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Differential disinhibition of the neonatal hypothalamic–pituitary–adrenal axis in brain-specific CRH receptor 1-knockout mice

M. V. Schmidt,[1] J. M. Deussing,[1] M. S. Oitzl,[3] F. Ohl,[5] S. Levine,[4] W. Wurst,[2] F. Holsboer,[1] M. B. Müller[1] and E. R. de Kloet[3]

[1]Max Planck Institute of Psychiatry, RG Molecular Stress Physiology, Kraepelinstr. 2–10, 80804 Munich, Germany
[2]GSF-National Research Center for Environment and Health, Neuherberg, Germany
[3]Leiden–Amsterdam Center for Drug Research/Leiden University Medical Center, Leiden, the Netherlands
[4]Department of Psychiatry, University of California, Davis, USA
[5]University of Utrecht, the Netherlands

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Abstract

In the adult, corticotropin-releasing hormone (CRH) is the key mediator for the behavioural and neuroendocrine response to stress. It has also been hypothesized that, during postnatal development of the stress system, CRH controls the activity of the HPA axis and mediates the effects of early disturbances, e.g. 24 h of maternal deprivation. In the current study we investigated the function of specific brain corticotropin-releasing hormone receptor type 1 (CRHR1) subpopulations in the control of the HPA axis during postnatal development under basal conditions as well as after 24 h of maternal deprivation. We used two conditional CRHR1-deficient mouse lines which lack this receptor, either specifically in forebrain and limbic structures (Cam-CRHR1) or in all neurons (Nes-CRHR1). Basal circulating corticosterone was increased in Nes-CRHR1 mice compared to controls. Corticosterone response to maternal deprivation was significantly increased in both CRHR1-deficient lines. In the paraventricular nucleus, Cam-CRHR1 animals displayed enhanced CRH and decreased vasopressin expression levels. In contrast, gene expression in Nes-CRHR1 pups was strikingly similar to that in maternally deprived control pups. Furthermore, maternal deprivation resulted in an enhanced response of Cam-CRHR1 pups in the brain, while expression levels in Nes-CRHR1 mouse pups were mostly unchanged. Our results demonstrate that brainstem and/or hypothalamic CRHR1 contribute to the suppression of basal corticosterone secretion in the neonate, while limbic and/or forebrain CRHR1 dampen the activation of the neonatal HPA axis induced by maternal deprivation.

Introduction

In rats and mice, the development of the stress system is characterized by a low activity of the hypothalamic–pituitary–adrenal (HPA) axis (the 'stress-hyporesponsive period'; Levine, 1994). Disruptions of this stress-hyporesponsive period have been shown to result in long-term consequences for HPA function and behaviour (Plotsky & Meaney, 1993; Sutanto *et al*., 1996; Anisman *et al*., 1998; Caldji *et al*., 1998). In addition, disturbance of normal stress system development has been shown to be a risk factor for a number of psychiatric diseases in humans (Heim *et al*., 2002; Rinne *et al*., 2002). Corticotropin-releasing hormone (CRH) drives the HPA axis and is the key mediator of the stress system, orchestrating the body's response to stress on various levels in the adult animal and during development (Vale *et al*., 1981; Holsboer, 1999). It is therefore a major candidate for mediating the short- and long-term consequences of disrupted HPA development.

In addition to driving the HPA axis, CRH is involved in food intake (Richard, 1995; Bovetto *et al*., 1996), immune suppression (De Souza, 1995; Karalis *et al*., 1997) and the regulation of anxiety-related behaviour (Dunn & Berridge, 1990; Steckler & Holsboer, 1999). The main receptor for CRH in the brain is the corticotropin-releasing hormone receptor type 1 (CRHR1), while the other known member of the CRH receptor family, CRH receptor type 2, seems to be main endogenous receptor for urocortin 1, urocortin 2 or urocortin 3 (Reul & Holsboer, 2002). The function of CRH has recently been further characterized by the use of transgenic mice (for review see Contarino *et al*., 1999b; Müller & Keck, 2002; Deussing & Wurst, 2005). CRH overexpression has been shown to increase anxiety-like behaviour (Stenzel-Poore *et al*., 1994; Van Gaalen *et al*., 2002) and HPA activity (Groenink *et al*., 2002). Furthermore, mice deficient in CRHR1 display impaired endocrine responsiveness to stress and reduced anxiety-like behaviour (Smith *et al*., 1998; Timpl *et al*., 1998; Contarino *et al*., 1999a). By using conditional CRHR1-knockout mice in which the receptor is deleted postnatally in selected brain areas, Müller and colleagues reported that the latter effect is mainly dependent on CRHR1 in forebrain or limbic structures (Müller *et al*., 2003).

CRH also plays an important role during the postnatal development of the stress system (Brunson *et al*., 2001; Plotsky *et al*., 2005). Baram and colleagues demonstrated that CRHR1 mediates the excitatory actions of CRH in the developing brain (Baram *et al*., 1997). Additionally, early changes in hypothalamic CRH gene expression

Correspondence: Dr Mathias V. Schmidt, Max Planck Institute of Psychiatry, RG Molecular Stress Physiology, Kraepelinstr. 2–10, 80804 Munich, Germany.
E-mail: mschmidt@mpipsykl.mpg.de

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may play a critical role in the mechanism by which early life experiences influence long-term HPA function (Avishai-Eliner *et al*., 2001; Chen *et al*., 2004). It has been demonstrated that CRH expression in the paraventricular nucleus (PVN) is rapidly induced by mild stressors during postnatal development (Dent *et al*., 2000). However, this CRH induction was not reflected in an activation of the HPA axis, as the adrenal glands are stress-hyporesponsive during that time. Thus, it can be hypothesized that CRH plays a prominent role in the regulation of the function and development of the postnatal brain.

To further elucidate the role of the CRHR1 during development, we recently investigated the HPA axis activity of conventional CRHR1-deficient mice at postnatal day (P)9 (Schmidt *et al*., 2003b). We demonstrated that CRHR1 is essential for the regulation of HPA axis activity under nondeprived conditions. Furthermore, maternal deprivation was largely without effect in CRHR1-knockout animals. However, due to the widespread distribution of CRHR1 in the brain, the anterior pituitary and peripheral tissues (De Souza, 1995), no differentiation could be made in regard to the population of CRHR1 responsible for the observed effects.

