn1 mRNA signal densities in the EW in non-stressed WT and CRF-OE mice compared to stressed WT (SWT) and stressed CRF-OE (SCRF-OE) mice. Vertical bars represent the means ± SEM (n=4 mice/group). * P <0.01.

2). A similar increase was seen when comparing the mean numbers of cells per section in control WT (31±3) with that in stressed WT mice (36±1) (Fig. 2), which is in concert with our previous findings in the rat (Kozicz et al., 2001b).

Also, Ucn1 mRNA was much higher in challenged than in non-challenged CRF-OE animals (Fig. 1B,D). The intensity of the Ucn1 mRNA signal appeared to be three times higher in challenged CRF-OE mice (SSD=35.0±3.4) than in non-challenged ones (SSD=11.8±2.0; P<0.01; $F_{3,22}$:49.94) (Fig. 3). However, this higher SSD was clearly lower than in challenged WT mice (SSD=95.2±2.7; P<0.01; $F_{3,22}$:114.31; Figs 1C,D, 3). These results provide evidence that despite the down-regulation of the Ucn1 mRNA level in the EW, in a state of a chronically high level of CRF the EW urocortinergic system still responds to an acute challenge threatening homeostasis.

Finally, we tested whether the EW neurons that had been activated by acute ether stress, are urocortinergic. Double-immunofluorescence labelling for anti-Ucn1 and anti-Fos showed that an average of 17 out of 23 (74%) Ucn1 neurons colocalized Fos-immunoreactivity in stressed CRF-OE mice, whereas in the stressed WT animals an average of 24 out of 35 (69%) Ucn1 neurons exhibited Fos-immunoreactivity (Fig. 1G,H). Ucn1-negative neurons recruited by acute ether stress were only occasionally seen in either CRF-OE or WT mice. In non-stressed animals no colocalization of Fos and Ucn1 was observed.

Discussion

General methodological considerations

In this study we used non-radioactive *in situ* hybridisation to assess the relative intensity of changes of Ucn1 mRNA expression in the EW. Since we did not aim to study absolute mRNA levels but were only interested in relative differences, no standard curve for DIG labelling had to be generated. In view of the strong differences found, and the methodological rigor we followed, we feel confident as to the reliability of our conclusions with respect to the existence of differences in expression strengths of Ucn1 mRNA between the experimental groups.

Ucn1 in CRF-OE mice

In CRF-deficient mice EW Ucn1 is upregulated (Weninger et al., 2000), suggesting an inverse relationship between CRF and Ucn1. Our observation that EW Ucn1 is down-regulated in CRF-OE mice, not only strengthens this hypothesis, but moreover supports the notion that besides CRF, the well-known central regulator of HPA axis activity, a second pathway is involved in coordinating stress responses in which EW Ucn1 may play a central role (Weninger et al. 1999; Skelton et al., 2000a). However, the exact mechanisms downregulating EW Ucn1 in CRF-OE mice remains at future issue. One possible

mechanism could be that increased glucocorticoid levels in CRF-OE mice (Groenink et al., 2003), via a direct glucocorticoid feed-back on mesencephalic neurons downregulate EW urocortinergic neurons.

Ucn1 has been suggested to be an important neuropeptide involved in the brain control of various physiological aspects of the stress response (Skelton et al., 2000b). Acute stressors activate EW urocortinergic neurons, with a peak of Ucn1 mRNA expression at 2-4 hours after acute challenge (Weninger et al., 2000; Kozicz et al., 2001b). Similar activation of EW neurons was seen following various physiological challenges, such as restraint and immobilization stress as well as psychological stressors, such as ether and immune challenge. Acute ether challenge resulted in the strongest activation of EW Ucn1 perikarya (Kozicz et al., 2001a). These data suggest that EW neurons are sensitive to both physiological and psychological stressors, and may play a role in the regulation of physiological responses during stress. However, up to now it remained to be determined whether acute stressors can activate the down-regulated EW urocortinergic system in a state of CRF-overexpression. Here, we hypothesize that in such a condition the responsiveness of EW neurons to acute challenges is changed. We used acute ether stress (the strongest activator of EW Ucn1 neurons), and found the recruitment of EW Ucn1-containing neurons in CRF-OE mice. The ratio of activated *vs.* non-activated Ucn1 neurons was very similar in WT and CRF-OE animals. Furthermore, it is intriguing that the specific signal density of Ucn1 mRNA compared to baseline levels of non-stressed animals increased with a greater magnitude in stressed CRF-OE than in stressed WT mice. Taken together, these results indicate a maintained acute responsiveness of CRF- and Ucn1-stress adaptation pathways in the state of chronically increased CRF.

Several studies suggest that peptides belonging to the CRF peptide family play biologically distinct roles in generating a stress response, as they act on either the CRF receptor 1 (CRF1) or the CRF receptor 2 (CRF2). For instance, Ucn1 and CRF bind to CRF1 with similar affinities, but the affinity of Ucn1 for CR2 is approximately 40 times stronger than the affinity of CRF itself (Vaughan et al., 1995). Interestingly, Skelton et al. (2000a) showed that chronic administration of the anxiolytic drug alprazolam results in an increased Ucn1 mRNA expression in the EW, whereas Ucn1-deficient mice showed increased anxiety-like behaviour (Vetter et al., 2002). Consistent with this finding, CR2-null mutant mice are also hypersensitive to stressors, and reveal an increased anxiety-like response (Bale et al., 2000; Coste et al., 2000). From these observations it has been concluded that the CR2 receptor mediates central anxiolytic responses, thereby opposing the anxiogenic effects evoked by the CRF1 receptor (Coste et al., 2000). Furthermore, Coste et al. (2001) put forward a model in which CRF1 played a role in the initiation of the stress response, whereas CRF2 was acting during the recovery phase. Interestingly, compared to WT mice, CRF-OE mice showed an enhanced stress-like behaviour after pre-stress (Groenink et al., 2003), suggesting alterations in CRF2 functioning and the possible involvement of urocortin. In CRF-OE mice, we found increased mRNA levels of CRF2 in dorsal raphe nucleus (Korosi et al., 2006). Based on these observations we

hypothesize that interactions among CRF, Ucn1 and CRF receptors may also play a role in the down-regulation of the EW Ucn1 message in CRF-OE mice. However, the nature of these interactions remains at future issue, and so does the identification of neuronal targets on which CRF acts to exert the observed changes.

In summary, our results support the notion that CRF and Ucn1 neuronal systems represent two separate but interrelated systems that inversely regulate adaptation to conditions with chronically elevated levels of CRF (such as during chronic stress and anxiety), but work in concert in response to acute challenges.

Chapter 6

Chronic ether stress-induced response of urocortin 1 neurons in the Edinger-Westphal nucleus in the mouse

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Brain Research (2005) 1046: 172-179

Abstract

Urocortin 1 (Ucn1) neurons, most abundantly expressed in the Edinger-Westphal nucleus (EW), respond to various acute challenges. In a recent study, we found that acute ether stress resulted in the strongest activation of EW Ucn1 cells, as revealed by immunohistochemistry for Fos (often used as a marker for neuronal activation). Although the acute stress responsiveness of EW Ucn1 neurons has been widely studied, the activation pattern of Fos in these neurons in response to repeated challenges has not yet been investigated. Therefore, we quantitatively studied Fos activation in EW neurons, and measured Ucn1 mRNA levels in EW neurons after acute and chronic ether stress in mice. Acute stress resulted in a robust Fos response and an increase in Ucn1 mRNA as compared to non-stressed mice. In the chronic stress paradigm, Fos expression was unchanged, whereas after 2 and 3 weeks of daily ether exposure Ucn1 mRNA expression had strongly declined in the EW. Fos and Ucn1 mRNA were co-expressed in EW neurons in both acutely and chronically stressed animals.

This paper is the first to demonstrate that Ucn1 mRNA-expressing neurons in the EW show a non-habituating Fos response to a chronic homotypic ether challenge that also resulted in a reliable down-regulation of EW Ucn1 mRNA levels vs. acutely stressed animals. Based on these results, we propose that the EW-Ucn1 system represents a novel stress adaptation pathway, which may play an important role in coping with chronic challenges.

Introduction

External and internal stimuli challenging body homeostasis activate distinct neuron populations in the mammalian brain. Analysis of stimulus-induced expression patterns of immediate early genes (IEG) such as c-fos, has proven to be useful in revealing differential recruitment of stress-sensitive brain areas in response to various types of acute and chronic stressors (Bullit, 1990; Chan et al., 1993; Cullinan et al., 1995). Activation of the corticotropin-releasing factor (CRF)-driven hypothalamo-pituitary-adrenal (HPA) axis is a critical component of the stress response. Parvocellular CRF-expressing neurons in the hypothalamic paraventricular nucleus (PVN) display an immediate and robust c-fos response after an acute challenge that lasts for some hours (Stamp and Herbert, 1999; Viau and Sawchenko, 2002). In contrast, repeated exposure to the same stressor (chronic 'homotypic stressor') leads to a complete habituation of PVN c-fos response (Stamp and Herbert, 1999; Viau and Sawchenko, 2002).

Besides CRF itself, other members of the CRF neuropeptide family, such as urocortin 1 (Ucn1; Vaughan et al., 1995), have been implicated in the brain control of physiological adaptation to stress. Central administration of Ucn1 induces a variety of physiological responses (for reviews, see Skelton et al., 2000b; Gysling et al.,2004) that resemble those occurring during stress. Ucn1 shares many structural and pharmacological properties with its family members (including CRF, sauvagine, urotensins, and Ucn2 and Ucn3) (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001), and its distribution in the rat brain has been described in detail (Kozicz et al., 1998; Bittencourt et al., 1999).

The most dominant site of Ucn1 expression is the Edinger-Westphal nucleus (EW) (Kozicz et al., 1998; Bittencourt et al., 1999). This compact, cholinergic nucleus is involved in oculomotor adaptation (Westphal, 1887) but the complexity of ascending and descending connections between the EW and non-oculomotor brain areas (Klooster et al., 1993; Loewy and Saper, 1978; Loewy et al., 1978) and its role in alcohol consumption and thermoregulation (Bachtell et al., 2002a,b) strongly suggest that the EW regulates these important physiological processes. Moreover, the expression of the stress-related neuropeptide, Ucn1 in this midbrain structure has drawn renewed attention to the EW. Indeed, EW-Ucn1 neurons also appear to be involved in the modulation of the acute stress response, since they are recruited by various acute stressors (Kozicz et al., 2001; Gaszner et al., 2004) and their Ucn1 mRNA expression is up-regulated during acute pain and restraint stress (Weninger et al., 1999; Kozicz et al., 2001). Furthermore, data from transgenic animals have revealed that EW-Ucn1 mRNA levels are strongly up-regulated in CRF-deficient mice (Weninger et al., 1999) and down-regulated in CRF-over-expressing mice (Dirks et al., 2002a; Groenink et al., 2002; Kozicz et al., 2004). Based on these data an important role for EW Ucn1 in stress adaptation can be postulated.

Although the acute stress responsiveness of the EW Ucn1 system is well established, little is known about its activation pattern in response to chronic challenges. Therefore,

the aims of this study were: 1) to assess the activation pattern of EW neurons by Fos-immunocytochemistry and 2) to determine the dynamics of EW Ucn1 mRNA expression in response to acute ether and a chronic homotypic ether challenges.

Materials and Methods

Animals

Forty, eight week-old male mice (C57BL/6J; Charles River, Sützfeld, Germany) were housed in standard plastic cages (4 mice/cage), in a temperature- and humidity-controlled environment, and maintained on a 12/12-h light/dark cycle (lights on: 06:00). The animals had permanently free access to food and water. All procedures were conducted in accordance with the Declaration of Helsinki and the Dutch law for animal welfare.

Acute and chronic ether stress

Prior to stressing animals, they were handled daily (placed near but not inside the container, allowing physical contact with it), for 4 days. Then, one group of animals (n=8) was acutely stressed by placing them into a closed glass container (diameter 30 cm; height 20 cm), in which they could freely move around on a metal grid 3 cm above the bottom. The container had been previously saturated with ether vapor with an ether-infiltrated piece of cotton placed under the grid. As soon as animals collapsed and frequency of breathing slowed down to 25-30/min, which occurred typically after 1-2 min of ether exposure, they were taken out of the container, and returned to their home cage. Animals of three other groups (n=8) were repeatedly challenged. For this purpose, they were handled and processed in the same way as the acutely stressed ones, but subjected for 7, 14 or 21 consecutive days to one daily ether exposure. Control mice (n=8) were treated in the same way as stressed animals, including placement for 2 min in a glass container without ether.

Blood sampling and hormone assays

Animals were deeply anesthetized with nembutal (100 mg/kg body weight; Sanofi-Synthelabo, Maassluis, The Netherlands) and after opening the chest cavity a small incision was made in the left ventricle. Within two min after anesthetization, a 30 and a 120 min post-stress blood sample (1 ml/animal) was collected into an ice-chilled tube containing 5 mg EDTA, and centrifuged (3,000 rpm), for 10 min. Plasma aliquots of 50 µl were stored at –20 °C until assay. The serum corticosterone titer was determined by radioimmunoassay, as follows. A mixture of 5 µl serum and 100 µl assay buffer (ASB, *i.e.*, 0.5 M sodium phosphate-buffered saline, PBS, containing 1 mg/ml gelatin and 1 mg/ml sodium azide, pH 7.4) was extracted with 1 ml diethyl ether. The air-dried extract was reconstituted with

1.25 ml ASB and equally divided into two test tubes. Each tube contained 500 μl extract (equivalent to 2 μl serum), tritiated corticosterone (12,000 cpm; NET-399, 90-120 Ci/ml; NEN, Hungary) and 15 nl/tube CS-RCS-57 antiserum (Sigma Chemical, St Louis, MO, USA; final dilution 1:47,000) in a total volume of 700 μl. As a standard, corticosterone was used in a 9-step series ranging from 15 to 4,000 fmol. After 16 h of incubation at 4 ^{o}C, bound and free steroids were separated with dextran-coated charcoal. Radioactivity was measured in a two-phase liquid scintillation system. The characteristics of the assay have been previously determined (Gaszner et al., 2004).

Tissue fixation

Immediately after taking the 120 min blood samples, animals were perfused transcardially with 20 ml 0.1 M PBS (pH 7.4), followed by 100 ml 4% ice-cold paraformaldehyde for 10 min. After dissection, brains were post-fixed in the same fixative, for 24 h, at 4 ^{o}C.

Fos immunocytochemistry

Material from challenged and from control animals was processed simultaneously for immunocytochemistry on free-floating sections. Coronal sections of the forebrain were cut at 25 μm with a VT 1000S vibratome (Leica, Solms, Germany). After 4x15 min washes in PBS, antigen penetration was enhanced with 0.1% Triton X-100 solution (Sigma Chemical) in PBS, for 10 min. After 4x15 min washes in PBS, sections were placed for 1 h into a solution of 2% bovine serum albumin and 10 % heparin (Leo Pharma, Weesp, The Netherlands) to block non-specific binding sites. After a brief wash in PBS, they were incubated in vials containing the primary polyclonal (rabbit) anti-c-Fos serum (1:400; #sc 52; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), for 48 h. After 4x15 min washes in PBS, incubation followed in secondary anti-rabbit serum (1:200; Vector ABC Elite Kit; PK-6101; Vector Labs, Burlingame, CA, USA), for 1 h at 20 ^{o}C. After 3x15 min washes in PBS, sections were treated with ABC reagent (1:50) from the ABC Elite Kits (Vector Labs), for 1 h at 20 ^{o}C. To develop the immunostaining, 10 mg 3-3'-diaminobenzidine (D 5637; Sigma) in 50 ml Tris buffer (pH 7.6) was applied for 10 min. The reaction was controlled under a microscope, and was stopped in Tris buffer. Some of the sections were further processed for *in situ* hybridization of Ucn1 mRNA.

In situ hybridization

Radioactive *in situ* hybridization was carried out using ^{33}P-labeled antisense and sense cRNA probes encoding for Ucn1. The Ucn1 probe (generous gift from Dr. P.E. Sawchenko, Salk Institute, La Jolla, CA, USA) was synthesized from a full length 579 bp Ucn1 cDNA subcloned in pBluescript-SK+ (Stratagene, La Jolla, CA, USA). Paraformaldehyde-fixed sections (see above) were mounted onto pretreated (Superfrost/Plus, Menzel-Glazer,

Braunschweig, Germany) slides and then incubated in 4% paraformaldehyde in 0.1 M borax buffer, for 30 min. After rinsing 5x3 min in PBS and drying in a vacuum desiccator, for 1 h, they were preincubated with 10 μg/ml proteinase K (25530, Invitrogen, Breda, The Netherlands), for 10 min at 37 °C, dehydrated, and dried again under vacuum, for 2 h. Probes were applied for hybridization at about 10^7 cpm/ml, for 16 h at 58 °C, in a solution containing 25 ml deionized formamide, 3 ml 5 M NaCl, 100 μl 0.5 M EDTA (pH 8.0), 1 ml 50x Denhardt's solution, 10 ml 50% dextransulfate and 1.6 ml MQ water. Then sections were treated with 10 μg/ml ribonuclease A (Roche, Mannheim, Germany), for 30 min at 37 °C. Rinses in 2x, 1x and 0.5x SSC containing 1 mM DTT were followed by rinsing in 0.1x SSC, for 30 min at 65 °C. Sections were then dehydrated, defatted in xylene, rinsed in absolute ethanol, air-dried, coated with Kodak NTB-2 liquid autoradiographic emulsion, and exposed in a desiccated, light-tight box, for 5 days at 4 °C. They were developed in Kodak D-19 (Sigma Chemical) developer, for 3.5 min at 14 °C, rinsed briefly in distilled water, fixed with Kodak fixer (Sigma Chemical), rinsed in running tap water for 30 min, dehydrated, and coverslipped with Entellan.

