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Site-specific overexpression of corticotropin-releasing hormone (CRH) in the mouse brain – modelling central CRH system hyperactivity

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Abbreviation list

A

A	Ambiguus nucleus
ACTH	Adrenocorticotropic hormone
AMY	Amygdala
AP	Anterior pituitary
ARH	Arcuate nuclei of the hypothalamus
AVP	Arginine vasopressin

B

BLA	Basolateral nucleus of amgdala
BNST	Bed nucleus of the stria terminalis
BSA	Bovine serum albumin

C

Cb	Cerebellum
CeA	Central nucleus of the amygdala
CNS	Central nervous system
CIP	Calf Intestinal Phosphatase
Con	Control
COR	CRH overexpression from the ROSA26 locus
COR-Cam	COR-CamKIIα
COR-Nes	COR-Nestin
CP	Choroid plexus
CRH	Corticotropin-releasing hormone
CRH-BP	CRH binding protein
CRH-R1	CRH type I receptor
CRH-R2	CRH type II receptor
CRH-Tg	CRH transgenic
CS	Conditioned stimulus
Ctx	Cortex

D

DEPC-H_2O	Diethyl pyrocarbonate-treated H_2O
del	Deletion band
DG	Dentate gyrus
DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
DT	Diphteria toxin
DTT	Dithiothreitol
DVC	Dorsal vestibular column

E

E	Epinephrine
E.coli	*Escherichia coli*
EDTA	Ethylendiamintetra acetate
EGTA	Ethylenglycoltetraacetate
EMFI	Embryonic mouse fibroblasts
ES cell	Embryonic stem cell
EtBr	Ethidium bromide

F

FCS	Fetal calf serum
FST	Forced swimming test

G

GI	Gastrointestinal
GR	Glucocorticoid receptor

H

h	Hour
HC	Hippocampus
Het	Heterozygote

Hom	Homozygote
HPA axis	Hypothalamic-Pituitary-Adrenal axis

I

icv	Intracerebroventricular
ISH	In situ hybridization
ISI	Inter-stimulus intervals

K

kDa	Kilodalton
Ki	knock in
KO	knock out

L

LC	Locus coeruleus
LD	Lateraldorsal nucleus of thalamus
LDT	Lateral dorsal tegmentum
LHA	Lateral hypothalamus area
LIF	Leukemia inhibitory factor
LP	Lateral posterior nucleus thalamus
LS	Lateral septum
LSi	Intermediate lateral septal nucleus
LSv	Ventral lateral septal nucleus
LV	Lateral ventral nucleus of thalamus

M

M	mol/l
MeA	Medial amygdaloid nucleus
MeAp	Medial nucleus of amygdala posterior part
min	Minute
MMC	Mytomycin C
MR	Mineralocorticoid receptor
MS	Medial septal nucleus
mt	Mutant

N

NaAc	Sodium acetate
NE	Norepinephrine
NP40	Nonidet P-40

O

OB	Olfactory bulb
OD	Optical density
OE	Overexpression

P

pA	polyadenylation sequence
PAG	Periaqueductal gray
PBN	Parabranchial nucleus
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Pontine gray
Pir	Piriform
POMC	Proopiomelanocortin
PPI	Prepulse inhibition
PVN	Paraventricular nucleus
PVT	Paraventricular nucleus thalamus

R

R26	ROSA26
RIA	Radioimmunoassay
RN	Retical nucleus
RT	Reticular nucleus
RNA	Ribonucleic acid
rpm	Rounds per minute

S

s	Second
SCP	Stresscopin
SDS	Sodium dodecyl sulfate
SON	Supraoptic nucleus
SRP	Stresscopin-related peptide
Sp	Spinal cord
SW	Slow wave
SVG	Sauvagine

T

TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEA	Triethanolamine
Thala	Thalamus
tRNA	Transfer RNA
Tris	Tris-(Hydroxylmethyl-) aminomethan
TST	Tail suspension test

U

u	Unit
UCN	Urocortin
UCN2	Urocortin 2
UCN3	Urocortin 3
URO	Urotensin I
US	Unconditioned stimulus

V

V	Voltage
v/v	Volume per volume
VL	Ventral lateral nucleus of thalamus
VMH	Ventromedial nucleus hypothalamus

W

WT	Wild type
wt	wild type band

X

X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. Introduction

1.1. Corticotropin-releasing hormone (CRH)

During recent years, a large body of evidence has emerged linking stressful life events with an increased vulnerability for affective and anxiety disorders. Stressful events often precede the onset of depression and stress has also been associated with the severity of the illness (Dunner et al., 1979) (Brown et al., 1987) (Hammen et al., 1992). Stress is a protective mechanism that allows the organism to react to threatening situations. Acute responses to stress are necessary in order to maintain homeostasis in the organism. However, chronic stress or dysregulation of this system can lead to mood and affective disorders, and other diseases. CRH has also been implicated in allostasis, the ability of an organism to maintain stability through change, as a critical component by which organisms actively deal with stress in their environment.