To circumvent these problems, in the current study two different lines of brain-specific, conditional CRHR1-deficient mice were used (Müller *et al*., 2003). Using a Cre/lox-mediated inactivation of the CRHR1 under the control of a cell type-specific promoter, these two mouse lines lack CRHR1 only in specific brain regions. For the Cam-CRHR1 line, CRHR1 expression is deleted postnatally (starting on P5) in forebrain and limbic structures, but not in the hypothalamus (including the PVN), the hindbrain, the anterior pituitary or other peripheral target tissues (Kelly *et al*., 1987; Xue *et al*., 2002). In the Nes-CRHR1 line, CRHR1 receptors are knocked out prenatally in all neurons, sparing only expression sites in the periphery (including the anterior pituitary; Tronche *et al*., 1999; Reichardt *et al*., 2000; Michalczyk & Ziman, 2005). We therefore addressed the question of whether CRHR1 in specific brain areas contribute to the regulation of the basal HPA axis activity in neonatal mice. Further, in order to elucidate the mechanism by which maternal deprivation affects the function of the HPA axis in neonates, the role of brain CRHR1 in mediating gene expression changes in response to maternal deprivation was investigated.

Materials and methods

Animals

Mice with a homozygous mutation of the CRHR1 gene, where exons 9–13 are flanked by two loxP sites, were generated at the Max Planck Institute of Psychiatry in Munich, Germany (Müller *et al*., 2003). CRHR1^{loxP} is sensitive to the Cre recombinase, which catalyses the site-specific recombination between the two loxP sites, thereby inactivating the CRHR1 gene in any cell expressing Cre recombinase (for review see Lewandoski, 2001). CRHR1$^{loxP/loxP}$ mice were crossed with one of two different effector mouse lines, which expresses Cre recombinase in a region- and cell-specific manner under the control of either the calcium–calmodulin kinase IIα (CaMKIIα) promoter or the nestin promoter. In the resulting mouse lines, the CRHR1 gene is inactivated depending on the expression profile of the specific promoter. Genes under the control of the CaMKIIα promoter are activated postnatally only in neurons of forebrain and limbic brain structures (Kelly *et al*., 1987; Xue *et al*., 2002), while the expression of genes controlled by the Nes-CRHR1 promoter starts prenatally in all neuronal cells (Tronche *et al*., 1999; Michalczyk & Ziman, 2005). We will further use the term Cam-CRHR1 to describe CRHR1$^{loxP/loxP}$ CaMKIIαCre mice and Nes-CRHR1 to describe CRHR1$^{loxP/loxP}$ NesCre mice. CRHR1$^{loxP/loxP}$ mice without Cre expression will be referred to as the respective controls.

Genotyping of the offspring was performed by Southern blot analysis of Xbal-digested tail DNA using an internal CRHR1 probe and a Cre recombinase-specific probe. Only homozygous Cam-CRHR1, Nes-CRHR1 or the corresponding control pups (no Cre expression) were used for data analysis. All mice in this study were kept on a mixed 129/SV × C57BL/6 background. Conditional knockouts and controls were obtained from the same litters, thereby excluding interlitter effects.

Pregnant females were transferred to clear polycarbonate cages containing sawdust and two sheets of paper towels for nest material during the last week of gestation. These females were checked for litters daily at 08.00 h. The day on which litters were found, the day of birth was defined as P0 for that litter. The litters remained undisturbed until the day of testing (P9). All animals were housed under a 12-h light, 12-h dark cycle (lights on at 06.00 h) and constant temperature (23 ± 2 °C) conditions. Food and water were provided *ad libitum*. This experiment was carried out at the animal facility of the Max Planck Institute of Psychiatry in Munich, Germany, and all the procedures were carried out in accordance with European Communities Council Directive 86/609/EEC. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.

Deprivation procedure

Maternal deprivation took place in a separate room in the animal facility under similar light and temperature conditions as mentioned previously. Mothers were removed from their home cages 24 h prior to the day of experimentation. The home cage, containing the litter, was then placed on a heating pad maintained at 30–33 °C for 24 h. Neither food nor water was available during the deprivation period. Non-deprived litters remained undisturbed with their mothers until the time of testing. Maternal deprivation or non-deprivation are referred to as 'condition'.

Experimental design

All mice were tested at P9. Each nest was assigned randomly to one of the two conditions, maternal deprivation or nondeprived control. Testing took place between 08.00 and 11.00 h. At the end of testing, all pups from a litter were killed immediately by decapitation. Experiments with the two different conditional CRHR1-deficient mouse lines were carried out separately.

Sampling procedure

Trunk blood from all pups was collected individually in labelled 1.5-mL EDTA-coated microcentrifuge tubes. All blood samples were kept on ice and later centrifuged for 10 min at 3300 ***g*** at 5 °C. Plasma was transferred to clean, labelled 1.5-mL microcentrifuge tubes. All plasma samples were stored frozen at −20 °C until the determination of adrenocorticotropic hormone (ACTH) and corticosterone. A minimum of 10 animals per group was analysed. ACTH and corticosterone were measured by radioimmunoassay (ACTH: sensitivity 10 pg/mL, intra-assay variation 4.1%, interassay variation 4.4%; corticosterone: sensitivity 12.5 ng/mL, intra-assay variation 4.4%, interassay variation 6.5%; both from MP Biomedicals Inc.). Whole heads (without skin and jaw) were removed, frozen in

isopropane and stored at −80 °C for *in situ* hybridization. Tail tips were removed and frozen for determination of the genotype of the pups.

In situ *hybridization*

The brains of 8–10 animals per group were used for *in situ* hybridization. Frozen brains were sectioned at −20 °C in a cryostat microtome at 16 μm in the coronal plane through the level of the hypothalamic PVN and dorsal hippocampus. The sections were thaw-mounted on poly L-lysine-coated slides, dried and kept at −80 °C.