Antiserum characterizations and controls

The specificity of the Fos antiserum was shown by absorbing it with the native Fos peptide (sc-52P; Santa Cruz), which completely abolished its immunoreactivity. Similarly, when the Fos antiserum was omitted from the protocol or replaced by non-immune goat serum (at the same serum dilutions), no immunoreaction was observed.

Image analysis

Digital images were taken at a resolution of 1200x1600 dpi. Amounts of Fos protein in the EW were estimated by counting Fos-positive cell nuclei, using the Leica DMRBE optical system with a Leica DC 500 digital camera (Leica Microsystems, Heerbrugg, Switzerland) connected to an IBM computer running Scion Image software (version 3.0b; NIH, Bethesda, MR, USA). Counts of Fos-positive cell nuclei were made at the midlevel of the EW (Bregma -3.2 to -3.6 mm; Paxinos and Franklin, 2001) in one series of 4 sections (25 μm) per animal interspaced by 75 μm. In sections that had been stained for Fos (immunocytochemistry) and Ucn1 mRNA (*in situ* hybridization), double-labeled neurons were counted when a clear Fos nuclear labeling was accompanied by a silver grain density of >5 times the silver grain density of the background (measured outside the EW). The mathematical correction factor for section thickness of cell counts according to Floderus (1944) was nearly 1 (0.965); therefore, no correction of counts was applied.

Digital images of *in situ* hybridization material were taken and analysed as described above. To quantify Ucn1 mRNA levels, the optical density (OD) was determined, and corrected for the silver grain density of the background as described above.

Statistical analysis

Values of the blood corticosterone level were tested with Student's t-test (α=5%). As to the analysis of Fos and Ucn1 mRNA data four representative sections of the EW per animal were used to calculate a single value for the average number of Fos-positive nuclei per section, and for the optical density (OD) of the Ucn1 mRNA autoradiographic signal. Data of each experimental group (n=4) were expressed as means ± standard error of the mean (SEM). A random selection procedure was maintained throughout the experiments. Fos and Ucn1 mRNA data were tested by one-way analysis of variance (ANOVA) followed by multiple comparisons of individual groups with Tukey's *post hoc* test (α=5%) using Statistica (StatSoft, Tulsa OK, USA), after testing for normality (Shapiro-Wilk test; Shapiro and Wilk, 1965) and for homogeneity of variance (Bartlett's Chi-square test; Snedecor and Cochran, 1989).

Digital imaging of illustrations

Digital images of sections were taken at a resolution of 1,200x1,600 dpi using the software supplied with the Leica DC 500 digital camera. They were imported into Adobe Photoshop 7.0, if necessary digitally adjusted as to brightness, contrast and sharpness, and assembled into plates at a resolution of 400 dpi.

Results

Plasma corticosterone responses

In control animals low plasma corticosterone levels were found, with no difference ($P>0.05$) between samples taken at 30 and 120 min (Fig. 1). Thirty min after initiation of the acute stress the corticosterone level was strongly increased (x8 vs. basal; $P<0.001$; Fig. 1). The level remained elevated ($P<0.001$) 30 min post-stress in animals exposed to repeated ether stress, but an attenuation appeared with increasing challenge period; after 3 weeks of challenge the increase was about 30% lower than in acutely stressed mice (Fig. 1). The corticosterone levels of stressed animals had returned to basal levels 120 min post-stress, as indicated by the fact that they did not differ from those observed in non-stressed controls (Fig. 1).

Fos response to acute ether challenge

In control mice, the level of Fos expression in the EW was very low, as judged from the very small numbers of Fos-positive cell nuclei (Fig. 2A). Acute ether stress recruited EW neurons, since in control animals only 6.3±0.4 cell nuclei showed Fos-labelling, but 2 h post-stress about 6 times (36.4±3.0) more Fos-positive neurons were observed ($F_{4,20}=89.4$;

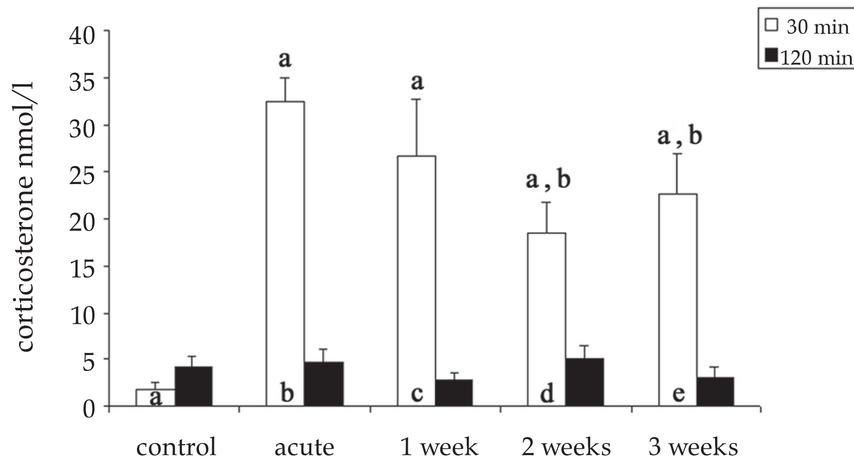

Figure 1. Plasma corticosterone concentration 30 min (white bars) and 120 min (black columns) after ether stress exposure in the various experimental groups. Bars represent the means ± S.E.M (n=4 mice/group). Letters on top of column indicate the experimental group with which significant difference (P<0.001) exists in Student's t test.

P<0.0001; Figs. 2B, 3). The vast majority of EW neurons showed co-expression of Fos and Ucn1 mRNA (Fig. 4A).

Fos response to repeated ether challenge

The number of Fos-positive nuclei in the EW remained clearly elevated throughout the 3-week period of repeated ether stress (32.5±4.0 at 7 days, 36.1±2.3 at 14 days and 34.0±2.5 at 21 days) and no habituation took place (Figs. 2C-E, 3). The ANOVA showed differences across the experimental groups ($F_{4,20}$=89.4; P<0.01) indicating that the challenges had affected one or more exposed groups. To identify these groups, data were analysed with Tukey's test. This revealed that all groups of repeatedly challenged mice exhibited an about 6 times stronger Fos response than unchallenged mice (P<0.01; Fig. 3). However, no difference was observed between acutely and repeatedly challenged animals (P>0.05; Fig. 3). Most of the EW neurons expressing Ucn1 mRNA were also Fos-immunopositive in all chronically challenged groups (Figs. 4B,C)

Ucn1 mRNA expression in the EW

To determine, how the expression of Ucn1 mRNA in the EW is influenced by acute and repeated ether challenges, we first quantified mRNA expression in the EW nucleus. The ANOVA showed a strong difference ($F_{4,20}$=26.8; P<0.001) across the experimental groups, and Tukey's test demonstrated that the acute challenge had strongly increased the amount of Ucn1 mRNA (OD=68.7±12.4, *vs.* 31.2±14.2 in unchallenged mice; P<0.001; Figs. 2F,G, 5). Compared to the acutely stressed group, after one week of repeated stress the OD was not significantly lower (57.2±18; Fig. 3H) but after 2 and 3 weeks of repeated stress it had

strongly declined (P<0.001; 29.5±7.3 and 25.4±10.0, respectively; Fig. 2I,J) to a level that did not differ from control level (Fig. 5).

Finally, we counted the number of Ucn1 mRNA-expressing neurons per section of the EW. The ANOVA ($F_{4,20}$=8.9; P<0.05) followed by Tukey's test showed that the number of Ucn1 mRNA expressing cells increased from 23.7±0.4 (Fig. 6) in controls to 26.7±3.2 two hours after acute stress (Figs. 2G, 6), and reached a maximum of 31.2±2.3 after 1 week of repeated ether challenge (P<0.05; Figs. 2H, 6), and decreased after two (25.8±2.0) and three weeks (23.4±1.6) of repeated challenge to a level not different from the control level (Figs. 2I,J, 6).

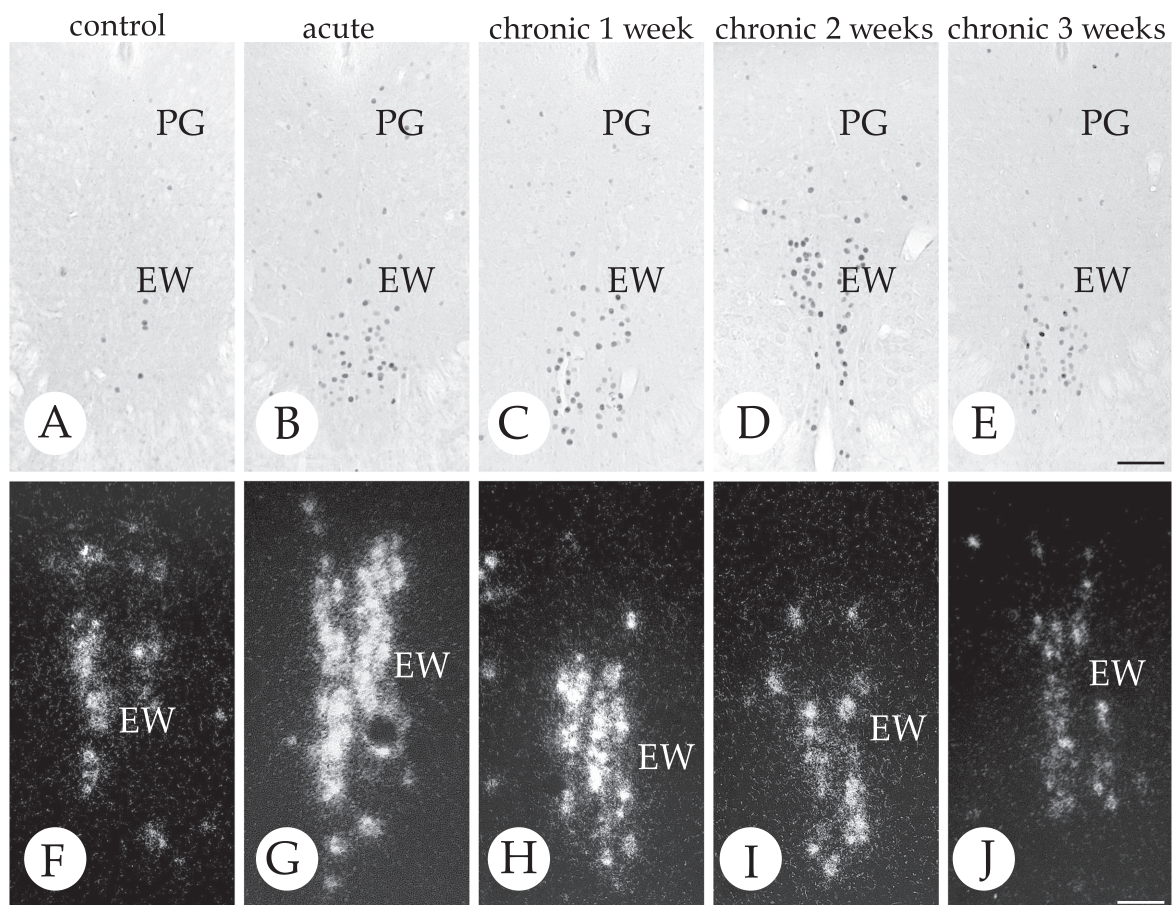

Figure 2. Fos-immunoreactive neurons in the EW (upper images). In control animals (A) only few EW neurons show immunoreactivity, which is in contrast to the high number of such cells after acute challenge (B), 1 week (C), 2 weeks (D) and 3 weeks (E) of daily ether challenge. Dark field images (lower images) show the expression pattern of Ucn1 mRNA in the EW nucleus in controls (F) and in acutely (G) and chronically (H-J) stressed mice. EW-Edinger-Westphal nucleus; PG-periaqueductal gray. Scale bar: A-E: 100 μm, F-J: 50 μm.

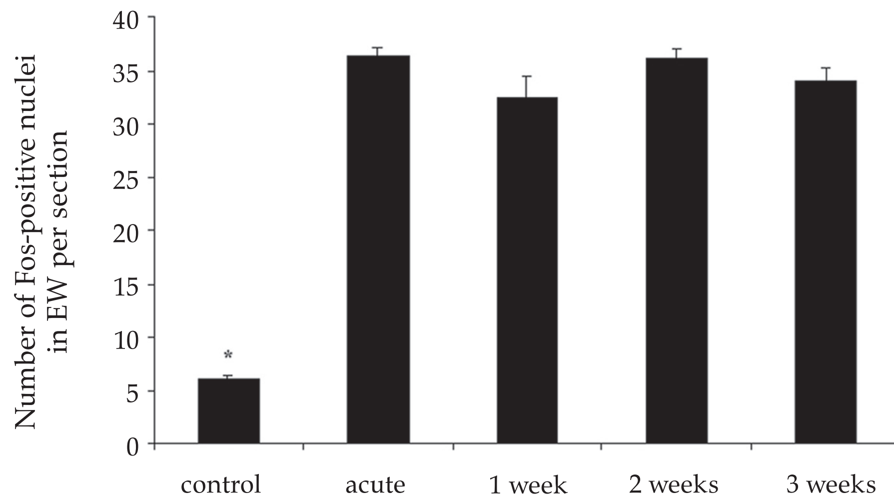

Figure 3. Number of Fos-immunopositive neurons in the EW 120 min after the last exposure to ether stress. Columns correspond to control, acutely challenged and chronically stressed animals for 1 week, 2 weeks and 3 weeks. Bars represent means ± S.E.M (n=4 mice/group). Asterisk indicates significant difference (P< 0.001) between control and each other group.

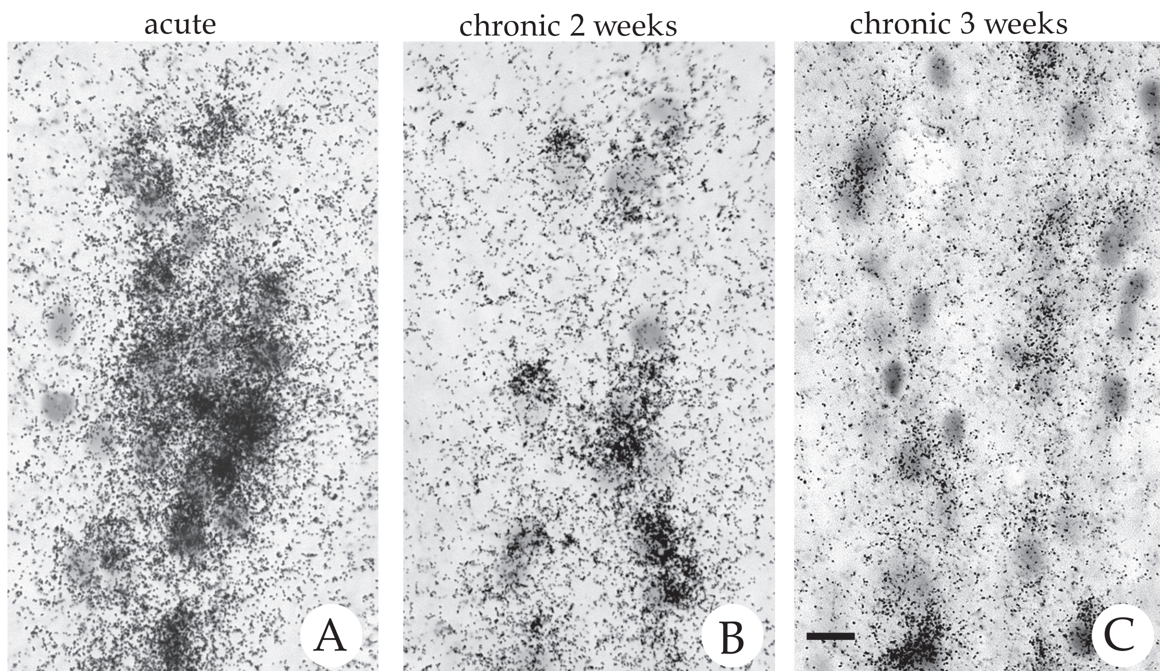

Figure 4. Many EW-Ucn1 mRNA-expressing neurons exhibit Fos-immunopositivity in acutely (A) and chronically (B and C) challenged mice. Scale bar: 10 μm.

Discussion

In this study we have demonstrated that a chronic, repeated ether challenge evoked an adaptation of the EW Ucn1 system, since the number of Fos-positive nuclei in the EW remained clearly elevated throughout the 3-week period of repeated stress. Moreover, EW Ucn1 neurons showed a complete down-regulation of the EW Ucn1 message in repeatedly stressed mice as compared to acutely challenged ones. This shows that in response to a chronic ether challenge, EW Ucn1 neurons reveal a non-habituating Fos response, suggesting that the EW Ucn1 system represents a novel stress adaptation system.