Results from experimental modulation of components of the hypothalamic-pituitary-adrenal (HPA) axis have helped to decipher specific roles for each of the receptors and ligands. Stress probably represents one of the main factors that lead to sustained hyperactivity of the HPA system and has been closely related to the etiology of depression. Stressful stimuli activate the HPA axis, which, together with other physiological responses, coordinate the behavioral response of the organism (Herman and Cullinan, 1997). Chronic stress or a chronic change in HPA axis activity can result in pathological consequences, such as psychiatric disorders. Depression and anxiety-related disorders are associated with chronic HPA axis deregulation (Holsboer and Barden, 1996). Treatment of depressed patients with anti-depressants restores HPA axis homeostasis, and this restoration is an important predictor for clinical improvement (Holsboer, 2000) (Holsboer and Barden, 1996).

CRH plays a pivotal role in the response of an organism to various stressors, coordinating neuroendocrine, autonomic, behavioral, and immunologic responses to stress (Dunn and Berridge, 1990b) (Holsboer, 1999) (Koob and Heinrichs, 1999) (Owens and Nemeroff, 1991) (Koob et al., 1993) (Pich et al., 1993). Rapid activation of CRH and its stimulation of the HPA axis constitute a primary system that promotes adaptive responses. Enhanced CRH signaling at various sites within the central nervous system (CNS) and activation of the HPA axis lead to enhanced attentive behaviors, suppressed reproductive and feeding behavior as well as physiological

changes that are aimed at mobilizing energy stores and redirecting oxygen and nutrients to sites of increased demand. Moreover, neuroendocrine studies strongly suggest that hyperactivity of central CRH circuits, resulting in a characteristic dysregulation of the HPA system, plays a causal role in the development and course of affective and anxiety disorders. The behavioral effects of centrally administered CRH can be reversed by CRH receptor antagonists and are independent of activation of the HPA axis. Furthermore, CRH receptor antagonists alone attenuate many of the behavioral consequences of stress, underscoring the role of endogenous CRH in mediating many stress-induced behaviors (Heinrichs et al., 1995).

For both experimental animals and humans, survival during chronic stress requires not only that increased secretion of corticosteroid hormones has to be maintained in the face of negative feedback control, but also that an additional corticosteroid response can be mounted to an incoming stressor when required. Such requirements dictate adaptive responses at all levels of the HPA axis (Checkley, 1996). However, continued and prolonged stress may disturb the HPA axis to such an extent that the negative feedback mechanisms are disrupted; and the adaptive responses of the HPA axis may then become maladaptive. For experimental animals, the pattern of HPA maladaptation depends both upon the nature of the chronic stressor, and upon the period and mode of stress application. Repeated homotypic stressors such as restraint, foot shock and intraperitoneal injections of hypertonic saline, are associated with consistent elevations of arginine vasopressin (AVP) mRNA expression in the parvocellular paraventricular nucleus (PVN) of the hypothalamus. CRH mRNA expression appears to be elevated only in those stress paradigms that are associated with conserved HPA responses to repeated activation, such as osmotic loading /dehydration or hypertonic saline injection (Aguilera et al., 2001). Some stress paradigms (e.g. adjuvant-induced arthritis) that continuously activate the HPA axis are actually associated with reductions in central CRH drive, but concomitant increases in AVP drive on the pituitary (Harbuz et al., 1997) (Shanks et al., 1998). The pattern of adrenocorticotrophic hormone (ACTH) release in chronic stress also depends upon the stressor, such that ACTH secretion may be maintained or reduced (Aguilera et al., 2001).