In situ hybridization using ^{35}S UTP-labelled ribonucleotide probes (CRH; glucocorticoid receptor, GR; mineralocorticoid receptor, MR) were performed as described previously (Schmidt *et al*., 2002). Briefly, for riboprobe *in situ* hybridization sections were fixed in 4% paraformaldehyde with 0.5% glutaraldehyde, and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine–HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes for CRH (full length), GR (1250 base pairs) and MR (750 base pairs) were transcribed from a linearized plasmid. Tissue sections (two brain sections per slide) were saturated with 100 μL of hybridization buffer containing $\sim 1.5 \times 10^6$ cpm ^{35}S-labelled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day the sections were rinsed in 2 × SSC (standard saline citrate), treated with RNAse A (20 mg/L) and washed in increasingly stringent SSC solutions at room temperature. Finally sections were washed in 0.1 × SSC for 1 h at 65 °C and dehydrated through increasing concentrations of alcohol.

For vasopressin (AVP), an oligo in-situ hybridization was performed. The oligonucleotides (sequence: 5′-gggcttggcagaatccacggactcttgtgtcccagccgctgtaccag-3′) were labelled with ^{35}S dATP using terminal transferase (Boehringer, Germany) and added to the hybridization mix. Brain sections were coverslipped and incubated overnight at 45 °C. The following day the sections were washed in 1 × SSC at 55 °C and dehydrated through increasing concentrations of alcohol.

The slides were exposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY, USA; CRH, 6 days; GR, 3 days; MR, 2 days) and developed. Autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion Corporation). The mean of 2–6 measurements was calculated from each animal. For AVP, slides were also dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY, USA) and exposed at 4 °C for 6 days. Slides were developed, counterstained with Cresyl Violet and examined under a light microscope with both bright- and dark-field condensers. For the quantitative analysis of AVP, dark-field pictures of the PVN were analysed with the Scion Image software. The grey-scale pictures were inverted and made binary (black or white) at a specific grey value threshold. Subsequently a circle of fixed size was superimposed on the PVN area and the average grey value was obtained.

Data analysis

The results were analysed using ANOVA with the level of significance set at $P < 0.05$. When appropriate, tests of simple main effects were made with Student's *t*-test. The initial analysis included sex as a factor; once it was determined that sex was not a significant factor, the data were collapsed across this variable. The data of Cam-CRHR1 and Nes-CRHR1 animals were analysed separately, as the experiments with these two conditional mouse lines were not performed simultaneously. Data are presented as mean ± SEM.

Results

Endocrine parameters

The peripheral activity of the stress system was tested under nondeprived conditions and in response to 24 h of maternal deprivation in both conditional mutant lines (Fig. 1). The results for corticosterone and ACTH in both mutant lines showed significant

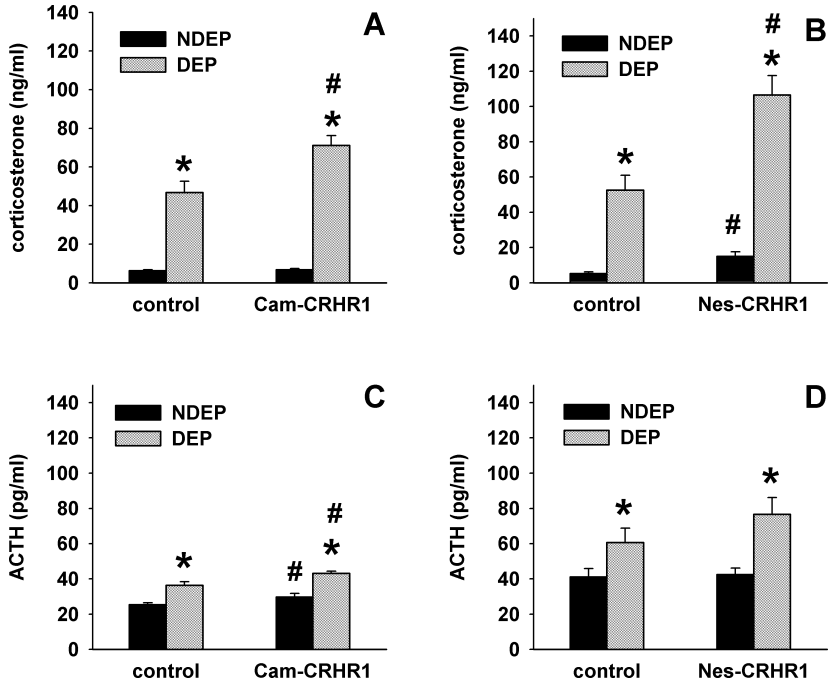

FIG. 1. Basal (NDEP)- and maternal deprivation (DEP)-induced (A and B) corticosterone and (C and D) ACTH plasma levels in Cam-CRHR1 (left) and Nes-CRHR1 (right) pups at P9; $n = 10$–22 for each condition. Data represent mean ± SEM; $^*P < 0.05$ vs. basal, $^{\#}P < 0.05$ vs. control.

differences of genotype (Cam-CRHR1: corticosterone: $F_{1,74} = 8.671$, $P < 0.004$; ACTH: $F_{1,56} = 8.38$, $P < 0.005$; Nes-CRHR1: corticosterone: $F_{1,47} = 20.829$, $P < 0.0001$), condition (Cam-CRHR1: corticosterone: $F_{1,74} = 152.557$, $P < 0.0001$; ACTH: $F_{1,56} = 40.584$, $P < 0.0001$; Nes-CRHR1: corticosterone: $F_{1,47} = 99.589$, $P < 0.0001$; ACTH: $F_{1,45} = 13.415$, $P < 0.001$) as well as for corticosterone a significant genotype × condition interaction (Cam-CRHR1: $F_{1,74} = 7.811$, $P < 0.007$; Nes-CRHR1: $F_{1,47} = 10.074$, $P < 0.003$). The data under nondeprived conditions showed no significant changes in Cam-CRHR1 animals, while Nes-CRHR1 pups showed higher basal corticosterone levels than controls. Maternal deprivation resulted in significant increases in ACTH and corticosterone in all tested groups. However, for corticosterone this increase was significantly greater in Cam-CRHR1 and Nes-CRHR1 mutants than in their respective controls.