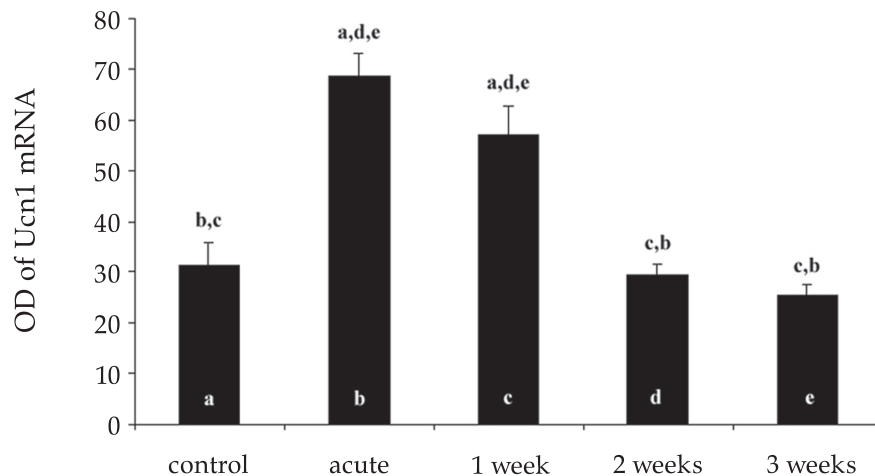

Figure 5. Optical density (OD) of Ucn1 mRNA in the EW 120 min after the last exposure to ether stress. Columns refer to control (a), acutely challenged (b), and chronically challenged mice for 1 week (c), 2 weeks (d) and 3 weeks (e). Bars represent means ± S.E.M (n=4 mice/group). Letters on top of column indicate the experimental group with which significant difference (P<0.001) exists.

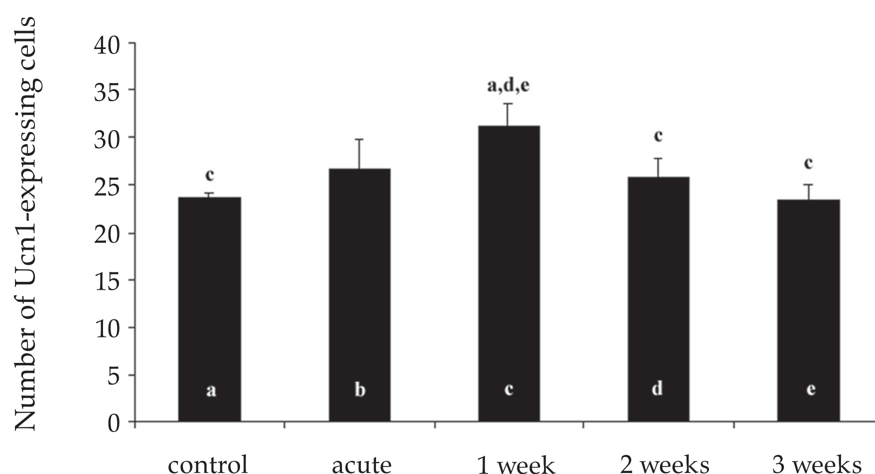

Figure 6. Number of Ucn1 mRNA-expressing neurons in the EW 120 min after the last exposure to ether stress. Columns correspond to control (a), acutely (b) and chronically (c-e) stressed mice for 1 week (c), 2 weeks (d) and 3 weeks (e). Bars represent the means ± S.E.M (n=4 mice/group). Letters on top of column indicate the experimental group with which significant difference (P<0.001) exists.

Analysis of stimulus-induced patterns of IEG expression, such as of the c-fos gene, are useful in showing different recruitment patterns of stress-responsive neuron populations in the brain in response to acute and chronic stressors (Bullit, 1990; Chan et al., 1993; Cullinan et al., 1995). Indeed, various acute stressors activate c-fos both in the PVN-CRF neurons (Viau and Sawchenko, 2002; Dunn et al., 2004) and in the EW-Ucn1 neurons (Kozicz et al., 2001; Gaszner et al., 2004). In PVN-CRF perikarya acute restraint exposure provokes a surprisingly rapid c-fos activation that is first detected at 30 min after stress, whereas EW-Ucn1 neurons show a somewhat delayed activation in response to stress (with a maximum 2-3 hours after stress initiation) and remain active for up to 18-24 hours (Kozicz et al., 2001; Weninger et al., 2000; Gaszner et al., 2004). Interestingly, repeated exposure to stressors often modifies the pattern of c-fos expression seen after acute stress, and the tendency for c-fos responses to habituate to repeated challenges is commonly found in various stress-sensitive brain centers (Melia et al., 1994; Chen et al., 1995; Viau and Sawchenko, 2002). Some areas (*e.g.* the lateral septum) show a slowly diminishing strength of the response, whereas in other areas, such as in the PVN, adaptation of IEG expression is more rapid and complete (Stamp and Herbert 1999; Viau and Sawchenko, 2002). Our observation of the non-habituating Fos response of EW Ucn1 neurons in a chronic homotypic stress paradigm is in contrast with this general phenomenon.

The question arises as to the mechanism responsible for the difference in response dynamics between the PVN and the EW in chronic stress. One possible mechanism could be the distinct dynamics of catecholaminergic inputs to these brain areas. In rat, central catecholaminergic neurons are activated by various types of stressor and they strongly innervate CRF neurons in the PVN (Kitazawa et al., 1987; Liposits and Paull, 1989), as well as Ucn1 neurons in the EW (Gaszner and Kozicz, 2003). The role of ascending cathecholaminergic inputs in the activation of IEG in the PVN in acute stress is well established (Murakami et al., 1997; Senba and Ueyama, 1997), and their habituation in chronic stress (Sawchenko et al., 1992; Laucher et al., 1994) may well contribute to the habituated response of PVN CRF neurons. In contrast to their role in the PVN, depletion of catecholaminergic terminals does not attenuate the Fos response of EW Ucn1 neurons to an acute challenge (Gaszner and Kozicz, 2003). Thus, it is unlikely that ascending catecholaminergic pathways play a role in the stress-response of the EW. Similarly, Bachtell et al. (2002b) concluded that dopamine did not play a role in alcohol-induced Fos expression in the EW. Therefore, we assume that ascending brainstem catecholaminergic terminals do not contribute to activation of EW neurons, neither in an acute nor in a chronic stress paradigm.

The biological actions of the members of the CRF-neuropeptide family are mediated via two types of G-protein-coupled receptor, CRF1 and CRF2, which have distinct expression patterns and physiological functions (Chen et al., 1993; Chalmers et al., 1995; Lovenberg et al., 1995a,b). Thus, in the mammalian brain, the CRF/Ucn receptors comprise two receptors and four ligands, two of which (Ucn2 and Ucn3) are pharmacologically monogamous and CRF and Ucn1 are promiscuous, acting on both CRF1 and CRF2 (Vaughan et al., 1995).

A prevailing theory claims a temporal difference in activation patterns of these receptors, *i.e.*, CRF1 would mediate the initiation phase of the stress response while CRF2 would act during the recovery phase (Bale et al., 2000, 2004; Coste et al., 2001; Reul and Holsboer, 2002; Strohle and Holsboer, 2003). The balanced activation of CRF1 and CRF2 pathways is thought to be essential for mental health (Reul and Holboer, 2002). Given the binding properties of Ucn1 for each CRF receptor and the delayed and maintained activation of EW-Ucn1 neurons in response to stress (see above), we hypothesize that Ucn1 plays an important role in shifting the initiation phase to the recovery phase, in a balanced manner, thereby promoting adaptation ('allostasis'; Sterling and Eyer, 1988; McEwen and Wingfield, 2003).

Our results show that Ucn1 cells in the EW exhibit a non-habituating Fos response to repeated ether challenge. On the other hand, this maintained Fos responsiveness is accompanied by a down-regulation of the EW Ucn1 message. This interesting adaptation of the EW Ucn1 system may be explained by an exhaustion of the secretory capacity of EW Ucn1 neurons upon perturbed activation. This could eventually end up in insufficient levels of Ucn1 peptide and an imbalance between CRF1- and CRF2-mediated stress-responses, resulting in physiological and mental disorders. In conclusion, here we present evidence indicating that the EW Ucn1 system adapts to chronic stressors and this process may play a role in adaptation to conditions that pose repeated challenges to the organism.

Acknowledgements

We thank Koen Weterings and Richard Feron (Department of Plant Cell Biology, Radboud University Nijmegen, The Netherlands) for their excellent technical expertise and support, and Kelly E. Jackson (Tulane University, New Orleans, LA, USA) for editing the manuscript.

Chapter 7

Urocortin 1 expression in the mouse Edinger-Westphal nucleus is regulated by chronically elevated corticosterone

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Submitted to European Journal of Neuroscience (2006)

Abstract

Successful stress adaptation requires activation of the hypothalamo-pituitary-adrenal (HPA-) axis, which releases corticotropin-releasing factor (CRF) and corticosteroids that help the organism to cope with the stressor. Glucocorticoids act through mineralocorticoid and glucocorticoid receptors (GR). Another brain nucleus assumed to play a role in stress adaptation is the Edinger-Westphal nucleus (EW). The EW is a main brain site containing the CRF-related peptide, urocortin 1 (Ucn1), and EW-Ucn1 expression is modulated by various acute and chronic stressors. This notion has led us to hypothesize that adaptation to stress is under the coordinate control of the HPA axis and the EW-Ucn1 system through corticosterone directly acting on EW-Ucn1 neurons. To support this hypothesis, we studied the colocalization of GR and Ucn1 in the mouse EW and tested the effect of 14 days of subcutaneous corticosterone administration on EW-Ucn1 neurons using immunohistochemistry, in situ hybridization and cytometry. At the single neuron level, the presence of GR and GR mRNA, the coexistence of GR with Ucn1, and changed patterns of Ucn1 peptide and Ucn1 mRNA expression were found, indicating that corticosterone may directly control activity of EW-Ucn1 neurons, providing a regulatory link between the HPA-axis and the EW during stress adaptation.

Introduction

Stress adaptation requires the activation of the hypothalamo-pituitary-adrenal (HPA-) axis, leading to the release of glucocorticoids that facilitate coping with the stressor (de Kloet et al., 1998; de Kloet, 2000). In rodents corticosterone regulates neurotransmitter levels (Azmitia, and McEwen 1974; Gottesfeld et al., 1978; de Kloet and Reul 1987), receptor densities (Biegon et al., 1985), intracellular signal transduction (Harrelson and McEwen 1987; Harrelson et al., 1987) as well as neuronal cell birth and death in the central nervous system (Sapolsky 1990; Gould and McEwen 1993; Sloviter et al., 1993a,b).

The effects of glucocorticoids on the brain are mediated via type I (mineralocorticoid receptor; MR) and type II (glucocorticoid receptor; GR) receptors (McEwen et al., 1986). Corticosteroids have a higher affinity for MR than for GR (Reul and de Kloet 1985). MRs are already occupied at basal corticosterone levels, whereas GRs become occupied only at high corticosterone levels, like during the peak of its circadian cycle or as a result of stressful events (Ratka et al., 1989; McEwen et al., 1992; Kawata 1995; de Kloet 2003). MRs are prevailing in limbic areas (van Eekelen et al., 1987; Chao et al., 1989) whereas GRs are more generally distributed throughout the brain (for review, see de Kloet et al., 1998).

The Edinger-Westphal (EW) nucleus is a dorsal midbrain nucleus. The EW displays a large number of neurons expressing GRs and GR mRNA (Morimoto et al., 1996). The EW is also the main site of urocortin 1 (Ucn1) expression in the brain and Ucn1-immunoreactive fibres of possible EW origin are observed mainly in lateral septum, dorsal raphe and spinal cord (Weninger et al., 1999; Skelton et al., 2000b; Weitemier et al., 2005). EW-Ucn1 has been implicated in the stress response (Weninger et al., 2000; Gaszner et al., 2004; Kozicz et al., 2004; Korosi et al., 2005). Administration of Ucn1 elicits similar physiological and behavioural responses as corticotropin-releasing factor (CRF) (Skelton et al., 2000b). For instance, intravenous injection of Ucn1 increases plasma adrenocorticotrope hormone level (Asaba et al., 1998; Oki and Sasano 2004), and intracerebroventricular injection of Ucn1 enhances anxiety-like behaviour (Spina et al., 1996; Moreau et al., 1997; Jones et al., 1998; Coste et al., 2000; Skelton et al., 2000b; Latchman 2002). Furthermore, Ucn1 expression in the EW is up-regulated in response to various acute challenges (Weninger et al., 2000; Bachtell et al., 2002a; Kozicz, 2003; Gaszner et al., 2004) and by benzodiazepines (Skelton et al., 2000a), whereas it is down-regulated by chronic stressors (Korosi et al., 2005). In addition, changed expression of EW-Ucn1 mRNA has been shown in mice with genetically modified CRF systems (Bale et al., 2000; Weninger et al., 2000; Kozicz et al., 2004).

Based on such observations it has been proposed that Ucn1-containing neurons in the EW cooperate with the HPA-axis to control stress adaptation processes (Weninger et al., 2000; Kozicz et al., 2004). However, the underlying mechanism of this cooperation is unknown. In this respect it is noteworthy that all conditions that evoke a change in Ucn1 expression in the EW also change plasma corticosterone levels (Skelton et al., 2000a; Weninger et al., 2000; Groenink et al., 2002; Kozicz, 2003; Gaszner et al., 2004). Therefore,

we suggest that corticosterone is part of a regulatory link between the HPA-axis and the EW-Ucn1 system during stress adaptation, by modulating not only hypothalamic CRF expression but also Ucn1 expression in the EW. More specifically, we hypothesize that adrenal corticosterone feeds back on Ucn1-containing EW neurons via GR, to change the neurons secretory activity. To test this hypothesis, we have examined in the EW of the mouse (1) the possible coexistence of GRs with Ucn1 and (2) the possible effects of chronic (two-weeks) corticosterone treatment on Ucn1 and Ucn1 mRNA expression, using immunocytochemistry, *in situ* hybridization and cytometry.

Materials and Methods

Animals

Eighteen, twelve weeks-old male mice (C57BL/6J; Charles River, Sutzfeld, Germany) were housed individually in standard plastic cages, in a temperature- and humidity-controlled environment, at a 12/12-h light/dark cycle (lights on: 06:00). Singular housing started 2 weeks and daily handling one week before implantation of either placebo 'sham' or corticosterone pellet (CORT) (see below). Animals had free access to food and water. All procedures were approved by the ethical committee for animal experimentation of the Departments of Pharmaceutical Sciences, Biology and Chemistry, Utrecht University (Dec-GNK-FSB), in accordance to the Dutch law for animal experimentation and the Declaration of Helsinki.

Corticosterone and placebo pellet implantations

Pellets of 100 mg (7 mm diameter) contained either 100% cholesterol (shams) or 80 % cholesterol (Acros Organics, Geel, Belgium) and 20 % corticosterone (ICN Biomedicals, Zoetermeer, The Netherlands) ('CORT'). They were implanted in adrenal-intact mice (n=9/group) under isofluorane anaesthesia. After a small incision was made under aseptic conditions into the skin at the nape of the neck, the pellet was pressed subcutaneously towards the side, so that the animal would be minimally disturbed in its movements. The incision was closed with suture material (vicryl rapid 5/0 45 cm; Ethicon Johnson and Johnson, Amersfoort, The Netherlands). Pellets were left in place for 14 days.

Hormone assays, body and organ weights

For plasma corticosterone measurements, blood samples were taken, every third day, between 09:00-10:00 a.m., starting the day before pellet implantation, during a period of 14 days. A sample (50-100 µl) was taken by tail incision, and collected into an ice-chilled capillaries containing 5 mg EDTA, and centrifuged (4,000 rpm) at 4 ˚C, for 15 min. Plasma aliquots of 50 µl were stored at –20 °C until assay. Corticosterone concentrations were

determined in duplicate, using a double-antibody radioimmunoassay (ICN Biomedicals). The characteristics of the assay have been previously determined (Groenink et al., 2002). All samples were taken by the experimenter who daily handled the mice. Body weights were determined every third day starting the day before the pellet implantation, during 14 days. At day 14, the adrenal glands and the thymus were dissected from all animals, freed from fat, and their wet weights determined.

Tissue processing

Animals (n=9/group) were deeply anesthetized with nembutal (60 mg/ml sodium pentobarbital, Sanofi Santé B.V., Maassluis, The Netherlands; 0.1 ml/mouse intraperitoneally), and perfused transcardially with sterile saline followed by freshly prepared 4% paraformaldehyde in RNAse-free 0.1 M sodium phosphate buffer (pH 7.4; 100 ml/mouse). Then, animals were decapitated and their brains removed, postfixed in 4% paraformaldehyde for 16 h at 4 °C, and stored in autoclaved 0.1 M sodium phosphate-buffered saline (PBS), at 4 °C. Brains were transferred to 30% sucrose in 0.1 M PBS for 16 h at 20 °C, and frozen with dry ice. Thirty µm coronal sections with 150-µm intervals were cut with a HM 440 E freezing microtome (Microm, Walldorf, Germany) and saved in sterile antifreeze solution (0.05 M PBS, 30% ethylene glycol, 20% glycerol) at -20 °C, until processing for immunocytochemistry and *in situ* hybridisation. For these procedures, sections of shams and CORT animals were processed simultaneously, at room temperature (20 °C), unless stated otherwise, as described below. Consecutive sections of the same animals were used for quantitative immunocytochemical and *in situ* hybridization studies.