1.2. The CRH family of neuropeptides

CRH is a 41-amino acid polypeptide derived from a 196-amino acid protein precursor (Vale et al., 1981). The human CRH gene contains two exons separated by one intron in its 5' untranslated region (Shibahara et al., 1983). The rat, ovine and mouse CRH gene have the same organization (Roche et al., 1988), the coding region of the CRH precursor is in exon 2, the 24 amino acids in the N-terminal contain a typical signal peptide sequences which is commonly associated with neuropeptides that undergo secretion (Thompson et al., 1987).

CRH is widely recognized as the major hypophysiotropic hormone in the mammalian stress response. In response to stress, neural input to the hypothalamus signals for increased synthesis of CRH in the PVN of the hypothalamus, which is then transported axonally to the median eminence and released into the portal circulation. CRH acts on pituitary corticotropes to stimulate proopiomelanocortin (POMC) synthesis and ACTH release. ACTH then induces the adrenal cortex to secrete glucocorticoids. Glucocorticoids mediate a variety of physiological and metabolic responses that are important for the survival of the organism, including negative feedback at multiple levels of the HPA axis to return the system to homeostasis (Arborelius et al., 1999) (Figure 1).

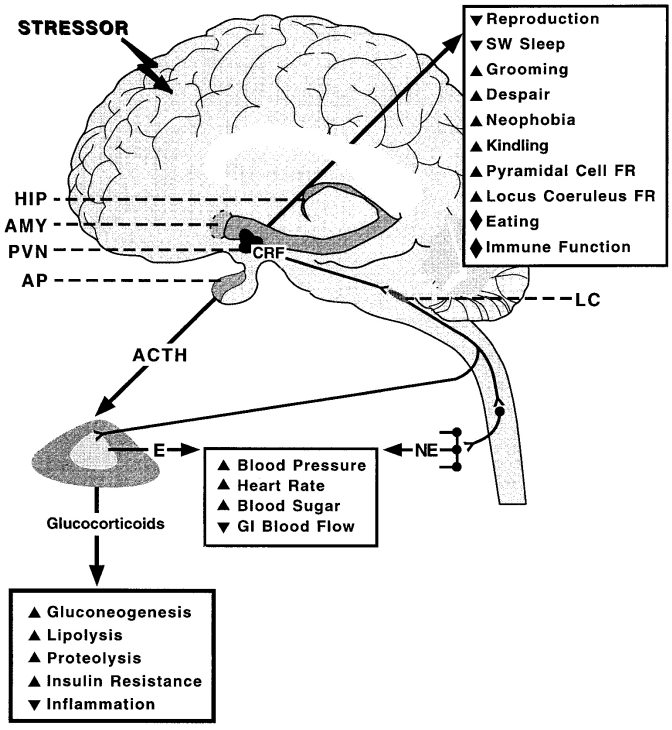

Figure 1. Schematic representation of the endocrine, behavioral, autonomic, and immunologic responses to stress mediated by central CRH neurons. ▲, increase; ▼, decrease; ♦, both increase and decrease. Abbreviations: AMY, amygdala; AP, anterior pituitary; E, epinephrine; HIP, hippocampus; LC, locus coeruleus; NE, norepinephrine; PVN, paraventricular nucleus; GI, gastrointestinal; FR, firing rate; SW, slow wave (picture adapted from (Arborelius et al., 1999)).

Histochemical studies indicate that CRH is distributed heterogeneously throughout the CNS and in peripheral tissues (Cummings et al., 1983) (Imaki et al., 1991) (Mezey and Palkovits, 1991) (Vamvakopoulos et al., 1990) (Figure 2). CRH has been found in the amygdala, bed nucleus of the stria terminalis (BNST), septum, Barrington's nucleus in the pons, cerebellum, cerebral cortex，and spinal cord in the brain. At these sites, CRH is thought to act as a neurotransmitter or neuromodulator, mediating a wide variety of responses including increased anxiety-like behavior, decreased food intake, enhanced learning, increased arousal, altered blood pressure, diminished sexual behavior and altered locomotor activity (Dunn and Berridge, 1990a) (Owens and Nemeroff, 1991). CRH was also found to express strongly in PVN of the hypothalamus in the brain. The hypothalamic CRH system is important for modulating endocrine and metabolic responses to stress while the amygdala, in concert with other extra-hypothalamic limbic regions has been important in manifesting the behavioral responses to stress (Davis et al., 1997b). In peripheral tissues, CRH is produced in the adrenal medulla, ovary, testis, heart, lung, liver, stomach, duodenum, pancreas, T-lymphocytes and placenta (Muglia et al., 1994) (Suda et al., 1984) (Herman et al., 1996).