Neuronal expression of HPA parameters

CRH expression was measured in the PVN (Fig. 2). For both genotypes ANOVA revealed a main effect of genotype (Cam-CRHR1: $F_{1,28} = 8.414$, $P < 0.008$; Nes-CRHR1: $F_{1,35} = 39.291$, $P < 0.0001$) and condition (Cam-CRHR1: $F_{1,28} = 59.197$, $P < 0.0001$; Nes-CRHR1: $F_{1,35} = 15.018$, $P < 0.001$) as well as an interaction between condition and genotype (Cam-CRHR1: $F_{1,28} = 8.474$, $P < 0.0077$; Nes-CRHR1: $F_{1,35} = 9.239$, $P < 0.005$). When compared to their respective controls, CRH expression was markedly elevated in Cam-CRHR1 mutants while being significantly decreased in Nes-CRHR1. Following maternal deprivation, CRH expression was decreased in control pups. Cam-CRHR1 animals also displayed a decrease in CRH expression following maternal deprivation, which was now indistinguishable from deprived control pups. In Nes-CRHR1 mutants, which already express low levels of CRH mRNA, no further effect of maternal deprivation on CRH expression was observed.

The expression of AVP was also studied in the PVN (Fig. 3). Significant effects of genotype (Cam-CRHR1: $F_{1,23} = 28.989$, $P < 0.0001$; Nes-CRHR1: $F_{1,35} = 4.471$, $P < 0.043$) and condition (Cam-CRHR1: $F_{1,23} = 6.37$, $P < 0.021$; Nes-CRHR1: $F_{1,35} = 7.18$, $P < 0.012$) were detected in both conditional CRHR1-deficient mouse lines. In addition, a genotype × condition interaction was observed in Nes-CRHR1 mutants ($F_{1,35} = 8.892$, $P < 0.006$). Under basal conditions Cam-CRHR1 mutants had significantly reduced expression levels of AVP in the PVN. In contrast, Nes-CRHR1-deficient mice showed an enhancement of AVP expression. Control pups of both conditional mouse lines responded to maternal deprivation with an increase in AVP expression. No change was observed in either of the CRHR1-deficient mice.

In the hippocampus, GR and MR expression were studied (Fig. 4). GR expression was measured in the CA1 area as, at that age, the expression levels in the other hippocampal areas are below the detection limit. Maternal deprivation resulted in a significant effect of condition in both mouse lines (Cam-CRHR1: $F_{1,30} = 10.152$, $P < 0.004$; Nes-CRHR1: $F_{1,38} = 71.846$, $P < 0.0001$). The expression levels of GR were decreased following maternal deprivation in all genotypes, with no significant differences between genotypes. MR expression was measured in the CA1, CA2, CA3 and dentate gyrus subregions of the hippocampus. For the Cam-CRHR1 mouse line, ANOVA revealed a significant effect of condition for all subregions (CA1: $F_{1,28} = 7.692$, $P < 0.011$; CA2: $F_{1,26} = 42.073$, $P < 0.0001$; CA3: $F_{1,26} = 20.823$, $P < 0.0001$; dentate gyrus: $F_{1,26} = 6.102$, $P < 0.022$). In addition, there were significant effects in the CA2 region for genotype ($F_{1,28} = 7.318$, $P < 0.013$) and a genotype × condition interaction ($F_{1,28} = 9.302$, $P < 0.006$). For the Nes-CRHR1

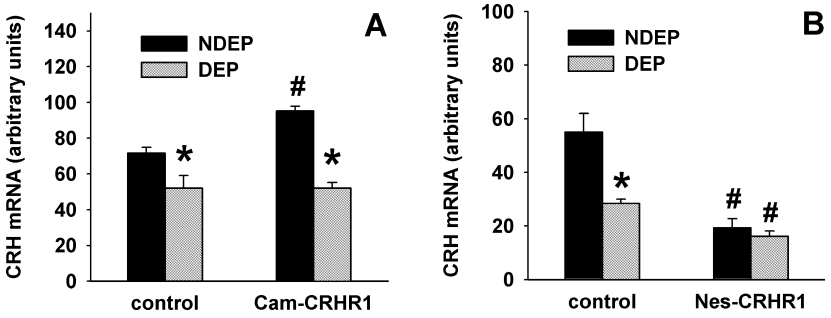

FIG. 2. Expression levels of CRH mRNA in the PVN in (A) Cam-CRHR1 (B) and Nes-CRHR1 mouse pups. Animals were either separated from their mother for 24 h (DEP) or left undisturbed (NDEP); $n = 8$–10 for each condition. Data represent mean ± SEM; $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.05$ vs. control.

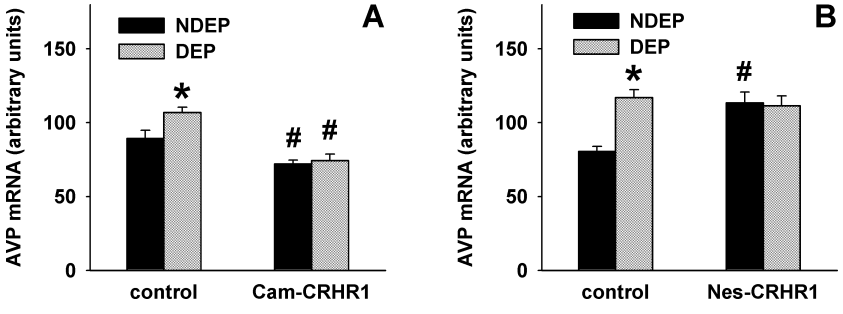

FIG. 3. Vasopressin (AVP) mRNA expression in the PVN of (A) Cam-CRHR1 and (B) Nes-CRHR1 mice at P9. Animals were either separated from their mother for 24 h (DEP) or left undisturbed (NDEP); $n = 8$–10 for each condition. Data represent mean ± SEM; $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.05$ vs. control.