Immunocytochemistry

For Ucn1 monolabeling, sections of CORT (n=9) and sham (n=9) animals were washed 3x10 min in 0.1 M PBS, and treated with 1% Triton X-100 (Sigma Chemical, Zwijndrecht, The Netherlands) in 0.1 M PBS, for 10 min. Then they were placed in 5% normal goat serum (Vector ABC Elite Kit; Vector Labs., Burlingame, CA, USA) in 0.1 M PBS, for 30 min, rinsed in 0.1 M PBS, and incubated in primary polyclonal (rabbit) anti-Ucn1 serum (1:5,000; gift from Dr. W.W. Vale, Salk Institute, La Jolla, CA, USA), for 16 h. After 3x10 min washes in 0.1 M PBS, incubation followed in secondary goat-anti-rabbit biotinylated serum (1:200; Vector Laboratories), for 1 h. After 3x10 min rinses in PBS, sections were treated with ABC reagent (1:100; Vector Laboratories) for 1 h, and with 10 mg 3-3'-diaminobenzidine (Sigma) in 50 ml Tris buffer (pH 7.6), for ca. 10 min.

For double immunofluorescence of Ucn1 and GR, the same procedure was followed, but after Triton X-100 treatment sections from shams (n=3) were washed 3x10 min in 0.1 M PBS and placed for 30 min into a solution of 0.5% blocking reagent (TSA Fluorescence System Kit; NEN Life Science Products, Renaissance; Boston, MA, USA) in 0.1 M PBS

(PBS-BT). Then, they were incubated in a mixture of primary polyclonal (goat) anti-Ucn1 serum (1:250; R-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and polyclonal (rabbit) anti-GR serum (1:10,000; M-20; Santa Cruz) in PBS-BT, for 16 h. After 3x10 min washes in 0.1 M PBS, incubation followed in secondary antiserum cocktail (Cy^2-conjugated donkey-anti-goat IgG; 1:80; Jackson Immunoresearch Laboratories, West Grove, PA, USA) and donkey-anti-rabbit biotinylated IgG (1:200; Vector Laboratories) in PBS-BT, for 2 h. After rinses of 3x5 min in 0.1 M PBS, sections were incubated in streptavidin-horseradish peroxidase (1:100; Renaissance) in PBS-BT for 30 min. Finally, after 3x5 min in 0.1 M PBS, sections were incubated in Cy^3 tyramide amplification reagent (1:50 in 1X amplification diluent; Renaissance), for 6 min. Reaction was stopped in 0.1 M PBS.

The high specificity of the Ucn1 antiserum has previously been reported (Bittencourt et al., 1999) and the high specificity of the GR antiserum has been tested before (Boyle et al., 2005). Furthermore, when the primary Ucn1 and GR antiserum was omitted, or replaced by non-immune rabbit serum at dilution of the primary antiserum, no immunoreaction was observed.

In situ hybridization

Radioactive *in situ* hybridization of Ucn1 mRNA was carried out in sham (n=9) and CORT animals (n=9) using ^{33}P (ICN Biomedicals) labelled antisense and sense cRNA probes encoding for Ucn1. Probes (gifts from Dr. P.E. Sawchenko, The Salk Institute, La Jolla, CA, USA) had been synthesized from a full length 579 bp Ucn1 cDNA subcloned in pBluescript-SK+ (Stratagene, La Jolla, CA, USA). Sections were mounted onto pretreated (Superfrost/Plus, Menzel-Glazer, Menzel, Germany) slides and then incubated in 4% paraformaldehyde in 0.1 M borax buffer, for 30 min. After rinsing 5x3 min in PBS and drying in a vacuum desiccator for 1 h, they were preincubated with 10 μg/ml proteinase K (25530, Invitrogen, Breda, The Netherlands), for 10 min at 37 °C, dehydrated, and dried again under vacuum, for 2 h. Probes were applied at about 10^7 cpm/ml, for 16 h at 58 °C, in a solution containing 25 ml deionized formamide, 3 ml 5 M NaCl, 100 μl 0.5 M EDTA (pH 8.0), 1 ml 50x Denhardt's solution, 10 ml 50% dextransulfate, and 1.6 ml MQ water. Then sections were treated with 10 μg/ml ribonuclease A (Roche, Mannheim, Germany), for 30 min at 37 °C. Rinses in 2x, 1x and 0.5x SSC containing 1 mM DTT were followed by rinsing in 0.1x SSC, for 30 min at 65 °C. Sections were then dehydrated, defatted in xylene, rinsed in absolute ethanol, air-dried, coated with Kodak NTB-2 liquid autoradiographic emulsion, and exposed in a desiccated, light-tight box for 5 days at 4 °C. They were developed in Kodak D-19 (Sigma Chemical) developer, for 3.5 min at 14 °C, rinsed briefly in distilled water, fixed with Kodak fixer (Sigma Chemical), rinsed in running tap water for 30 min, dehydrated, and coverslipped using Entellan.

Non-radioactive *in situ* hybridization of GR mRNAs was carried out on shams (n=3) with the free-floating section method according to Jessell (http://cpmcnet.columbia.edu/dept/neurobeh/jessell/insitu.html), with minor modifications, as follows. Antisense and

sense (control) RNA probes were generated using a full length 520 bp GR cDNA subcloned in pBluescript (Stratagene, La Jolla, CA, USA) labelled with DIG-11-UTP using a labelling kit from Roche Molecular Biochemicals (Basel, Switzerland). First, sections were fixed in 0.1 M borax-buffered 4% paraformaldehyde (pH 9.5), at 4 °C for 30 min, and rinsed 4x7 min in 0.1 M PBS. After rinsing in autoclaved MQ water, acetylation was performed with 0.25% acetic acid anhydride in 0.1 M tri-ethanolamine buffer (pH 8.0), for 10 min, followed by rinsing in 2 times concentrated (2X) standard saline citrate buffer (SSC; pH 7.0) for 5 min. Hybridization mixture (50% deionized formamide, 0.3 M NaCl, 0.001 M EDTA, Denhardt's solution, 10% dextran sulphate) together with 0.5 mg/ml tRNA and the mRNA-digoxigenin (DIG) probe (ca. 2.5 ng/ml), were placed into a water bath at 80 °C for 5 min, and then on ice for another 5 min. Sections were incubated in hybridization solution for 16 h at 60 °C, rinsed 4x7 min with 4X SSC, incubated for 30 min at 37 °C in preheated RNAse medium (0.5 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, 0.01 mg/ml RNAse A; pH 8.0) that had been added just before the start of incubation, and stringently washed in steps with decreasing SSC concentrations (2X, 1X, 0.5X, 0.1X), for 30 min at 58 °C. Dig label was detected with the alkaline phosphatase (AP) method with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate-toluidine salt (NBT/BCIP) as substrate. Briefly, after rinsing 4x5 min with buffer A (0.1 M Tris/HCl, 0.15 M NaCl; pH 7.5) sections were preincubated in buffer A containing 0.5% blocking agent (Roche) for 1 h, followed by 3 h incubation with sheep anti-DIG-AP (Roche; 1:5,000) in buffer A containing 0.5% blocking agent. Subsequently, sections were rinsed four times for 5 min in buffer A, followed by two times of 5 min rinsing in buffer B (0.1 M Tris/HCl, 0.15 M NaCl, 0.05 M $MgCl_2$; pH 9.5). After 16 h incubation in NBT/BCIP medium (10 ml buffer B, 2.4 mg levamisole, 175 µl NBT/BCIP mixture; Roche) in a light-tight box, the reaction was stopped by placing the sections in buffer C (0.1 M Tris/HCl, 0.01 M EDTA; pH 8.0).

Cytometry

For determinations of both Ucn1 and Ucn1 mRNA, 9 animals per experimental group were studied. Simple neuron counting was carried out by direct microscopic examination, using a Leica DMRBE microscope (Leica Microsystem, Heerbrugg, Switzerland). Per animal, counts of DAB-stained neurons (immunocytochemistry of Ucn1) and of hybridization-positive neurons, (Ucn1 mRNA) were made in 5 sections per animal, 150 µm interspaced, at the midlevel of the EW (Bregma: -3.2 to -3.6 mm; Paxinos and Franklin, 2001). Counts were made by an observer who was neuroanatomically experienced but unaware of the scientific aim of the study and the key of the encoded slides. Per animal, counts of the 5 sections were averaged, and the means of the 9 animals per group were used to calculate the group mean and the standard deviation (SD).

Of the Ucn1 mRNA-positive cells, the optical density (O.D.) of silver grains (expressed as a.u.) was determined, using digital images taken at a resolution of 1,200 x 1,600 dpi with the Leica DMRBE optical system and a Leica DC 500 digital camera (Leica Microsystem)

connected to an IBM computer running Scion Image software (version 3.0b; NIH, Bethesda, MR, USA). The outcomes were corrected for background O.D.

Statistical analysis

Plasma corticosterone data, and body weight gain were analysed with a repeated measures analysis of variance (ANOVA) (α=5%) with 'treatment duration' as 'within subjects' factor, and 'group' (CORT, sham) as 'between subjects' factor. Values of adrenal and thymus weights, numbers of Ucn1- and Ucn1 mRNA-positive cells and Ucn1 mRNA O.D. values were analyzed by one-way ANOVA (α=5%). All tests were performed after testing for normality (Shapiro and Wilk, 1965) and for homogeneity of variance (Barlett's chi-square test; see Snedecor and Cochran, 1989), and using the Statistica package (Statsoft, Tulsa OK, USA).

Results

Plasma corticosterone

To assess the efficacy of corticosterone pellet implantation to raise plasma corticosterone, blood samples were taken at 5 time points, starting the day before the surgery (day 0). As a result of the corticosterone pellet implantation the corticosterone level appears substantially higher in the CORT animals than in the shams, throughout the experimental period ($F_{1,16}$ = 300.34, $P<0.0001$; Fig. 1). Whereas at day 0 shams (11.0±7.7 ng/ml) and CORT mice (13.0±11.6 ng/ml) do not differ ($F_{1,16}$= 0.55, $P>0.05$), three days after pellet implantation the corticosterone level in CORT mice shows a peak being 21.8 times higher than in shams (283.8±47.4 ng/ml *vs.* 12.9±7.8 ng/ml; $F_{1,16}$ = 476.63, $P< 0.0001$). From day 3 onwards the corticosterone level in CORT mice slightly and steadily decreases but remains, by the end of the 14 days, 4 times higher than in the sham group (*e.g.* day 12: 76.0±26.9 ng/ml *vs.* 10.9±8.1 in shams; $F_{1,16}$= 80.84, $P< 0.0001$; day 14: 41.5±25.5 ng/ml *vs.* 13.5±10.72 ng/ml in shams $F_{1,16}$= 6.09, $P< 0.05$; Fig. 1).

Body, adrenal gland and thymus weights

To determine physiological effects of the chronically elevated plasma corticosterone level, we measured body weight during the 2 week experimental period, and assessed thymus and adrenal gland wet weights at the day of sacrificing the animals. Both shams and CORT animals slightly gain in body weight during the experimental period, but no effect of corticosterone implantation is detectable ($F_{1,16}$=0.91, $P>0.05$; Fig. 2A). In the CORT mice, adrenal gland weight, is ca. 1.5 times lower (1.8±0.3 mg) than in shams (2.8±0.6 mg; $F_{1,16}$ = 27.60, $P<0.0001$; Fig. 2B) and thymus weight is even 3 times lower (11.7±4.3 mg) than in shams (36.7±6.3 mg; $F_{1,16}$= 169.02, $P<0.0001$; Fig. 2C). Similar results are found when

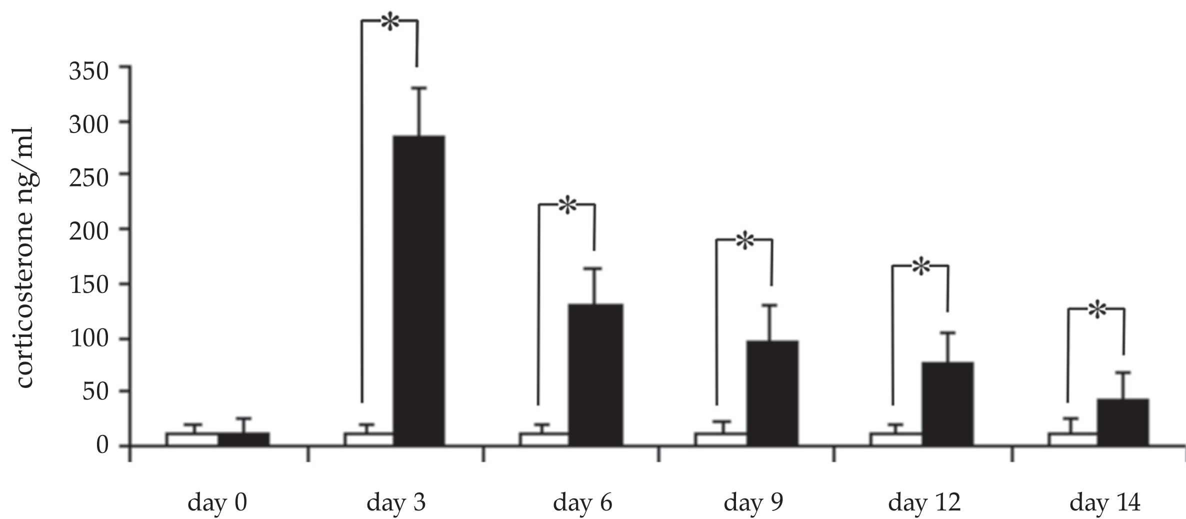

Figure 1. Plasma corticosterone concentration 30 min (white bars) and 120 min (black columns) after ether stress exposure in the various experimental groups. Bars represent the means ± S.E.M (n=4 mice/group). Letters on top of column indicate the experimental group with which significant difference (P<0.001) exists in Student's t test.

adrenal gland and thymus weights are related to body weights (ng/mg bw; P<0.0001 for both adrenal gland and thymus weight; data not shown).

GR and Ucn1 in the EW

Many EW neurons show clear *in situ* hybridization of GR mRNA (Fig. 3A), and have cell nuclei immunopositive with the anti-GR serum (Fig. 3C,F). The EW also contains a large number of strongly Ucn1 mRNA-positive neurons (Fig. 3B) as well as many Ucn1-immunoreactive (ir) neurons, revealing dendritic arborizations (Fig. 3D,G). In double immunofluorescence-labeled sections, all neurons in the EW that are immunoreactive for Ucn1 also reveal clear GR-ir in their nuclei (Fig. 3E,H). In case of both GR and Ucn1 mRNA detection, no appreciable background staining was noticeable, and also with the sense probes (control) no hybridization signal were seen.

Cytometry

To determine the effect of chronically elevated levels of the basal plasma corticosterone on the EW, we quantified the number of EW-Ucn1-ir neurons. At the end of the experimental period, the mean number of Ucn1-ir cells per section in CORT animals (27.7±2.3) is 23.9% lower than in shams (36.4±4.6; $F_{1,16}$= 20.43, P<0.001; Figs. 4A,B; 5A). Furthermore, we quantified the expression of Ucn1 mRNA by cell counting and measuring the O.D. of silver grains. Compared to shams (16.5±2.6 neurons/section), CORT mice revealed 24.8 % less Ucn1 mRNA-positive neurons (12.4±2.2 neurons/section; $F_{1,16}$ =11.56, P<0.01; Figs. 4C,D; 5B), but the overall O.D. of the Ucn1 mRNA signal, *i.e.* the summated O.D. of all

hybridization-positive cells in a section (expressed in arbitrary units, a.u.), did not differ between the two groups (shams: 17.6±4.5 a.u.; CORT: 16.5±3.4 a.u.; $F_{1,16}$=0.30; P>0.05; Figs. 4C,D; 5C). To approximate the amount of Ucn1 mRNA at the single neuron level (O.D./neuron), we divided the overall O.D. per section by the number of Ucn1 mRNA-expressing neurons in that section. It then appears that the OD/neuron in CORT mice (1.8±0.5 a.u.) is clearly higher (38.5 %) than in shams (1.3±0.2 a.u.; $F_{1,16}$=4.41, P<0.05; Fig. 5D).

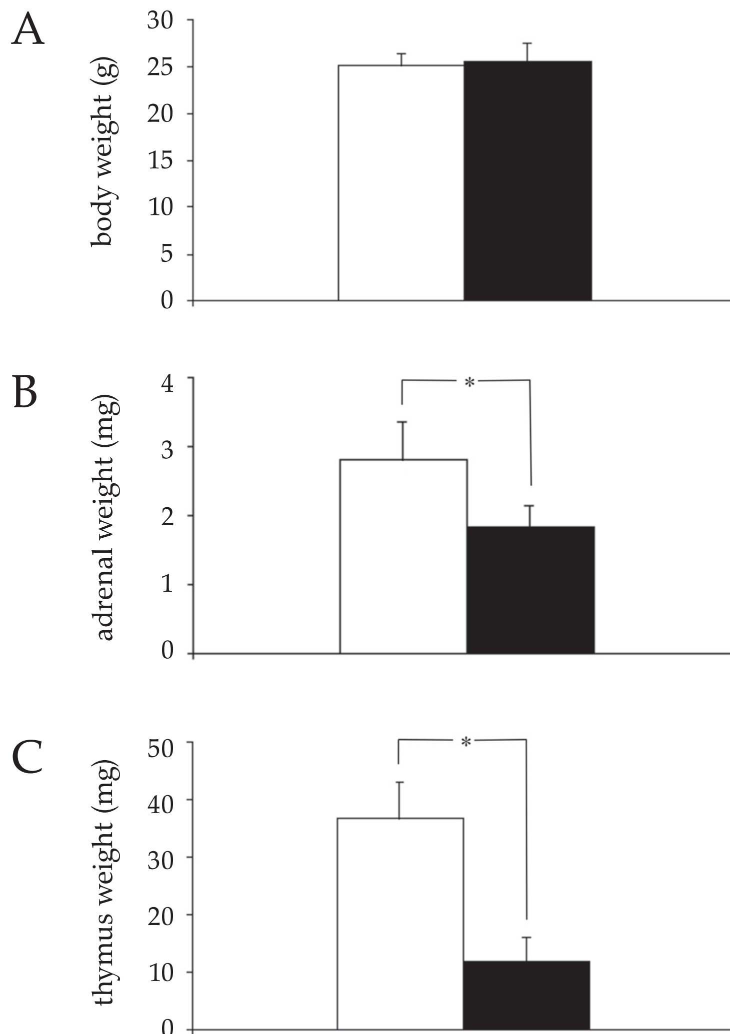

Figure 2. Effect of 14 days of corticosterone treatment on body weight (A) wet weights of adrenal gland (B) and thymus (C) of CORT mice (black bars; n=9) and of shams (white bars; n=9). No significant effect appears as to body weight, but adrenal and thymus weights are clearly reduced in CORT mice. Data are expressed as mean + SD; * P<0.0001.