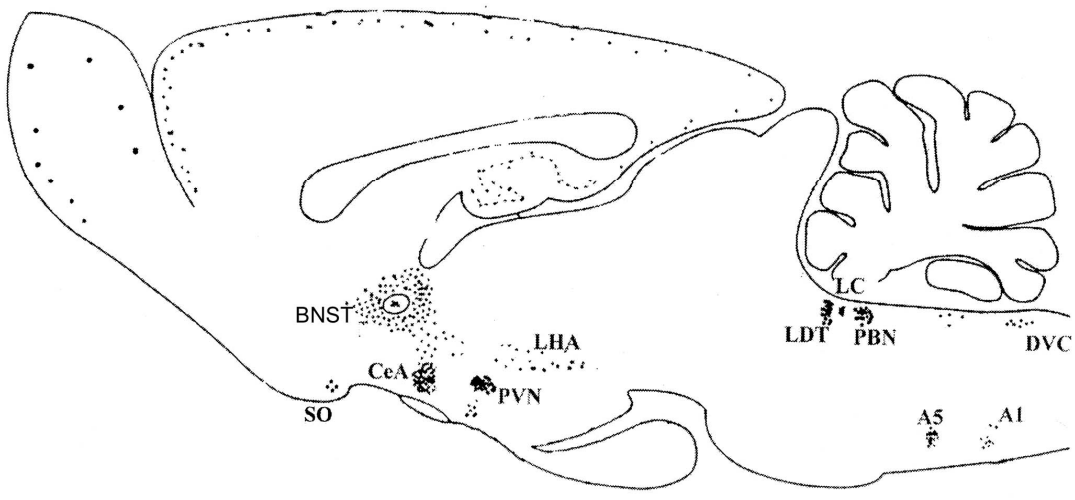

Figure 2. The distribution of CRH-immunoreactive cells in the rodent brain (picture adapted from (Smagin et al., 2001), data from (Behan et al., 1996) (Kozicz et al., 1998) (Yamamoto et al., 1998)). Abbreviations: A, nucleus ambiguus; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of amygdala; DVC, dorsal vestibular column; LC, locus coerulus; LDT, lateral dorsal tegmental; LHA, lateral hypothalamus area; PBN, parabranchial nucleus; PVN, paraventricular nucleus; SO, supraoptic nucleus.

Taken together, these results suggest that CRH not only controls the endocrine stress response, but also integrates the autonomic, behavioral, and immunologic responses to stress. Moreover, aberrant regulation of CRH expression and activity contributes to a number of psychiatric disorders including depression, anxiety disorders, and anorexia. Investigation on the expression and regulation of CRH activity is critical to our understanding of the pathophysiology of these clinical disorders (Salas et al., 1997).

CRH was first isolated and characterized from ovine hypothalamus in 1981 by Vale and colleagues (Vale et al., 1981). There is a high degree of homology between rat, mouse and human CRH genes (Herman et al., 1992) (Seasholtz et al., 1988) (Vamvakopoulos et al., 1990). Other CRH-like peptides were soon discovered in the urophyses of teleost fish (*Catostomus commersoni*) (Lederis et al., 1982) (Ichikawa et al., 1982) and skin of the tree frog (*Phyllomedusa sauvegei*) (Montecucchi and Henschen, 1981) (Broccardo et al., 1981). These peptides are known as urotensin I and sauvagine respectively, and exhibit ACTH releasing activity from pituitary with equal efficacy to human or rat CRH. However, it was until 1995 that a new CRH-like peptide was isolated and characterized in mammals by Vaughan and colleagues (Vaughan et al., 1995). This 40 amino acid amidated peptide, known as urocortin (UCN), shows 45 % amino acid identity to human CRH (Vaughan et al., 1995) and is a potent agonist for both receptors. *In situ* hybridization (ISH) and immuno-cytochemistry studies demonstrate a limited expression profile for UCN in the brain (i.e. Edinger Westphal, lateral septum, lateral superior olive, supraoptic nucleus of the hypothalamus, brainstem motor nuclei), with numerous sites of peripheral expression (Bittencourt et al., 1999) (Vaughan et al., 1995). In contrast to CRH, levels of UCN-immunoactivity are not high in the median eminence, suggesting that UCN is not an important endocrine factor in the regulation of ACTH or beta-endorphin release (Turnbull and Rivier, 1996). While intracerebroventricular (icv) administration of UCN has been shown to decrease food and water intake (Jones et al., 1998) (Spina et al., 1996), increase anxiogenic behavior (Jones et al., 1998) (Moreau et al., 1997), stimulate ACTH release (Vaughan et al., 1995), decrease mean arterial blood pressure (Vaughan et al., 1995), and inhibit injury-induced edema (Turnbull et al., 1996). The nucleotide sequence of the CRH gene is highly conserved throughout all vertebrates, including teleost fish, toad, chicken, golden hamster, rat, mouse, cow, sheep, pig, dog and humans. Recently, two new members of the CRH neuropeptide family have been