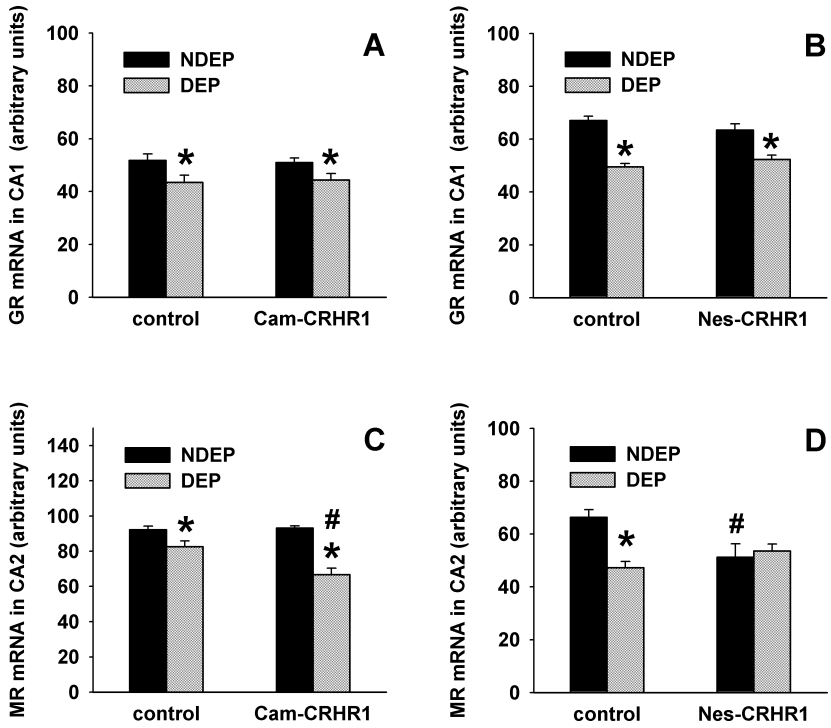

FIG. 4. Expression levels of (A and B) GR in the CA1 area and (C and D) MR in the CA2 area in (A and C) Cam-CRHR1- and (B and D) Nes-CRHR1-deficient mouse pups. The 9-day-old mouse pups were either maternally deprived (DEP) or left undisturbed (NDEP); $n = 8–10$ for each condition. Data represent mean ± SEM; $^{*}P < 0.05$ vs. basal, $^{\#}P < 0.05$ vs. control.

mouse line, ANOVA revealed a significant effect of condition ($F_{1,38} = 5.766$, $P < 0.022$) and a genotype × condition interaction ($F_{1,28} = 9.515$, $P < 0.004$) for the CA2 subregion. The strongest effects in both lines were observed in the CA2 area with similar, but less pronounced, effects in the other subregions. Under basal conditions MR expression was not altered in the Cam-CRHR1 line, while Nes-CRHR1 mutants showed a lower MR expression than did control pups. Following maternal deprivation, control pups of both lines responded with a decrease in MR expression. In Cam-CRHR1 pups this effect was significantly stronger than in control pups, while there was no effect in Nes-CRHR1 animals.

A summary of the effects of the lack of CRHR1 in various brain areas and the effects of maternal deprivation in the different CRHR1-deficient mouse lines is displayed in Table 1.

TABLE 1. The basal and maternal deprivation-induced HPA activity in control, Cam-CRHR1, Nes-CRHR1 and total CRHR1-deficient mice

Parameter	Control	Cam-CRHR1	Nes-CRHR1	CRHR1 total KO
Basal condition vs. control				
Corticosterone	–	0	↑	0
ACTH	–	0	0	n.d.
CRH	–	↑	↓↓	↑
AVP	–	↓	↑	↑
MR in hippocampus	–	0	↓	↓
GR in hippocampus	–	0	0	0
Maternal deprivation vs. own genotype				
Corticosterone	↑	↑↑	↑↑	0
ACTH	↑	↑	↑	n.d.
CRH	↓	↓↓	0	0
AVP	↑	0	0	0
MR in hippocampus	↓	↓↓	0	0
GR in hippocampus	↓	↓	↓	↓

Data for CRHR1 total KO mice are based on Schmidt *et al*. (2003b). Arrows indicate direction of regulation; ↑, up-regulation; ↓, down-regulation; double arrows, very strong effect; 0, no effect; n.d., not determined.

Discussion

In the current manuscript we describe the activity of the HPA axis in neonatal mice, which lack the CRHR1 in specific areas of the brain. The aim of this study was the functional differentiation of brain CRHR1 populations during postnatal development under nondeprived and maternally deprived conditions. Our results demonstrate that CRH in the neonate plays a major role in restraining peripheral HPA axis activity. Hindbrain and hypothalamic CRHR1 mainly suppress the basal HPA activity in the neonate, while limbic and forebrain CRHR1 dampen the maternal deprivation-induced activation of the neonatal HPA axis.

In addition to the distinct regional deletion of the CRHR1 in the two mouse lines a number of other differences exist and these might contribute to the observed phenotypes. First, due to the employed promoters the onset of CRHR1 deletion is different in Cam-CRHR1 and Nes-CRHR1 mice. While the receptors are already deleted very early during prenatal development in nestin-driven CRHR1-deficient mice, Cam-CRHR1 pups decrease receptor expression only postnatally a few days before testing. Thus, after birth Cam-CRHR1 mice undergo a short period of relatively uninhibited HPA responsiveness that the Nes-CRHR1 mice do not go through. This difference in early experience with endocrine substrates may also contribute to the observed differences between the two strains. Additionally, even though we have observed a distinct phenotype in the Cam-CRHR1 pups, the receptor in these mice might be only partially dysfunctional due to a long half-life. Furthermore, compensatory mechanisms in Nes-CRHR1 animals have to be taken into account.

Both forebrain and whole-brain CRHR1-deficient mice showed an increased corticosterone response to maternal deprivation, which represents a powerful stressor during postnatal development. This finding indicates that CRHR1 signalling from limbic and forebrain areas is mainly involved in the negative feedback regulation of the maternal deprivation-induced HPA activity. Thus, in mice with a fully functional brain CRH system the response to maternal deprivation is attenuated. This is in line with data from adult forebrain-deficient CRHR1 mice, which also show an enhanced stress response to restraint stress (Müller *et al*., 2003), indicating a diminished negative feedback. In addition, nestin-driven neonatal CRHR1-knockout mice also have higher basal corticosterone levels. As basal ACTH levels do not differ between control and knockout pups, a possible modulation of sympathetic adrenal innervation by CRHR1 signalling in the brainstem seems probable (Engeland & Gann, 1989). Thus, while brainstem CRHR1 signalling seems to be able to suppress the basal corticosterone secretion from the adrenals, forebrain and limbic CRHR1 circuits are involved in suppressing the maternal deprivation response of the neonate.