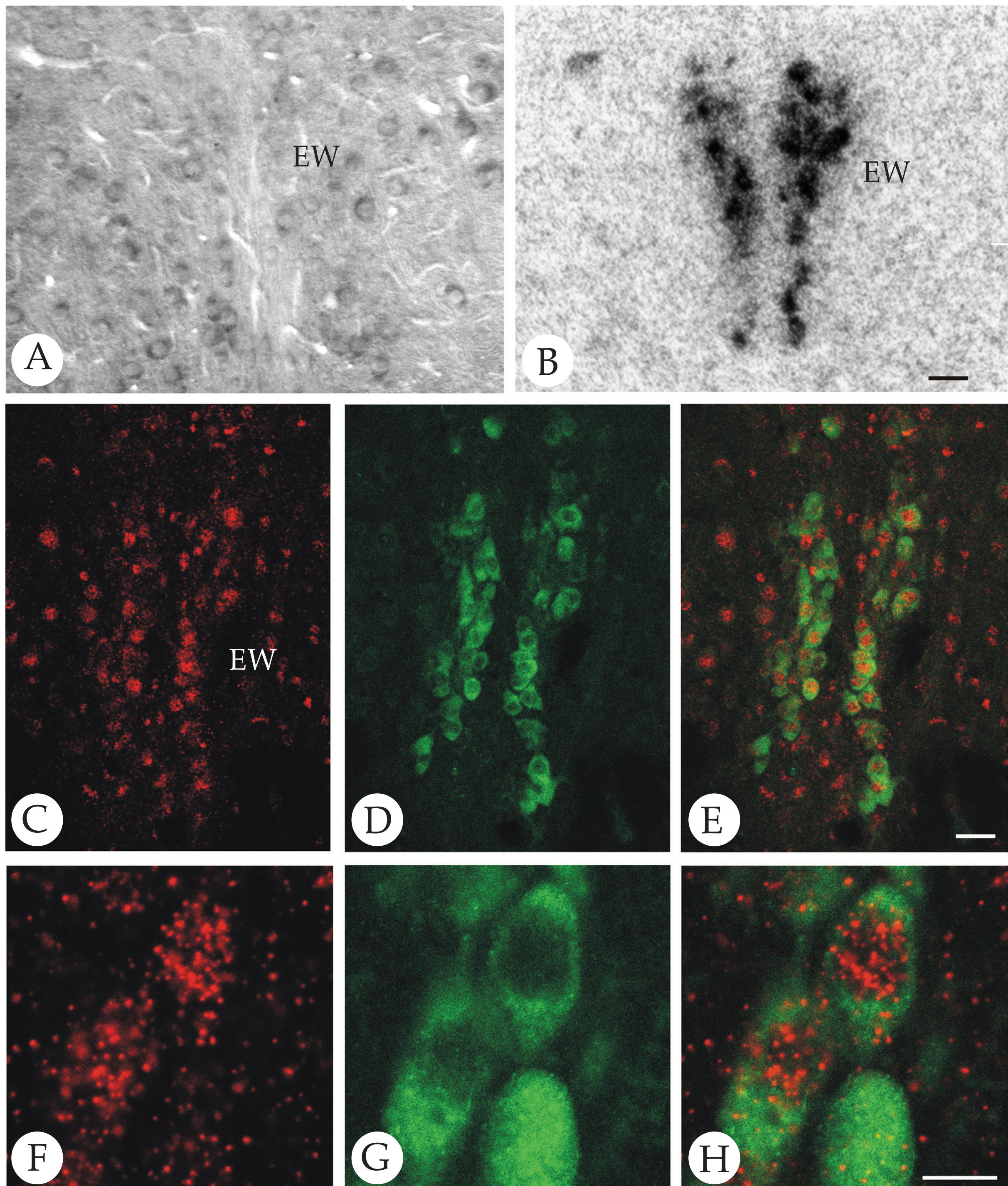

Figure 3. Neurons in the Edinger-Westphal nucleus, reveal by *in situ* hybridization (A) glucocorticoid receptor (GR) mRNA (non-radioactive labeling) and (B) urocortin1 (Ucn1) mRNA (radioactive labeling), and by immunofluorescence labeling, GR (C,F) and Ucn1 (D,G). Double-immunofluorescence labeling shows coexistence of these GR-and Ucn1-immunoreactivities in E and H. F-H are details of C-E. Scale bars in A-E: 50 μm, in F-H: 20 μm.

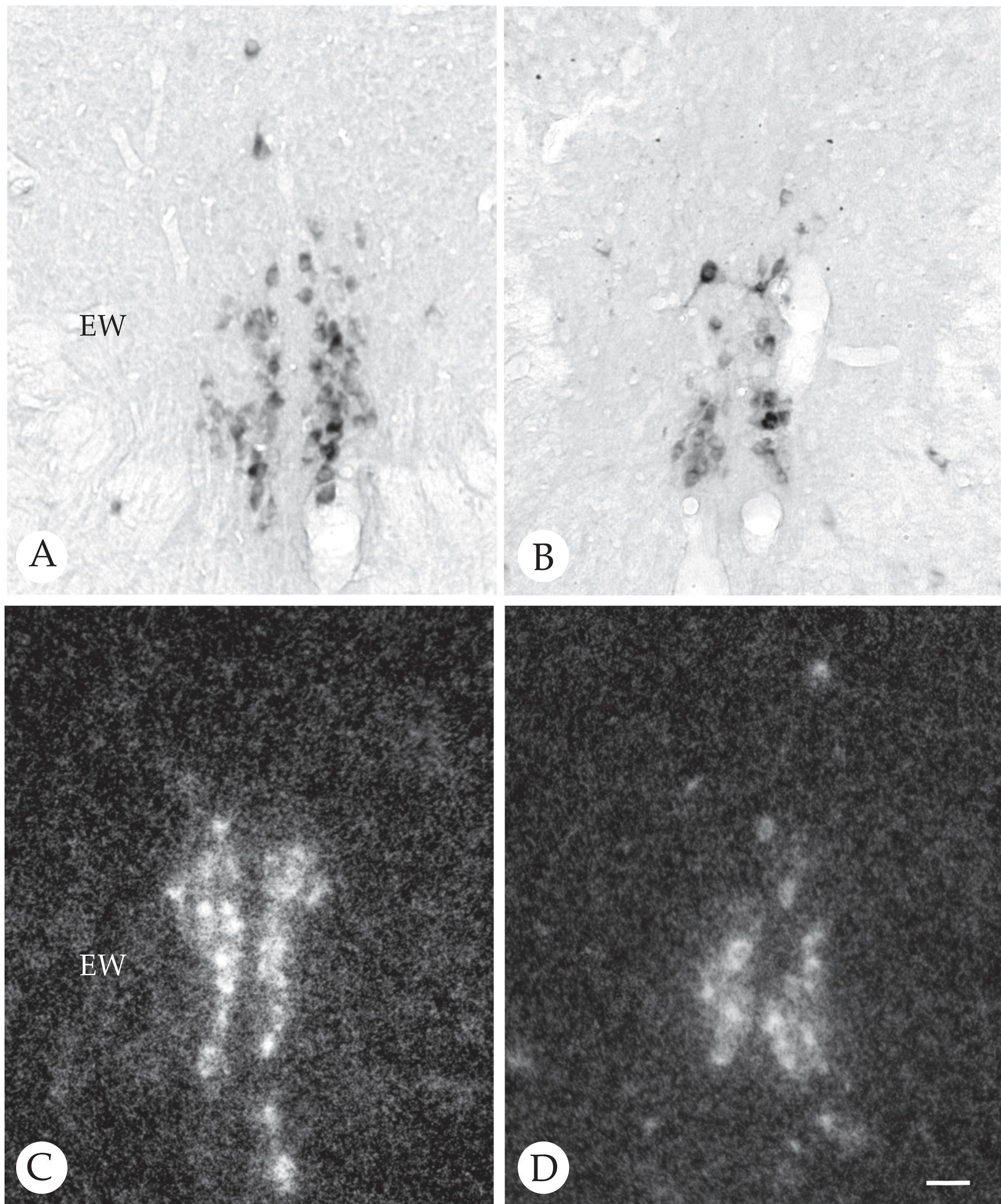

Figure 4. Effect of corticosterone treatment on Ucn1-immunoreactive neurons (A,B) and Ucn1 mRNA-positive neurons (C,D) in the EW. Compared to shams (A,C), clearly lower number of neurons are seen in CORT mice (B,D). Scale bar = 100 μm.

Discussion

General considerations

Corticosterone was administered via subcutaneously implanted pellets in adrenal-intact mice. This type of continuous administration avoids daily injections that might act as a chronic stressor and, moreover, would evoke only a transient increase in the plasma corticosterone level. We used adrenal-intact mice because adrenalectomy would affect plasma levels of adrenaline and of adrenal steroids other than corticosteroids. After chronic treatment with corticosterone we have found a strongly elevated plasma corticosterone level in CORT mice compared to the shams, throughout the experimental period of 14 days. This supraphysiological level was accompanied by a decrease in the relative and absolute weights of the adrenal gland and of the thymus, whereas no change in body weight gain was found. These data are consistent with previous observations of corticosterone-treated mice (Makino et al., 1994; van den Buuse et al., 2004; Prickaerts et al., 2005) and are clear evidence that corticosterone implantation specifically inhibited HPA-axis activity.

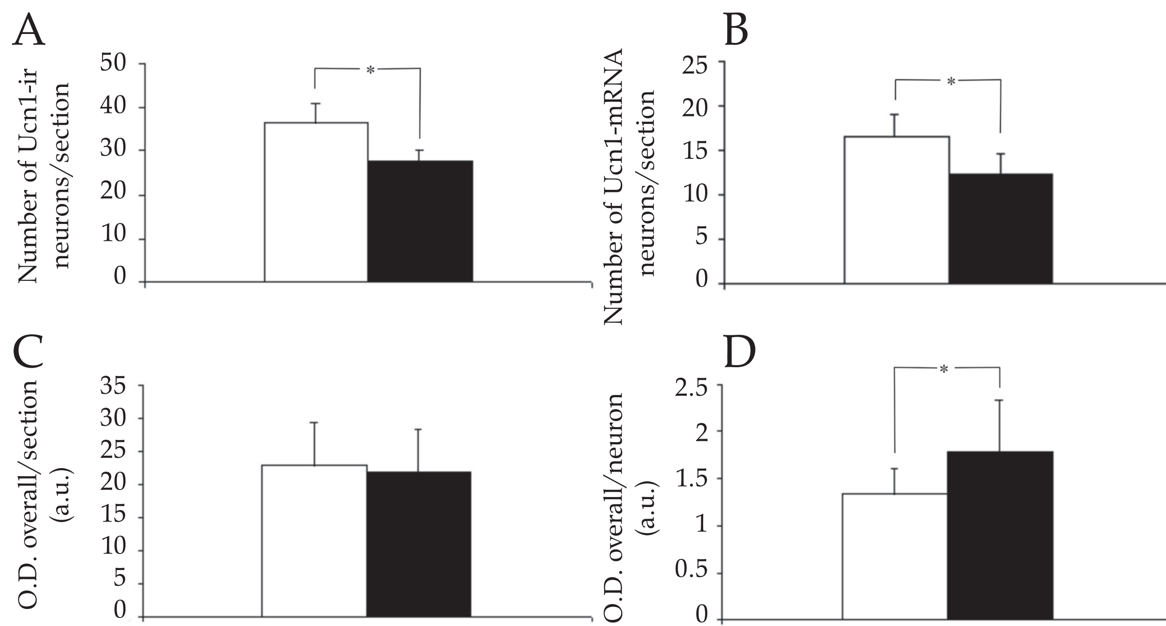

Figure 5. Quantitation of the effects of corticosterone treatment on Ucn1-neurons in the EW. Compared to shams (white bars; n=9), CORT-mice (black bars; n=9) have less Ucn1-immunoreactive (A) and less Ucn1 mRNA-positive (B) neurons. On the other hand, the optical density (O.D.) of their Ucn1 mRNA signal does not differ for the EW overall (C), but is higher per single neuron (D). Bars represent means + SD; * P<0.01.

Ucn1 neurons contain GR

In this study we have shown that the mouse EW contains a large number of Ucn1- and Ucn1 mRNA-containing neurons, which confirms previous findings (Kozicz et al., 2004; Korosi et al., 2005; Weitemier et al., 2005) that the EW is a rich source of Ucn1 in the mouse brain. Furthermore, we showed the presence of GRs for the first time in the mouse EW using *in situ* hybridization with a GR mRNA probe and GR-immunocytochemistry. More specifically, the double immunocytochemical study reveals that EW neurons that contain Ucn1 are also immunoreactive for GR, which is strong evidence for the presence of GR in Ucn1-producing neurons. These results strongly support our hypothesis that glucocorticoids can act directly on Ucn1-neurons in the mouse EW, and support our assumption that the presence of GR in EW-Ucn1 neurons is an important component of the link between the EW and the HPA-axis.

Ucn1 expression in the EW is modulated by chronically elevated corticosterone

The amount of peptide present in a neuron does not give information as to the secretory activity of that neuron if no information is available on the balance between biosynthesis, axonal transport, intracellular breakdown and exocytosis of the peptide. However, when the degree of immunoreactivity changes as a result of an experimental challenge, it may be concluded that the activity of the neuron changes as well, either by an increase or by a decrease. The same argument holds for the number of immunoreactive neurons present in a nucleus. Therefore, in our case, the fact that corticosterone treatment leads to a lower number of Ucn1-ir neurons in CORT mice than in shams, indicates that the secretory activity of these neurons has changed, and that corticosterone regulates this activity. This notion fits, obviously, with our demonstration of GRs in these neurons. At first glance, it seems surprising that CORT animals do not reveal a change in the overall O.D. of the Ucn1 mRNA signal, as this observation would seem to suggest that corticosterone does not affect Ucn1 mRNA production at all. A similar finding was made for the rat EW, where Ucn1 mRNA expression is not affected by 5-days implantation of a corticosterone pellet (Weninger et al., 2000). Theoretically, the steroid might have acted on other aspects of secretory activity, such as intracellular breakdown and/or exocytosis. However, closer examination of the EW, at the single cell level, shows that there is a corticosterone effect on Ucn1 mRNA indeed, in a dual way: whereas less neurons (24.8 %) show a hybridization signal with the Ucn1 mRNA probe, the remaining hybridizing cells show a higher O.D. than the shams, indicating an increase in Ucn1 mRNA production and, hence, in Ucn1 biosynthesis. These hybridization data at the single cell level clearly support our hypothesis that adrenal corticosterone feeds back via GR on Ucn1-containing neurons in the EW, to change their secretory activity. The differential reaction of individual neurons of the EW to the corticosterone treatment suggests that they are differentially recruited, some becoming hyperactive, others becoming inactivated or even disappearing. This type of

differential neuronal recruitment has been shown previously in the neuroendocrine CRF-expressing neurons in the PVN during the stress response (Viau and Sawchenko, 2002), and is a well-known phenomenon in secretory systems in general, such as in the pars intermedia of the amphibian pituitary gland (de Rijk et al., 1990; Gonzalez de Aguilar et al., 1999; Corstens et al., 2005). To obtain more insight into the mechanism of this differential recruitment, it is of interest to correlate the degree of Ucn1 mRNA expression with that of GR expression.

Possible way of action of corticosterone on the EW

Glucocorticoids, and more in particular corticosterone, also regulate other members of the CRF family of peptides, such as CRF and Ucn2 (Makino et al., 1995b; Pinnock and Herbert 2001; Chen et al., 2003). The presence of GR in CRF-expressing neurons suggests a direct effect of glucocorticoids on these neurons (Cintra et al., 1987; Ceccatelli et al., 1989a). It has been postulated that glucocorticoids regulate CRF transcription via a cAMP-responsive element (CRE) present in the promoter of the CRF-gene (Swanson and Simmons, 1989; Pinnock and Herbert 2001). Since CRE is also present in the promoter of the mouse Ucn1 gene (Zhao et al., 1998) it might be a target for glucocorticoids to regulate Ucn1-gene expression. On the other hand, the expression of Ucn2 in the mouse hypothalamus and brainstem (Reyes et al., 2001) appears to be controlled by glucocorticoids via a glucocorticoid-responsive element (GRE) in the Ucn2-gene promoter (Chen et al., 2003). The presence of different mechanisms through which glucocorticoids may regulate the expressions of different CRF-related peptides might explain how these peptides play their distinct roles in the neuroendocrine, autonomic and behavioural responses to various experimental challenges. In this respect, the mechanism by which corticosterone controls Ucn1-expression in the mouse EW during stress adaptation, deserves future attention.