Interestingly, we found strong differences in CRH mRNA basal expression and regulation in the PVN between the two conditional knockout mouse lines. While forebrain- and limbic system-limited CRHR1-deficient mice showed a higher CRH mRNA expression than control pups, Nes-CRHR1-knockout mice had a lower CRH expression in the PVN. There are a number of reports indicating that CRH expression can be regulated by at least two different mechanisms, via glucocorticoid feedback and via CRHR1-mediated feedback (Makino *et al*., 2002). We were recently able to show that neonatal total CRHR1-knockout mice had a higher expression of CRH in the PVN, while their plasma corticosterone levels were similar (Schmidt *et al*., 2003b). It was hypothesized that the basal CRH expression in these animals was regulated via CRHR1 in a corticosterone-independent mechanism. Based on our current data it seems probable that the same mechanism is responsible for the increased CRH expression in Cam-CRHR1-deficient mice. This direct CRH feedback could be mediated by forebrain or limbic brain nuclei, such as the bed nucleus of the stria terminalis or the central amygdala. In conclusion, under conditions of low corticosterone levels during the stress-hyporesponsive period, CRH expression in the neonate seems to be suppressed by CRHR1-mediated signalling in the forebrain and limbic system.

High corticosterone levels can result in decreased CRH expression in the adult (Viau *et al*., 1999). In addition, increases in basal corticosterone levels at the end of the stress-hyporesponsive period in the neonate or during maternal deprivation parallel the decrease in CRH expression (Schmidt *et al*., 2003a; Schmidt *et al*., 2004). Thus, the low CRH expression in the Nes-CRHR1-deficient mice might be caused by the enhanced basal levels of corticosterone in these animals. This would also imply that corticosterone negative feedback is able to overwrite the effects of CRHR1-mediated feedback on CRH expression. Alternatively, high corticosterone levels have been shown to activate CRH expression in the amygdala and the bed nucleus of the stria terminalis (Makino *et al*., 1994; Watts, 1996), which in turn could modulate PVN activity. However, the magnitude of the corticosterone increase in Nes-CRHR1 animals is rather small compared to the maternal deprivation-induced increase in control animals. It is therefore not clear whether the small increase in basal corticosterone in Nes-CRHR1 pups could be responsible for the diminished CRH expression in these animals.

The effects of the CRHR1 deficiency on CRH mRNA are partly counteracted by the cosecretagogue of CRH, AVP. CRH and AVP are coexpressed in the parvocellular neurons of the PVN. AVP secretion has been shown to exponentiate the effects of CRH on ACTH production and is critically involved in the regulation of HPA axis activity under various physiological states (Rivier & Vale, 1983; Antoni, 1993). In forebrain-specific CRHR1 knockouts basal AVP mRNA expression is decreased, while mouse pups with a CRHR1 deletion in the whole brain display a higher AVP expression. In the Nes-CRHR1 mutants AVP may partly compensate for the decrease in CRH expression in the PVN. This effect would be similar to the proposed mechanism following chronic stress, where AVP has been described as being the more important secretagogue for HPA function (Scaccianoce *et al*., 1991; Bartanusz *et al*., 1993). Alternatively, the altered AVP expression in both conditional mouse lines could be due to the fact that in Cam-CRHR1 mutants the CRHR1 is still present in the PVN while in Nes-CRHR1 the receptor is inactivated in this area.

Mouse pups lacking the CRHR1 in the whole brain show a decreased MR expression in the CA2 area compared to controls, but no effect of maternal deprivation. These results are very similar to what has been reported in conventional CRHR1-deficient mouse pups (Schmidt *et al*., 2003b). In contrast, forebrain-specific knockouts did not differ from their control counterparts in terms of basal MR expression, but responded to maternal deprivation with a greater decrease in the CA2 area. Although corticosterone has been shown to be able to regulate MR expression (Herman & Watson, 1995), there also seems to be a functional interaction of the CRHR1 system and the MR. In adult animals, Gesing *et al*. (2001) were able to demonstrate that the MR is under positive regulation of CRH. These findings are supported by adult forebrain CRHR1-deficient mice, where a functional interaction between the limbic CRH–CRHR1 signalling pathway and MR regulation was evident (Müller *et al*., 2003). The current data in the neonate support this hypothesis, as the lowest MR expression was found in pups with the lowest CRH expression (Nes-CRHR1 knockouts) and an MR response to maternal deprivation was only observed when a CRH response occurred (or vice versa).

It is interesting to note that the central HPA parameters of Nes-CRHR1 pups under nondeprived conditions closely resembled the control situation following maternal deprivation. These findings imply that CRHR1 deficiency in the brain mimics certain aspects of maternal deprivation in the absence of highly elevated glucocorticoid concentrations. Thus, the effects of maternal separation might be mediated not exclusively via corticosterone but also via a CRH-dependent mechanism. The exception to this hypothesis would be the regulation of GR expression, which decreases after maternal deprivation by a corticosterone- and CRH-independent mechanism (Schmidt *et al*., 2003b).

An additional factor, which should be regarded, is the role of CRHR1 in brainstem regions. CRH is strongly associated with the noradrenergic system, which in turn influences HPA function. Anatomical and functional connections between brainstem nuclei (e.g.the locus ceruleus) and the PVN or limbic structures have been demonstrated (Koob, 1999). While this brainstem CRH system is fully intact in Cam-CRHR1 mice, it is compromised in Nes-CRHR1 knockouts. It seems therefore probable that alterations of noradrenergic signalling in these animals will contribute to the observed phenotype.

In summary, we were able to show that CRH plays an important role in regulating the activity of the neonatal HPA axis. It is apparent that a misbalanced CRHR1 system due to a lack of this receptor in specific brain regions results in distinct neuroendocrine phenotypes. We have demonstrated that one of the main functions of CRH in the neonate is the stabilization of corticosterone secretion at a low level. Hindbrain and brainstem CRH signalling in the neonate contributes to the regulation of basal HPA axis activity, while the forebrain and

limbic CRHR1 system modulates a negative feedback inhibition when the HPA system is activated.