Possible role of BDNF in EW neuronal recruitment

The reduced number of neurons expressing Ucn1 and Ucn1 mRNA in CORT mice could be due to a immunoreactivity and hybridization level that is below the sensitivity of our immunocytochmeical and hybridization method, but it might also be due to a complete loss of Ucn1-containing neurons, as was shown for the neurodegenerative action of corticosterone on the rat hippocampus (Magarinos et al., 1996; Gould et al., 1997). A crucial factor in down-regulation of neuronal secretory activity is brain-derived neurotrophic factor (BDNF), which controls gene expression (Leibrock et al., 1989; Smith et al., 1995b). A decreased level of BDNF may result in atrophy or even loss of neurotransmitter phenotype so that neurochemical messengers in the cell become immunohistochemically undetectable (Sofroniew et al., 1993). BDNF coexists with Ucn1 in the rat PVN (Smith et al., 1995a) whereas our preliminary studies indicate that BDNF is present in Ucn1-containing neurons in the mouse and rat EW (T. Kozicz et al., unpubl. res.). These data suggest a role

for BDNF in regulating the expression of these two stress-related peptides. Interestingly, in the PVN, BDNF expression is negatively controlled by corticosterone (Smith et al., 1995a; Prickaerts et al., 2005). Therefore, we propose that in the EW, corticosterone does not (only) act directly on Ucn1 production, but (also) on BDNF production, and in this way controls the secretory activity of Ucn1-neurons during their participation in stress adaptation. This role of BDNF might hold for the Ucn1 neurons in the whole EW or, in the light of their possible differential recruitment, might be restricted to a subpopulation of neurons that become inactive as a result of corticosterone action.

Conclusions

With this study we provide evidence for the existence of a corticosteroid-mediated mechanism for the direct regulation of the activity of Ucn1-producing neurons in the mouse EW by corticosterone, which may act as a regulatory link between the HPA-axis and the EW during adaptation to, especially, chronic stressors that threaten the survival of the animal. It will be of interest to test if this mechanism also acts in other animal species, as Ucn1-production by the EW has been shown to occur in many other vertebrates, including amphibians (Calle et al., 2005), other rodents (Kozicz et al., 1998), and human (Ryabinin et al., 2005), and GR receptors have also been found in the rat EW (Morimoto et al., 1996).

Acknowledgements

We thank Dr. R.E. de Kloet (Department of Medical Pharmacology, University of Leiden, The Netherlands) for commenting on the manuscript.

Chapter 8

General Discussion

Introduction

The various components of the HPA-axis are key regulators mediating physiological and behavioral stress adaptation (for review see *e.g.* Chrousos and Gold, 1992). Failure of this system leads to failed adaptation ('maladaptation') and most likely underlies stress-related cardiovascular diseases and physiological and mental disorders such as anxiety and depression. Based on clinical and fundamental research data, alterations in the dynamics of the HPA-axis may often be causal to stress-related psychopathologies (for review see Arborelius et al., 1999). The importance of the HPA axis for normal survival, with the CRF family of ligands and their receptors as central regulators, has been shown throughout the animal kingdom, from lower vertebrates to primates and humans (*e.g.* Chrousos and Gold, 1992; Kozicz et al., 1998; Skelton et al., 2000b; Dautzenberg and Hauger, 2002; Calle et al., 2005; Ryabinin et al., 2005). Therefore, studying this axis and the CRF-related peptides CRF and Ucn1 in particular, may be expected to substantially increase our insight into the mechanisms of adaptation and into the causes of maladaptation and disease.

In this context, this thesis research has been carried out. It has focused on the roles of CRF, Ucn1 and their receptors in stress adaptation, and concerns the HPA axis as well as a second system that is assumed to be involved in stress adaptation, the Edinger-Westphal nucleus (EW). In this final chapter of the thesis, main conclusions and implications from this research will be discussed, and some perspectives for future research will be made.

Research strategy

Our research to increase insight into the functioning of CRF-related peptides in stress adaptation systems, has been carried out in two steps: 1. Localizing the peptides and their receptors, and 2. studying the expression of these peptides and their receptors under conditions of experimentally induced short-term and long-term regulatory changes in the stress adaptation systems. The experimental paradigms were chosen in such a way that they mimicked as much as possible natural situations of adaptation to natural stressors: a) over-expression of CRF in CRF-OE mice, to mimic a condition of chronic stress, b) administration of short-term and long-term stressors (*e.g.* ether stress), to mimic the action of natural short-term and long-term adaptation, respectively, and c) administration of corticosterone by implanted corticosterone-containing pellets, to mimic adrenal feedback to the adaptation systems as occurs during long-term adaptation.

Studies were carried out with a variety of techniques, gene mutation, immunocytochemistry and *in situ* hybridization being mostly used. Obviously, not all combinations of stressor, peptide, receptor and brain regulatory center could be tested. Choices were made on the basis of scientific priority and experimental feasibility. In the following paragraphs, the significance of the main methodological approaches and scientific results will be discussed, and some possible follow ups of the research will be proposed.

The CRF-OE mouse model

To assess the role of central CRF in stress adaptation, we decided to use an animal model in which CRF expression had been genetically manipulated. Such models have been produced to study the putative mechanisms underlying mental disorders. There is a strong link between hypersecretion of CRF and major depressive disorder (Nemeroff, 1996). In depressed patients elevated levels of CRF in the cerebrospinal fluid have been reported (Nemeroff et al., 1984) as well as increased numbers of CRF-expressing neurons in the paraventricular nucleus of the hypothalamus (PVN; Raadsheer et al., 1994).

One type of transgenic model is the CRF-knockout mice. This mutant has been used to assess the importance of CRF in adaptation and maladaptation processes as occur during stress and stress-related pathologies (Muglia et al., 1995). Although such mutants have shown that CRF is an important regulatory peptide in the functioning of the HPA-axis, they are not very suitable to study the functioning of the HPA-axis itself, nor the relation between this axis and other stress regulatory systems like the EW, because the HPA lacks its main regulator peptide, CRF in the hypothalamic PVN. For this reason, we have preferred to use a transgene animal model that still would posseses a functional HPA-axis and also presents the possibility to study the role of CRF in other stress-adaptation components in the brain. Two CRF-overexpressing transgene mouse models conform to these requirements, namely the so-called Stenzel-Poore mouse (Stenzel-Poore et al., 1992) and the CRF-OE mouse, developed by Olivier and collaborators (Dirks, 2001), both intended to mimic long-term central hypersecretion of CRF as is present in humans suffering from anxiety and depression. These two models differ, however, in the experimental way CRF is over-expressed, which has consequences for the suitability of the two models for our research.

To produce their CRF-overexpressing mouse, Stenzel-Poore et al. (1992) used the murine metallothionein promotor. This approach results in CRF gene over-expression in restricted areas of the brain, providing the possibility to relate the activation of these areas to specific changes in other components of the stress adaptation system. However, in addition to over-expression in the brain, CRF is also over-expressed in peripheral organs of this mouse, such as in heart, testis and lungs. This peripheral over-expression hampers the unambiguous interpretation of results obtained with this mutants in terms of a regulatory role of the brain and more in particular the hypothalamus. Moreover, in a Stenzel-Poore mouse, the HPA-axis is rather insensitive to stressors (Coste et al., 2001). The CRF-OE mouse mutant developed by Olivier and co-workers reveals CRF over-expression exclusively in the central nervous systems, making it more suitable to investigate the central effect of CRF excess on stress adaptation systems than the mutant developed by Stenzel-Poore et al. (1992). Furthermore, as we have shown, the CRF-OE mouse is still responsive to stressors. Therefore, we have chosen the CRF-OE mouse as a tool for investigating the significance of central CRF excess for stress adaptation. Both advantages of this mutant as a model for our studies will be considered into more detail

below.

Exclusive CRF over-expression in the brain

In the CRF-OE mutant, the Thy-1 promoter induces CRF gene over-expression starting shortly after birth, with a maximum level by postnatal day 14, and this over-expression is maintained throughout adult life (Morris and Grosveld, 1989; Vidal et al., 1990). In **Chapter 2** we show that this over-expression is restricted to the central nervous system. This situation neatly mimics the situation in depressed patients (Raadsheer et al., 1994), but as we show in **Chapter 2**, differs from it in that the CRF-OE mouse expresses CRF not only in the 'traditional' CRF-producing areas like the PVN, but also in areas that normally (in wild types and in human patients) do not express any CRF. This broad, non-specific CRF mRNA expression in 'novel brain areas' is due to the action of the Thy-1 promotor, which does not specifically induce CRF mRNA in normal-CRF expressing neurons, but more or less randomly in neurons throughout the brain (Luthi et al., 1997; Wiessner et al., 1999), irrespective their CRF production. Therefore, is important to know whether the CRF present in these novel areas is functional, *i.e.* is released to act on a functional receptor. Since we have shown in **Chapter 3** that these novel CRF areas are not associated with 'novel CRF receptor-expressing areas' indicating that this novel CRF is not functional unless it acts on targets already present in WT.

CRF-OE mice still respond to stress

Obviously, to investigate the role of excess brain CRF in the stress adaptation process using CRF-OE mice, it is necessary that the mutants are able to react to stressful stimuli. In contrast to the Stenzel-Poore mouse, in which the HPA axis is desensitized to stressor stimulation (Coste et al., 2001), the CRF-OE mice display normal corticosterone and PVN responses to acute stress (Groenink et al., 2002). Moreover, we show in **Chapter 5** that the other important component in the brain assumed to control stress adaptations, the EW-Ucn1 system, responds with a significant increase in Fos expression to a stressful challenge. Apparently, in the CRF-OE mouse, the central stress adaptation mechanisms are not disrupted and able to respond to stressors in both a neural and neuroendocrine fashion. Therefore, we consider this transgene mouse a valid model for investigating the role of chronically elevated CRF in the control of central stress adaptation.

Direct and indirect effects of CRF over-expression

Compared to WT mice, CRF-OE mice reveal a large number of differences in behavior, physiology and brain functioning (this thesis). Because the primary modification in the CRF-OE mouse is the mutation leading to CRF over-expression, we attribute these differences to an increased CRF release. The differences occur at different levels, viz. at the cellular

level, such as neuropeptide and receptor expression, and at the organismal level revealing autonomic, neuroendocrine and behavioral changes (Dirks et al., 2002b; Groenink et al., 2002). At the cellular level, the extensive and exclusively central over-expression of CRF (**Chapter 2**) is associated with down-regulation of CRF1 and up-regulation of CRF2 mRNAs in specific brain areas (**Chapter 3**) and with a strong down-regulation of Ucn1 peptide and Ucn1 mRNA in the EW (**Chapter 5**). These changes are accompanied by autonomic alterations like increased body temperature and increased heart rate (**Chapter 2**), whereas from a neuroendocrine point of view, the mutant shows increased levels of bioactive CRF in the hypothalamus (**Chapter 2**), hypercortisolism combined with adrenal gland enlargement, as well as dexamethasone nonsuppression (Groenink et al., 2002).

The question arises if CRF causes these changes in a direct way, or via other components of the stress adaptation systems. Moreover, some of the changes may even not be caused by CRF itself, but by the expression or inhibition of the expression of one or more other genes, as part of a compensatory process activated by CRF gene over-expression. Additional experiments are necessary to provide an insight into the exact role and action of CRF in stress adaptation in these mutant mice.

Is the CRF-OE mouse a good model for depression?

This thesis research is not primarily focused on the role of CRF in mental disorders like depression. Nevertheless, in view of the supposed major role of stress in mental disorders like depression, and of our aim to increase the insight in the relation between CRF and stress adaptation, the question of the suitability of this mutant for research of the mechanisms of depression deserves attention.

The autonomic and neuroendocrine changes revealed by the CRF-OE mouse resemble those present in human patients suffering from chronic stress and depression (*e.g.* increased body temperature, heart rate and hypercortisolism; **Chapter 2**; Groenink et al., 2002). Therefore, it may be hypothesized that in these mice CRF overproduction will result in anxiogenic-like behavior. However, this transgenic animal does not reveal an anxious or depressed phenotype (Groenink et al., 2003), showing instead altered sensory information processing like a reduced startle response and impaired prepulse inhibition (Dirks et al., 2002b). Similar abnormal sensory information processing has been observed in schizophrenia patients, which suggests that the CRF-OE mouse model might be useful to investigate a possible role of chronically elevated CRF in the genesis of schizophrenia rather than of depression.

In contrast to the CRF-OE mouse, at later age, the Stenzel-Poore mouse develops a Cushing's syndrome-like phenotype together with an increase in anxiety-related behaviors (Stenzel-Poore et al., 1994) and with impaired learning capacity (Heinrichs et al., 1996). This would seem to indicate that the Stenzel-Poore mouse is a good model for studies of depression. However, as in this mutant CRF-over-expression also occurs in peripheral organs, it cannot be excluded that the behavioral changes are at least partly due to excess

of peripheral CRF. In fact, peripherally overproduced CRF may pass the blood-brain barrier to eventually reach mood regulatory centres in the brain (Kastin and Akerstrom, 2002). Therefore, neither of the two CRF-OE mouse models may be suitable to study CRF-mediated induction of anxiety and depression.

Is the EW-Urocortin 1 neuronal system involved in stress adaptation?

At the start of this research, the assumption that the EW-Ucn1 is involved in the regulation of the stress adaptation response was based on the pharmacological properties, responsiveness to acute stress and behavioral effects of Ucn1. This peptide binds with similar affinities to both CRF1 and CRF2, and when centrally administered, it induces a variety of physiological responses (for review see Skelton et al., 2000b) that resemble those occurring during stress. Furthermore, the Ucn1-containing EW neurons respond to acute stressful stimuli with neuronal activation and increased Ucn1 expression (Weninger et al., 2000; Gaszner et al., 2004). Other type of approaches to support the supposed role of the Ucn1 and the EW in stress adaptation, such as lesion of the EW (Bachtell et al., 2004) and the use of Ucn1-deficient mice (Vetter et al., 2002; Wang et al., 2002) have not been successful. The lesion study mainly focused on the role of Ucn1 in feeding, thermoregulation and in ethanol consumption rather than on its role in stress adaptation, and the investigations studying Ucn1 deficient mice, gave contrasting results. In fact, from the two available Ucn1-knockouts, one displays a normal stress-induced anxiety behaviour and normal autonomic control (Wang et al., 2002), whereas the other transgene model does show increased anxiety-like behavior that is accompanied by an apparently unrelated, atypical hearing impairment (Vetter et al., 2002). The present research, however, provides strong support for the hypothesis that Ucn1 in the EW plays a substantial role in stress adaptation, showing its response in various chronic stress-like conditions. We first demonstrate that EW-Ucn1 neuronal activity is changed under chronic stress and chronic stress-like conditions (**Chapter 2**). In fact, there is down-regulation of Ucn1 expression after 3 weeks of chronic homotypic ether stress when compared with the expression level after acute stress. This expression pattern is accompanied by a non-habituating neuronal activation as apparent from a maintained Fos response during the three weeks of chronic challenge (**Chapter 6**). Furthermore, 2 weeks of corticosterone treatment reduces the number of neurons expressing Ucn1 peptide and mRNA (**Chapter 7**) and, finally, CRF-OE mice present a strong Ucn1 mRNA down-regulation (**Chapter 5**). Conclusive proof for the involvement of the EW in stress regulation might come from demonstrating the targets of Ucn1 release from this nucleus under stress-inducing conditions.

Comparing the EW-Ucn1 and the HPA-axis dynamics

One of the main points of attention in this research is the relationship between Ucn1 in the EW and the HPA-axis. In this paragraph we will compare first the response of both

systems under similar conditions (*e.g.* acute or chronic challenges), and then discuss their involvment in the different phases of the stress response 'initiation and recovery'.

CRF-PVN and Ucn1-EW in response to stress

As to the EW, after acute stress there is an increase in Fos response and in Ucn1 mRNA expression in WT mice (Weninger et al., 2000; Gaszner et al., 2004) and, despite of the chronically high level of CRF, in CRF-OE mice (**Chapter 5**). Furthermore, under chronic stress (-like) conditions (*e.g.* chronic homotypic ether stressor or CRF-OE) EW-Ucn1 expression is down-regulated (**Chapter 5 and 6**). Finally, during chronic corticosterone treatment a reduced number of neurons expressing Ucn1 peptide and mRNA was found (**Chapter 7**).

In contrast, the PVN shows quite different responses to such stress-related challenges. Parvocellular CRF-expressing neurons mount a robust and immediate Fos response after an acute challenge (Stamp and Herbert, 1999) associated with an increase in CRF expression (Makino et al., 1995a; Viau and Sawchenko, 2002). Repeated exposure of the PVN to the same stressor (chronic 'homotypic stressor'), such as chronic restraint or immobilization stress, leads to a complete habituation of the PVN Fos response (Stamp and Herbert, 1999; Viau and Sawchenko, 2002) associated with elevated CRF expression (Makino et al., 1995a; Viau and Sawchenko, 2002). Furthermore, also when comparing the effects of a homotypic chronic stressor *vs.* an acute stressor on the PVN-CRF and EW-Ucn1 systems, clear differences between the two systems appear. While the expressions of both CRF in the PVN (Makino et al., 1995b) and Ucn1 in the EW (Gaszner et al., 2004) increase after acute stress, chronic stress increases CRF mRNA expression in the PVN (Makino et al., 1995b) but decreases EW Ucn1 mRNA expressions compared to acute stress (**Chapter 6**). Furthermore, in response to a chronic homotypic challenge, CRF neurons do, but EW neurons do not habituate (**Chapter 6**). The inverse relationship between CRF and Ucn1 under chronically stressful conditions is also clear from the observation that EW-Ucn1 expression is upregulated in CRF-deficient mice (Weninger et al., 2000) whereas in the CRF-OE mice Ucn1 expression is down-regulated, both at the mRNA and peptide level (C**hapter 5**).

These data indicate that, topographically and phenotypically different stress-sensitive effector neuron populations (PVN and EW) react in a dynamically distinct manner to long-term stressful experiences. Apparently, opposite stress response of PVN-CRF and EW-Ucn1 only appears under chronic stress-like conditions, because under acute conditions the two systems react in a similar way. This suggests that the EW-Ucn1 system and the classical PVN-CRF represent two separate but interrelated entities that regulate in an opposite way adaptation to conditions with chronically elevated levels of CRF, but work in concert in response to acute challenges.

How do CRF and Ucn 1 act in the different phases of the stress response?

Both CRF and Ucn1 act on both CRF1 or CRF2, but whereas Ucn1 binds to CRF1 with a similar affinity as CRF (Vaughan et al., 1995), the affinity of Ucn1 for CRF2 is approximately 40 times higher than that of CRF itself. Recently, a model was proposed in which CRF1 are involved in the acute phase of the stress response (when the HPA-axis becomes transiently strongly activated) while CRF2 act during the so-called 'recovery phase', in which the activity of the HPA axis gradually lowers to a more continuous, moderately elevated level (Coste et al., 2001; Reul and Holsboer, 2002). This idea is corroborated by the fact that CRF2-knockout mice are hypersensitive to stressors and reveal increased anxiety-like behaviour (Bale et al., 2000; Coste et al., 2000). We suggest that Ucn1, acting through the CRF2, is central in the recovery phase of the stress response by facilitating long-term adaptation to stressful stimuli. In this way, Ucn1 might also dampen stress-induced anxiety (anxiolytic effect) thereby counteracting the anxiogenic actions of CRF on the CRF1 (Strohle and Holsboer, 2003; Bale and Vale, 2004; Muller et al., 2004). In this view, not only CRF but also Ucn1 is an important player in controlling the actions of the PVN- and EW stress-responsive circuits.

How do the EW-Ucn1 system and the HPA-axis communicate?

If the PVN- and EW- systems are involved in stress adaptation in order to co-ordinately control stress adaptation, they need to have one or more sites of interaction. Theoretically, these sites can be of various kinds, *e.g.* at the level of a) external hormonal inputs like glucocorticoids, and/or b) external neural inputs like serotonin. These two possibilities will be considered into some detail below.

Glucocorticoid receptors

Glucocorticoid feed-back may represent another link between the HPA-axis and the EW. More specifically, the presence of GR in the PVN (Cintra et al., 1987; Ceccatelli et al., 1989a) and in EW-Ucn1 neurons (demonstrated in **Chapter 7**) points to GR as possible sites of communication between the two systems. Corticosterone administration can affect the expressions of both CRF mRNA (Makino et al., 1995b; Pinnock and Herbert, 2001; Chen et al., 2003) and Ucn1 mRNA (**Chapter 7**) as well as of another member of the CRF family of peptides, Ucn2 (Chen et al., 2003). This type of interaction between the EW and the PVN might represent the mechanism by which Ucn1 in the CRF-OE mice is down-regulated. A direct action of glucocorticoids on the EW in this mutant is in line with the observation that these animals possess an elevated level of corticosterone (Groenink et al., 2002).

It has been proposed that glucocorticoids regulate CRF transcription via a cAMP-responsive element (CRE) present in the promoter of the CRF-gene (Swanson and

Simmons, 1989; Pinnock and Herbert, 2001). Since CRE is also present in the promoter of the mouse Ucn1 gene (Zhao et al., 1998) it might be a target for glucocorticoids to regulate Ucn1-gene expression. On the other hand, the expression of Ucn2 in the mouse hypothalamus and brainstem (Reyes et al., 2001) appears to be controlled by glucocorticoids via a glucocorticoid-responsive element (GRE) in the Ucn2-gene promoter (Chen et al., 2003). The presence of different mechanisms through which glucocorticoids may regulate the expressions of different CRF-related peptides might explain how these peptides play their specific roles in the neuroendocrine, autonomic and behavioural responses to various experimental challenges. Interestingly, when the common ancestral gene of CRF-related peptides is traced back (Lovejoy and Balment, 1999) it is clear that CRF/Ucn1 and Ucn2/Ucn3 are two separate evolutionary branches with unique functional characteristics (Zorrilla and Koob, 2005). This suggests that Ucn1 gene is regulated rather via the CRE then via GRE, although GRE is present in the Ucn1 gene promoter (A. Chen, personal communication).

Serotonergic input

A third possible site of communication between the PVN-CRF system and the EW-Ucn1 system could be via a regulatory (common) neural input. The best candidate for such a regulator would seem to be serotonin (5-HT). 5-HT is well-known to regulate HPA-axis activity during stressful challenges. Disturbed functioning of serotonergic neurotransmission seems to play a key role in the aetiology of depression (for reviews see *e.g.* Graeff et al., 1996; 1997; Carrasco and Van de Kar, 2003). A neuroanatomical basis for this relationship is formed by the presence of 5-HT in the dorsal raphe (DR) (Dahlstrom and Fuxe, 1964; Abrams et al., 2004) where CRF cell bodies and fibres (Swanson et al., 1983) as well as a dense distribution of Ucn1-fibres have been observed (Bittencourt et al., 1999). Moreover, we have shown that the DR contains a high level of CRF2 expression (**Chapter 3**; see also Van Pett et al., 2000). Thus, in turn, the CRF and Ucn1 systems are in a unique topographic position to influence serotonergic neurotransmission at the level of the raphe nuclei, providing the possibility of a regulatory, looped control system.

In this respect it is also noteworthy that the only difference in CRF2 expression observed in CRF-OE mice when compared to WT is a remarkably higher number of mRNA-positive cell body profiles in the DR nucleus (**Chapter 3**). Microinjection of CRF into the DR alters raphe neuronal activity *in vivo* (Kirby et al., 2000) and *in vitro* (Lowry et al., 2000; Kirby et al., 2000; Price et al., 2002), and the tone of the raphe-serotonin system is regulated in a dynamic manner through CRF2 activation (Pernar et al., 2004). Therefore, our result of an increased expression of CRF2 mRNA in the DR of CRF-OE mice (**Chapter 3**) may contribute to the identification of the neuronal networks involved in stress-related diseases such as anxiety and major depression. To test this possibility it needs to be investigated whether Ucn1 is actually released in the DR and whether there is direct interaction between Ucn1 terminals and 5-HT in this nucleus.

Is there a sympathetic role for Ucn1 in stress adaptation?

Till now this General Discussion has been focusing on the central role of Ucn1, and more in particular on the relation of Ucn1 in the EW with the HPA-axis. However, our finding of CRF1 and CRF2 receptor mRNA expressions in the spinal cord (**Chapter 4**) points to still another target for Ucn1, viz. the spinal cord and, hence, peripheral organs. To get some clues as to which targets would be under the control of Ucn1 released inside the spinal cord, it is helpful to take a close look at the projecting areas of the EW-Ucn1 neurons. Urocortinergic neurons in the brain, beside sending main projections from the EW to the lateral septum and to the dorsal raphe, have major projections to the spinal cord (Bittencourt et al., 1999), which indicates that Ucn1 (also) plays a role in the stress response through the sympathetic system. In support of this idea, in **Chapter 4** we demonstrate the presence of the CRF receptor mRNA in the mouse spinal cord, revealing a distribution largely similar to that of Ucn1. The strongest coexistence appears in lamina VII and in the intermediolateral column, structures both containing spinal preganglionic autonomic neurons (Grant and Koerber, 2004). In this area of the spinal cord there is a large abundance of both receptor mRNAs and Ucn1-immunoreactive terminals (Bittencourt et al., 1999). Therefore we propose that Ucn1 actions via the spinal cord is involved in the control of sympathetic stress responses (**Chapter 4**).

For the future

Apart from rendering new data that increase our insight in the roles of the HPA-axis and the EW and especially of the CRF-related peptides in stress adaptation, the studies carried out in this thesis research have generated new questions, some of which will discussed briefly below.

First, to increase our insight into the role and action of CRF in stress adaptation in mutant mice, experiments with adrenalectomy followed by corticosterone replacement in the CRF-OE mice could reveal if the adrenals play a crucial role in the regulation of the EW and therefore in the communication between the HPA-axis and the EW.

In addition to the valuable data that the CRF OE mouse has provided, new genetic models may help pinpointing the role of CRF and Ucn1 in the control of adaptation. These models should lack the disadvantages of the CRF-OE mouse, such as unpredictable compensatory and adaptive alterations at various levels, which make the CRF-OE less suitable to study complex behaviors such as anxiety and major depression. To date novel approaches (*e.g.* conditional knock-out and RNA silencing) seem to be more promising, as they provide the possibility to control the expression of a compound in a site-specific as well as in a time-limited fashion.

To further explore the possible regulatory link(s) via corticosterone between the HPA axis and EW the responsiveness of EW-Ucn1 neurons to various corticosterone levels and with different exposure times, needs to be tested, in combination with specific

GR antagonists. Furthermore, the exact signal transduction mechanism(s) via which glucocorticoids control EW-Ucn1 expression need(s) to be addressed in detail, also with respect to the interesting possibility that in addition to these steroids other plasticity-inducing factors like brain-derived neurotrophic factor may regulate Ucn1 EW expression (Smith et al., 1995a; Prickaerts et al., 2005)

Finally, the changed activity of EW-Ucn1 during changes in HPA-axis activity, could contribute to the occurrence of anxiety and depression. To test this possibility it would be important to measure EW-Ucn1 mRNA levels in human brains (*e.g.* by means of RT- PCR) and to see whether similarly to CRF, it is possible to detect Ucn1 in the cerebrospinal fluid.

Given our knowledge about the CRF family of peptides (CRF, Ucn1, Ucn2 and Ucn3) and their receptors (CRF1 and CRF2), it will be a challenge to obtain a complete understanding of the distinct roles of CRF-related peptides in the stress adaptation process. Such an understanding would be crucial to develop novel drugs for the prevention and treatment of stress-related pathologies.

Final considerations

This thesis research has increased our knowledge about the roles of CRF-related peptides and their receptors in stress adaptation, especially via the HPA-axis and the EW-Ucn1 systems. The clear effects of CRF over-expression on the EW nucleus, and the intriguing relationship between EW and the HPA-axis, found in these studies, warrant increased future attention to the role of EW nucleus in adaptation and in maladaptation processes, two sides of the same coin of physiology of the healthy and diseased subject.

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List of frequently used abbreviations

ACTH	adrenocorticotrope hormone
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CNS	central nervous system
CRF	corticotropin-releasing factor
CRF1	corticotropin-releasing factor receptor type 1
CRF2	corticotropin-releasing factor receptor type 2
CRF-OE	CRF over-expression
DR	dorsal raphe nucleus
EW	Edinger-Westphal nucleus
GR	glucocorticoid receptor
HPA-axis	hypthalamus-pituitary-adrenal-axis
LS	lateral septum
O.D.	optical density
PVN	paraventricular nucleus of the hypothalamus
Ucn1	urocortin 1
Ucn2	urocortin 2
Ucn3	urocortin 3
WT	wild type

Summary

The body's ability to adapt to external and internal factors that challenge homeostasis is essential for survival, and depends on stress adaptation systems. Failure of these systems may lead to the development of stress-related physiological and mental disorders. The traditionally best-known adaptation system in vertebrates including human, is the hypothalamo-hypophyseal adrenal (HPA-) axis, in which hypothalamic corticotropin-releasing factor (CRF) plays a central role. The discovery of the CRF-related peptide, urocortin 1 (Ucn1) in the Edinger-Westphal nucleus (EW), acting like CRF through CRF receptors, raises the question how CRF-related peptides, the HPA-axis and the EW collaborate in stress adaptation. This question underlies this thesis research.

We have focused on the presence and dynamics of CRF, Ucn1 and their receptors CRF1 and CRF2, using various experimental approaches including a mouse model of central CRF over-production (CRF-OE), acute and chronic stress paradigms, and chronic corticosterone treatment. Determinations were both at the transcriptional and translational level, especially by *in situ* hybridization and immunocytochemistry, respectively. In **Chapter 2** we show that the mouse lines CRF-OE_{2122} and CRF-OE_{2123} have increased amounts of CRF peptide and mRNA, restricted to the central nervous system. Only in CRF-OE_{2122} mice this CRF over-expression is associated with an increased level of bioactive CRF in the hypothalamus, increased body temperature and heart rate, and increased food and water consumption, as compared to wild-type (WT) mice. Therefore, this transgene mouse line has been selected, as a valid model to study the role of central CRF in stress adaptation. In **Chapter 3** it is shown that CRF differentially controls the two CRF receptors, CRF1 and CRF2, with regard to mRNA expression and expression sites in the brain: CRF over-production leads not only to a reduced number of CRF1 mRNA-expressing neurons in the subthalamic nucleus, globus pallidus, lateral septum, substantia nigra, primary somatosensory cortex and principal sensory nucleus, but also to an increased number of neurons expressing CRF2 mRNA in the dorsal raphe. In **Chapter 4** we support our hypothesis that CRF receptors, besides mediating actions of CRF and CRF-related peptides within the brain, also mediate such actions at the level of the spinal cord. The presence of both CRF receptor mRNAs is demonstrated throughout the spinal cord. These expressions differ from each other in strength and as to the site (laminae of Rexed) in the spinal cord. These results lead us to assume that CRF-related-peptides exert their actions at peripheral components of the stress adaptation system, via distinct spinal cord receptors.

In **Chapters 5, 6 and 7** support has been gathered for the presumed role of the EW in stress adaptation. In **Chapter 5** it is demonstrated that chronic CRF excess strongly down-regulates EW-Ucn1 neurons, both at mRNA and peptide level. Furthermore, these neurons in CRF-OE mice respond, like the HPA-axis, to an acute challenge by increased Fos and Ucn1 mRNA expression. These findings indicate that the HPA-axis and the EW respond in concert to acute challenges but act in opposite ways during chronic stress.

We further investigated the EW during chronic activation of the HPA-axis, and show in **Chapter 6** that, in contrast to the HPA-axis, the EW does not habituate to a chronic homotypic ether challenge and shows down-regulation of Ucn1 mRNA levels *vs.* acutely stressed animals. Finally, in **Chapter 7** studies are described that indicate that chronic stress-induced activation of the adrenals results into inhibition of the EW-Ucn1 system, most likely through a direct action of corticosterone on EW-Ucn1 neurons. This notion is supported by the demonstration that glucocorticoid receptors coexist with Ucn1 in EW-neurons, and that chronic corticosterone treatment reduces the number of mRNA- and peptide-containing EW-Ucn1 neurons.

In **Chapter 8** the results obtained in the thesis are put into a broader perspective, leading to the conclusion that the CRF-OE mouse model is a suitable tool for investigating the role of chronically elevated CRF in the control of stress adaptation. Furthermore, we propose that Ucn1 in the EW plays a substantial role in stress adaptation, and suggest a series of studies to further elucidate the roles of CRF in the HPA-axis and Ucn1 in the EW, in stress adaptation.

Samenvatting in het Nederlands

Het vermogen van het lichaam om zich aan te passen aan steeds veranderende uitwendige en inwendige omstandigheden die homeostasis bedreigen, en dat essentieel is om te overleven, berust op de werking van stress-adaptatiesystemen. Wanneer deze systemen tekort schieten, ontstaan door stress veroorzaakte ziekten van lichaam en geest. Het traditioneel best bekende stress-adaptatiesysteem in gewervelden inclusief de mens, is de hypothalamus-hypofyse-bijnier- (HHB-) as, waarin het hypothalame corticotropin-releasing factor (CRF) een centrale rol speelt. De ontdekking van het aan CRF-verwante peptide, urocortine 1 (Ucn1) in de Edinger-Westphal nucleus (EW), dat net als CRF aangrijpt op CRF-receptoren, heeft de vraag doen rijzen hoe CRF-achtige peptiden in de HBB-as en in de EW samenwerken bij stress-adaptatie. Deze vraag ligt ten grondslag aan dit promotieonderzoek.