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Abbreviations

ACTH, adrenocorticotropic hormone; AVP, vasopressin; Cam-CRHR1, $CRHR1^{loxP/loxP}$ CaMKIIαCre mice; controls, $CRHR1^{loxP/loxP}$ mice without Cre expression; CRH, corticotropin-releasing hormone; CRHR1, CRH receptor type 1; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; MR, mineralocorticoid receptor; Nes-CRHR1, $CRHR1^{loxP/loxP}$ NesCre mice; P, postnatal day; PVN, paraventricular nucleus.

References

Anisman, H., Zaharia, M.D., Meaney, M.J. & Merali, Z. (1998) Do early-life events permanently alter behavioral and hormonal responses to stressors? *Int. J. Dev. Neurosci.*, **16**, 149–164.

Antoni, F.A. (1993) Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Front. Neuroendocrinol.*, **14**, 76–122.

Avishai-Eliner, S., Eghbal-Ahmadi, M., Tabachnik, E., Brunson, K.L. & Baram, T.Z. (2001) Down-regulation of hypothalamic corticotropin-releasing hormone messenger ribonucleic acid (mRNA) precedes early-life experience-induced changes in hippocampal glucocorticoid receptor mRNA. *Endocrinology*, **142**, 89–97.

Baram, T.Z., Chalmers, D.T., Chen, C., Koutsoukos, Y. & De Souza, E.B. (1997) The CRF1 receptor mediates the excitatory actions of corticotropin releasing factor (CRF) in the developing rat brain: in vivo evidence using a novel, selective, non-peptide CRF receptor antagonist. *Brain Res.*, **770**, 89–95.

Bartanusz, V., Jezova, D., Bertini, L.T., Tilders, F.J., Aubry, J.M. & Kiss, J.Z. (1993) Stress-induced increase in vasopressin and corticotropin-releasing factor expression in hypophysiotrophic paraventricular neurons. *Endocrinology*, **132**, 895–902.

Bovetto, S., Rouillard, C. & Richard, D. (1996) Role of CRH in the effects of 5-HT-receptor agonists on food intake and metabolic rate. *Am. J. Physiol.*, **271**, R1231–R1238.

Brunson, K.L., Avishai-Eliner, S., Hatalski, C.G. & Baram, T.Z. (2001) Neurobiology of the stress response early in life: evolution of a concept and the role of corticotropin releasing hormone. *Mol. Psychiatry*, **6**, 647–656.

Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P.M. & Meaney, M.J. (1998) Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. *Proc. Natl Acad. Sci. USA*, **95**, 5335–5340.

Chen, Y., Bender, R.A., Brunson, K.L., Pomper, J.K., Grigoriadis, D.E., Wurst, W. & Baram, T.Z. (2004) Modulation of dendritic differentiation by corticotropin-releasing factor in the developing hippocampus. *Proc. Natl Acad. Sci. USA*, **101**, 15782–15787.

Contarino, A., Dellu, F., Koob, G.F., Smith, G.W., Lee, K.F., Vale, W. & Gold, L.H. (1999a) Reduced anxiety-like and cognitive performance in mice lacking the corticotropin-releasing factor receptor 1. *Brain Res.*, **835**, 1–9.

Contarino, A., Heinrichs, S.C. & Gold, L.H. (1999b) Understanding corticotropin releasing factor neurobiology: contributions from mutant mice. *Neuropeptides*, **33**, 1–12.

De Souza, E.B. (1995) Corticotropin-releasing factor receptors: physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. *Psychoneuroendocrinology*, **20**, 789–819.

Dent, G.W., Smith, M.A. & Levine, S. (2000) Rapid induction of corticotropin-releasing hormone gene transcription in the paraventricular nucleus of the developing rat. *Endocrinology*, **141**, 1593–1598.

Deussing, J.M. & Wurst, W. (2005) Dissecting the genetic effect of the CRH system on anxiety and stress-related behaviour. *C. R. Biol.*, **328**, 199–212.

Dunn, A.J. & Berridge, C.W. (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res. Brain Res. Rev.*, **15**, 71–100.

Engeland, W.C. & Gann, D.S. (1989) Splanchnic nerve stimulation modulates steroid secretion in hypophysectomized dogs. *Neuroendocrinology*, **50**, 124–131.

Gesing, A., Bilang-Bleuel, A., Droste, S.K., Linthorst, A.C., Holsboer, F. & Reul, J.M. (2001) Psychological stress increases hippocampal mineralocorticoid receptor levels: involvement of corticotropin-releasing hormone. *J. Neurosci.*, **21**, 4822–4829.

Groenink, L., Dirks, A., Verdouw, P.M., Schipholt, M., van der Veening, J.G., G.J. & Olivier, B. (2002) HPA axis dysregulation in mice overexpressing corticotropin releasing hormone. *Biol. Psychiatry*, **51**, 875–881.

Heim, C., Newport, D.J., Wagner, D., Wilcox, M.M., Miller, A.H. & Nemeroff, C.B. (2002) The role of early adverse experience and adulthood stress in the prediction of neuroendocrine stress reactivity in women: a multiple regression analysis. *Depress. Anxiety*, **15**, 117–125.

Herman, J.P. & Watson, S.J. (1995) Stress regulation of mineralocorticoid receptor heteronuclear RNA in rat hippocampus. *Brain Res.*, **677**, 243–249.

Holsboer, F. (1999) The rationale for corticotropin-releasing hormone receptor (CRH-R) antagonists to treat depression and anxiety. *J. Psychiatr. Res.*, **33**, 181–214.

Karalis, K., Muglia, L.J., Bae, D., Hilderbrand, H. & Majzoub, J.A. (1997) CRH and the immune system. *J. Neuroimmunol.*, **72**, 131–136.

Kelly, P.T., Shields, S., Conway, K., Yip, R. & Burgin, K. (1987) Developmental changes in calmodulin-kinase II activity at brain synaptic junctions: alterations in holoenzyme composition. *J. Neurochem.*, **49**, 1927–1940.

Koob, G.F. (1999) Corticotropin-releasing factor, norepinephrine, and stress. *Biol. Psychiatry*, **46**, 1167–1180.