We hebben onze aandacht gericht op de aanwezigheid en de dynamiek van CRF en Ucn1, en van hun receptoren CRF1 en CRF2. Daarbij is gebruik gemaakt van verscheidene experimentele modellen en benaderingswijzen, waaronder een transgene muis die CRF tot overproductie brengt (CRF-OE muis), acute en chronisch stress paradigmata, en chronische behandeling met corticosteron. Waarnemingen zijn meestal zowel op transcriptioneel als op translationeel niveau uitgevoerd, met respectievelijk, *in situ* hybridizatie en immunocytochemie. In **Hoofdstuk 2** wordt gedemonstreerd dat de muizenlijnen CRF-OE_{2122} en CRF-OE_{2123} uitsluitend in de hersenen CRF- en CRF mRNA-gehalten bezitten die ten opzichte van wildtype muizen verhoogd zijn. Alleen in CRF-OE_{2122} muizen gaat deze CRF overexpressie samen met een vermeerdering van bioactief CRF in de hypothalamus, met een verhoogde lichaamstemperatuur en hartslag, en met toegenomen voedselopname en waterconsumptie. Daarom is deze transgene muizenlijn in de rest van dit onderzoek gebruikt als onderzoekmodel om de rol van centraal CRF in stress-adaptatie te onderzoeken. In **Hoofdstuk 3** laten we zien dat CRF de CRF1 and CRF2 receptoren op verschillende wijzen controleert, zowel wat betreft de mate van receptor mRNA expressie als wat betreft de plaats waarop de receptoren in de hersenen tot expressie komen: CRF overproductie leidt tot een afname van het aantal neuronen dat CRF1 mRNA tot expressie brengt in de subthalame nucleus, de globus pallidus, het laterale septum, de substantia nigra, de primaire somatosensorische cortex en de principale sensorische nucleus, maar tot een toename van het aantal neuronen dat in de dorsale raphe CRF2 mRNA tot expressie brengt. In **Hoofdstuk 4** onderbouwen we onze hypothese dat CRF-receptoren de werking van CRF en Ucn1 niet alleen in de hersenen maar ook in het ruggenmerg mediëren. Beide CRF-receptor mRNAs komen in het ruggenmerg voor, maar hun individuele expressies verschillen in sterkte en in plaats (laminae van Rexed). Op grond van deze vinding veronderstellen we dat CRF-peptiden hun werking op perifere componenten van het stress-adaptatiesysteem uitoefenen via locale concentraties van CRF1 en CRF2 in het ruggenmerg.

In de **Hoofdstukken 5, 6** en **7** wordt beschreven hoe steun is verkregen voor de veronderstelling dat de EW betrokken is bij stress-adaptatie. In **Hoofdstuk 5** wordt aangetoond dat in EW-Ucn1 neuronen in CRF-OE muizen worden geactiveerd door chronisch verhoogd CRF, zowel op mRNA- als op peptide-niveau. Ook reageren deze EW-neuronen, net als de HBB-as, op een acute stressor met een toename van de expressie van Fos en van Ucn1 mRNA. Dit suggereert dat de HBB-as en de EW samenwerken in de adaptatieresponse van het dier op acute stressoren maar tegengesteld reageren bij chronische stress. In **Hoofdstuk 6** zien we dat de EW, in contrast met de HBB-as, niet habitueert bij chronische homotypische ether stress en daarbij een verlaagd Ucn1 mRNA-gehalte vertoont vergeleken met acute stress. Tenslotte wijzen onze studies erop dat activering van de bijnier door chronische stress tot remming leidt van de EW, waarschijnlijk door een directe actie van corticosteron op EW-Ucn1 neuronen (**Hoofdstuk 7**). Dit idee wordt ondersteund door de demonstratie dat deze neuronen glucocorticoid-receptoren bevatten en dat experimentele chronische verhoging van corticosteron het aantal van deze neuronen verlaagt.

In **Hoofdstuk 8** zijn de verkregen resultaten in een breder perspectief geplaatst, wat ons tot de conclusie heeft gebracht dat het CRF-OE muizenmodel een zeer nuttig ‘gereedschap’ is om de rol te onderzoeken van chronisch verhoogd CRF in de controle van het stress-adaptatieproces. Voorts veronderstellen we dat ook Ucn1 in de EW een substantiële rol speelt in stress-adaptatie en suggereren we een serie toekomstige experimenten om de functies van CRF in de HBB-as en van Ucn1 in de EW in het stress-adaptatieproces verder op te helderen.

Összefoglalás

Az alkalmazkodás képessége a homeosztázist veszélyeztető különböző külső és belső kihívásokhoz (stressz) alapvető fontosságú az egyed túlélése szempontjából. Ezen alkalmazkodási képességet “stressz adaptációnak” nevezzük, mely különböző idegrendszeri mechanizmusokon keresztül valósul meg. Ezen mechanizmusok hibás müködése különböző stressz-kiváltotta fiziológiai, testi és szellemi elváltozásokhoz vezet. A legismertebb stressz-adaptációs mechanizmus a gerincesekben, beleértve az embert, a hipotalamusz-agyalapimirigy-mellékvese (HAM-) tengely, melynek a legfontosabb hírvivője a hipotalamikus corticotropin-releasing faktor (CRF). Egy a CRF-hez hasonló fehérje, az urocortin 1 (Ucn1) felfedezése az Edinger-Westphal (EW) magban, e fehérje a CRF receptorokon keressztül kifejtett, a CRF-hez hasonlatos hatásai, felvetik azt a kérdést, hogy valyon a CRF fehérje család különböző tagjai miként működnek közre a stressz-adaptációban. E kérdés megválaszolása állt a jelen tézisben összefoglalt kutatásaim középpontjában.

A CRF, az Ucn1 és ezek receptorai (CRF1 és CRF2) jelenlétének és expressziójuk dinamikájának vizsgálata adja kutatásaink fő témáját. Ezen kérdések tanulmányozására különböző kisérleti megközelítéseket alkalmaztunk, azaz, CRF over-expresszáló egér modelt (CRF-OE), akút és krónikus streszz paradigmákat, és krónikus kortikoszteron kezelést. A gén transzkripció és fehérje átírás meghatározása főleg *in situ* hibridizáció és immunocitokémiai technikák alkalmazásával történt. Tézisem **második** felyezetében bemutatom, hogy mindkét CRF over-expresszáló egér vonalban, a CRF-OE_{2122} és a CRF-OE_{2123}, a CRF peptid és mRNS szintek megemelkedése csak a központi idegrendszer területén volt megfigyelhető. Ezen a CRF over-expresszió a CRF-OE_{2122} egerekben, összehasolítva a vad típussal (WT) eggyütt jár egy megemelkedett bioaktív hipotalamikus CRF szinttel, megnövekedett test hőmérséklettel, szívritmussal, étel és víz fogyasztással. Ezen adatok alapján feltételeztük, hogy a CRF-OE egér alkalmas a CRF fehérje, a stressz adaptációban betöltött szerepének további tanulmanyozására. A **harmadik** felyezetben részletezzük, hogy a CRF különféleképpen szabályozza a két CRF receptor mRNS expresszióját illetve előfordulását a különböző agyterületeken. CRF over-expresszió hatására a következő magokban csökkent a CRF1 mRNS-t expresszáló neuronok száma: nucleus subthalamicus, globus pallidus, septum laterale, substantia nigra, elsődleges szomatoszenzoros kéreg és a háromosztatú agyideg fő szenzoros magja. Ezzel ellentétben a dorsal raphe magban megnövekedett a CRF2 mRNS expresszáló idegsejtek száma. A **negyedik** felyezetben leírt adatok alátámasztják azon hipotézisünket, hogy a CRF receptorok, nemcsak az agyban, hanem a gerincvelő területén is szerepet játszhatnak a CRF fehérje család hatásainak közvetítésében. Ebben a felyezetben írjuk le mindkét CRF receptor mRNA előfordulását a gerincvelőben. A két receptor expressziójának intenzitása és eloszlása a gerincvelői Rexed laminákban jelentősen különbözött. Ezen eredmények alapján feltételezzük, hogy a CRF- peptid család tagjai ezen gerincvelői receptoraikon keresztül képesek befolyásolni a stressz-adaptáció perifériás folyamatait.

Az **ötödik, hatodik és hetedik** felyezetben leírt adatok alátámasztják az EW mag feltételezett szerepét a stressz-adaptációban. Az **ötödik** felyezetben bemutatjuk, hogy krónikusan megemelkedett CRF fehérje szint hatására jelentősen csökkent az EW mag idegsejtjeiben az Ucn1 fehérje és mRNS expressziója. Továbbá, mind a CRF-OE egerekben, mind a vad típusban aktiválódnak ezen idegsejtek, akút stressz hatására, és emelkedett Ucn1 mRNS expresszióval reagálnak. Ezen eredményeink alapján megállapíthatjuk, hogy a HAM tengely és az EW mag hasonlóan válaszol akút stressz hatásokra, de ellenkező reakciót mutat krónikus kihívásokra. A továbbiakban az EW mag aktivitásának változásait vizsgáltuk egy krónikusan aktiv HAM tengely mellett. Ezen eredményeinket a **hatodik** felyezetben az alábbiakban foglaltuk össze. A HAM tengellyel ellentétben az EW mag mindvégig megtartja válaszkészségét krónikus kihívásokkal szemben, és egy csökkent Ucn1 mRNS expresszióval válaszol. Végül a **hetedik** felyezetben leírt kisérletek arra mutatnak rá, hogy a mellékvese krónikus-stressz által előidézett aktvációja, az EW-Ucn1 rendszer gátlásához vezet, valószínűleg az EW-Ucn1 neuronokon való direkt kortikoszteron visszacsatoláson keresztül. A kortikosteron receptor és az Ucn1 együttes előfordulását az EW mag neuronjaiban bebizonyítottuk, eggyütt azzal, hogy krónikus kortikoszteron kezelés csökkenti az EW-neuronok számát.

A **nyolcadik** felyezet a munkám során elért eredményeim összefoglalását, tágabb értelemben vett megbeszélését tartalmaza. Ebben a fejezetben arra a következtetésre jutottam, hogy a CRF-OE egér model alkalmas a krónikusan megemelkedett CRF, a stressz adaptatció szabályozásában betöltött szerepének vizsgálatára. Továbbá, felvetjük, hogy az Ucn1 az EW magban lényeges szerepet kap a stresszhez való alkalmazkodás folyamatában, és olyan jövőbeli kutatások terveit vázoljuk fel, amelyek a CRF-HMA tengely és a Ucn1-EW rendszer, a stressz adaptációban játszott szerepének további és pontosabb megismeréséhez segíthetnek hozzá.

Acknowledgements

I think I can say that during the last four years I participated in a very special and particularly long ‘inburgeringcursus’. The ‘course’ led to this ‘proefschrift’ (thesis) and of course to much more than that. It has been a great adventure and I would like to thank all the people that have contributed either directly or indirectly.

I would like to begin by acknowledging my ‘numerous’ (co-)promotores. Eric, with absolute certainty this thesis wouldn’t have existed without your help. I’m very grateful for the trust you had in me. From the very beginning you invested an incredible amount of time and energy in teaching me how to become an independent researcher. You have always been ready, without hesitation, to help me solve a multitude of problems. You taught me how important it is to set my own standards and helped me through the tedious writing phase. Thank you very much for all this. Berend, thank you for giving me the opportunity to take part in this project, and to participate at many scientific meetings. Even though we did not work in direct contact on a daily basis, you have been a solid presence that I could count on, always with very rapid replies especially during the last stage of my project.
Tamás, hálásan köszönöm kutatási munkámhoz nyújtott segítségedet, ötleteidet, amelyek nagyban hozzájárultak disszertációm elkészítéséhez. Sokat jelentett számomra a veled való együttműködés és együttgondolkozás. Szívből köszönöm a jövőbeni lépéseim tervezésében nyújtott segítségedet is.
Jan V, besides all the help that you have always been ready to give me, I’m most grateful for the time we spent together at the microscope. During this time you transmitted to me a lot of your knowledge, and your love of the neuroanatomy. Jan vdG, thank you for your support particularly in arranging bureaucratic matters like the famous DEC protocols and in making it possible I could undertake my experiments in Utrecht.

Of course many more people contributed to this thesis and to making the last four years special.

Jos, thank you for your, always kind and patient, help in all technical problems. I enjoyed all the time spent working in your lab and chatting with you.
I would like to thank all the Students that I worked with; Sietske, Nazza, Jakub, Marloes and Anouk, you all contributed so much to the results of this thesis and it was great fun to work together with all of you.
Collegues, thank you for the very nice and ‘gezellig’ time at work .
Geert and Maarten, it was great to have you as corridor-room mates. I had a lot of fun while working, and that is in large part thanks to you two.What I liked the most is that we became friends and that we could discuss work, talk about other things including a lot of nonsense. Bruce, thanks to you I did not feel lost at the huge conferences, and it was so much nicer and much more fun to be there with you. Debbie (Depc), thank you for

teaching me how to make probes and for sharing with us your experience of becoming a mama twice. Peter C, your kind help and enthusiasm made work in the lab much more fun and Frouwke, thanks for the 'opsleepen' sessions and for the nice chats we had during those times. Richard and Koen, thanks for your advice with the radioactive in situ. Gideon, Wout, Eric-Jan, Juriaan and Edwin, thank you for all the crazy borrels and other fun things, also outside the lab, that you organized. I have a great memory of our weekend in Terschelling. Wim S, Adhanet, Hongyan, Maartje Nicole, Gea, Frank, Kari, Maria Angelica Tony, Ron, Daisy, Peter K Wim A, Liesbeth P, Liangchun, Angela, Erwin, Gert, Else, Kim, Raffa, Noemi Ruben, Jos, Najara you all contributed a great deal to the nice atmosphere in the lab. Peter W, Iain and Coco, thanks for all the nice times we had at the various dinners and at Merelyn. Peter, I like it that we became closer friends and that we can always talk about our super nice aquariums.
I would also like to thank all the collegues from Utrecht; Lucianne, Marga, Monika, Marijke, Philip, Meg, Jhonny, Megane and Annaloes for helping me out and for making the time I spent there always pleasurable. From the lab in Leiden, I would like to thank Sergiu for helping me with my pellet experiment, and Peter S for his dedicated help with the 'not supposed to be floating' in situ.

A very special thanks goes to my 'Paranimfen' Marinella and Virginie. I`m sooo happy that you two are beside me at my promotion. Through these years you both really became my sisters. We did so many beautiful things together that I cannot even begin to list them here. You two are my home away from home.
Mari, piccolina mia, sei completamente parte integrante della mia vita, nel vero senso della parola...a lavoro, fuori da lavoro, in italia, in olanda...hi hi hi. Ogni volta che ci penso mi sembra incredibile. La nostra amicizia mi riempie il cuore ogni giorno. Ti ringrazio per essermi stata vicina in tutti i momenti difficili e per tutti i super bei momenti che abbiamo passato assieme e per tutti quelli che verranno. Ti voglio tanto tanto bene.
Virginie 'Princi' after the erasmus I couldn`t imagine that our lives could get close again, but they did and it has been sooo nice. I share with you all the crazy passions, like the one for spongebob, the presidents, the swimming, even in late September, in the freezing lakes...and our new favourite, cooking...in dutch...hi hi. We have a lot a lot of fun, but also share all the difficult or sad moments. I love you for all of this.

A special thanks goes to all the other friends that made me feel at home in Nijmegen; Kasper, Rutger, Huub, Anton, Gaelle being with you is always nice for your sweetness and craziness at the same time , Liesbeth I like a lot our long long talks. Trynke I enjoyed all our chats about work and life, and the way you involved me in your life, for example the great sailing weekends. I appreciated that a lot, and I had a lot of fun with you at conferences and it was an honour being your paranimf. Arne, a very special thank you for your help in the layout of my thesis.
Un ringraziamento speciale va ai miei amici di Torino; Chiaretta nonostante la distanza, la

nostra amicizia e rimasta un punto fermo nella mia vita e lo rimarra per sempre, Fracchia adoro le nostre interminabili chiacchierate, Robi, Tizzi, Sergio, Andre e Daniele, grazie per avermi fatto stare bene e a casa, ogni volta che tornavo a Torino.
I would like to thank Marianne and Piet for all the relaxing sundays in Blerick, in which they spoiled me with a lot of delicious food, beautiful walks in the woods and of course fishing. Petra I enjoyed every time we spend together, the long chats we had about all sort of things and of course our super-duper diving holiday and sailing weekends.
Drága Mucsi, Apu és Gyöngyi 'Tittiiii'! Az utolsó négy évben nagyon nagyon hiányoztatok, de a távolság ellenére, mindig mellettem álltatok és közel voltatok hozzám. Akármilyen messze vagytok mindig biztonságban éreztem magam annak tudatában, hogy mindig számíthatok rátok.
Bart, you have been with me in this adventure from the very beginning till the end. When I think of this four years spend toghether all the fun we always have and our super-nice trips come to my mind. With you I dived for the first time and this was one of the most amazing experience of my life. I cannot wait to see where our next adventure takes us.

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Curriculum vitae

Aniko Korosi was born on March 14 1977 in Budapest, Hungary. In 1996 she received her secondary education at the Liceo Scientifico Leonardo da Vinci in Turin, Italy and proceeded to study biology. During her study, via an EC-Erasmus program, she spent 6 months in Nijmegen at the Catholic University of Nijmegen. Here she investigated the morpho-functional changes in the forebrain of the WAG/Rij rat model for absence epilepsy. In November 2001 she graduated in biology at the Turin's University (Universita degli Studi di Torino). The research leading to this thesis was started in april 2002 at the department of Psychopharmacology, Utrecht University in collaboration and in with the Department of Cellular Animal Physiology, Radboud University Nijmegen under the supervision of Prof. Prof. Dr. E.W. Roubos, Dr. B. Olivier, Dr T. Kozicz, Dr. J.G. Veening and Dr J. van der Gugten. The results obtained during this project were presented at various national and international scientific conferences and finally presented and discussed in this thesis.