Levine, S. (1994) The ontogeny of the hypothalamic-pituitary-adrenal axis. The influence of maternal factors. *Ann. NY Acad. Sci.*, **746**, 275–88. [Discussion, 289–293]

Lewandoski, M. (2001) Conditional control of gene expression in the mouse. *Nat. Rev. Genet.*, **2**, 743–755.

Makino, S., Gold, P.W. & Schulkin, J. (1994) Corticosterone effects on corticotropin-releasing hormone mRNA in the central nucleus of the amygdala and the parvocellular region of the paraventricular nucleus of the hypothalamus. *Brain Res.*, **640**, 105–112.

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

Michalczyk, K. & Ziman, M. (2005) Nestin structure and predicted function in cellular cytoskeletal organisation. *Histol. Histopathol.*, **20**, 665–671.

Müller, M.B. & Keck, M.E. (2002) Genetically engineered mice for studies of stress-related clinical conditions. *J. Psychiatr. Res.*, **36**, 53–76.

Müller, M.B., Zimmermann, S., Sillaber, I., Hagemeyer, T.P., Deussing, J.M., Timpl, P., Kormann, M.S., Droste, S.K., Kuhn, R., Reul, J.M., Holsboer, F. & Wurst, W. (2003) Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. *Nat. Neurosci.*, **6**, 1100–1107.

Plotsky, P.M. & Meaney, M.J. (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. *Brain Res. Mol. Brain Res.*, **18**, 195–200.

Plotsky, P.M., Thrivikraman, K.V., Nemeroff, C.B., Caldji, C., Sharma, S. & Meaney, M.J. (2005) Long-term consequences of neonatal rearing on central corticotropin-releasing factor systems in adult male rat offspring. *Neuropsychopharmacology*, **30**, 2192–2204.

Reichardt, H.M., Tronche, F., Bauer, A. & Schutz, G. (2000) Molecular genetic analysis of glucocorticoid signaling using the Cre/loxP system. *Biol. Chem.*, **381**, 961–964.

Reul, J.M. & Holsboer, F. (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr. Opin. Pharmacol.*, **2**, 23–33.

Richard, D. (1995) Exercise and the neurobiological control of food intake and energy expenditure. *Int. J. Obes. Relat. Metab. Disord.*, **19** (Suppl. 4), S73–S79.

Rinne, T., De Kloet, E.R., Wouters, L., Goekoop, J.G., DeRijk, R.H. & Van den Brink, W. (2002) Hyperresponsiveness of hypothalamic–pituitary–adrenal axis to combined dexamethasone/corticotropin-releasing hormone challenge in female borderline personality disorder subjects with a history of sustained childhood abuse. *Biol. Psychiatry*, **52**, 1102–1112.

Rivier, C. & Vale, W. (1983) Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. *Nature*, **305**, 325–327.

Scaccianoce, S., Muscolo, L.A., Cigliana, G., Navarra, D., Nicolai, R. & Angelucci, L. (1991) Evidence for a specific role of vasopressin in sustaining pituitary–adrenocortical stress response in the rat. *Endocrinology*, **128**, 3138–3143.

Schmidt, M.V., Enthoven, L., van der Mark, M., Levine, S., De Kloet, E.R. & Oitzl, M.S. (2003a) The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. *Int. J. Dev. Neurosci.*, **21**, 125–132.

Schmidt, M.V., Enthoven, L., Van Woezik, J.H., Levine, S., De Kloet, E.R. & Oitzl, M.S. (2004) The dynamics of the hypothalamic-pituitary-adrenal axis during maternal deprivation. *J. Neuroendocrinol.*, **16**, 52–57.

Schmidt, M.V., Oitzl, M.S., Levine, S. & De Kloet, E.R. (2002) The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. *Brain Res. Dev. Brain Res.*, **139**, 39–49.

Schmidt, M.V., Oitzl, M.S., Müller, M.B., Ohl, F., Wurst, W., Holsboer, F., Levine, S. & De Kloet, E.R. (2003b) Regulation of the developing hypothalamic-pituitary-adrenal axis in corticotropin releasing hormone receptor 1-deficient mice. *Neuroscience*, **119**, 589–595.

Smith, G.W., Aubry, J.M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., Sawchenko, P.E., Koob, G.F., Vale, W. & Lee, K.F. (1998) Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron*, **20**, 1093–1102.

Steckler, T. & Holsboer, F. (1999) Corticotropin-releasing hormone receptor subtypes and emotion. *Biol. Psychiatry*, **46**, 1480–1508.

Stenzel-Poore, M.P., Heinrichs, S.C., Rivest, S., Koob, G.F. & Vale, W.W. (1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. *J. Neurosci.*, **14**, 2579–2584.

Sutanto, W., Rosenfeld, P., De Kloet, E.R. & Levine, S. (1996) Long-term effects of neonatal maternal deprivation and ACTH on hippocampal mineralocorticoid and glucocorticoid receptors. *Brain Res. Dev. Brain Res.*, **92**, 156–163.

Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F. & Wurst, W. (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat. Genet.*, **19**, 162–166.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R. & Schutz, G. (1999) Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat. Genet.*, **23**, 99–103.

Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science*, **213**, 1394–1397.

Van Gaalen, M.M., Stenzel-Poore, M.P., Holsboer, F. & Steckler, T. (2002) Effects of transgenic overproduction of CRH on anxiety-like behaviour. *Eur. J. Neurosci.*, **15**, 2007–2015.

Viau, V., Chu, A., Soriano, L. & Dallman, M.F. (1999) Independent and overlapping effects of corticosterone and testosterone on corticotropin-releasing hormone and arginine vasopressin mRNA expression in the paraventricular nucleus of the hypothalamus and stress-induced adrenocorticotropic hormone release. *J. Neurosci.*, **19**, 6684–6693.

Watts, A.G. (1996) The impact of physiological stimuli on the expression of corticotropin-releasing hormone (CRH) and other neuropeptide genes. *Front. Neuroendocrinol.*, **17**, 281–326.

Xue, J., Li, G., Bharucha, E. & Cooper, N.G.F. (2002) Developmentally regulated expression of CaMKII and iGluRs in the rat retina. *Dev. Brain Res.*, **138**, 61–70.