cloned: stresscopin-related peptide (SRP)/urocortin II (UCN2) and stresscopin (SCP)/ urocortin III (UCN3) (Hsu and Hsueh, 2001) (Lewis et al., 2001) (Reyes et al., 2001). SRP/UCN2 shows moderate to low similarity with human/rat CRH (34 %), human urocortin (43 %), SCP/UCN3 (37-40 %), urotensin I (34 %), and sauvagine (< 25 %). However, SCP/UCN3 is more distant from other members of the CRH family (18-32 % identity) (Figure 3). SRP/UCN2 mRNA is highly expressed in the paraventricular, supraoptic and arcuate nuclei of the hypothalamus (ARH), the locus coeruleus, and motor nuclei of the brain stem and spinal cord. In the periphery, SRP/UCN2 mRNA is detected in the heart, adrenal gland and peripheral blood cells (Reyes et al., 2001) (Hsu and Hsueh, 2001). The posterior part of the BNST, the lateral septum (LS) and the medial amygdaloid nucleus (MeA) are important brain sites that express SCP/UCN3 mRNA; high levels of SCP/UCN3 mRNA expression have been detected in the gastrointestinal tract, muscle, adrenal gland and skin. Their terminal fields innervate hypothalamic and brain stem areas matching CRH type 2 receptor (CRH-R2) distribution (Li et al., 2002). Because of their selectivity for the CRH-R2, UCN2 and UCN3 have been described as 'stress-coping' peptides (Hsu and Hsueh, 2001). Both peptides are capable of reducing anxiety, blood pressure and arousal. The ability of UCN2 to increase exploration on the elevated plus maze (Valdez et al., 2002) strongly suggests a role for the CRH-R2 in mediating anxiolytic effects, which suggest that CRH and UCN are two anti-parallel stress systems that function as organizers of the sympathetic and parasympathetic response respectively.

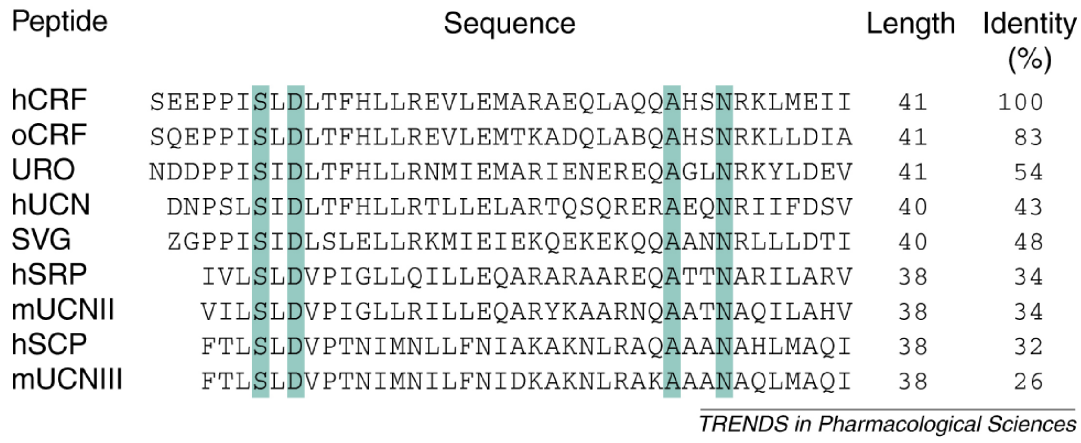

Figure 3. Alignment of members of the CRH family of neuropeptides. The amino acids that are homologous between the CRH peptides were boxed. Abbreviations: h, human; m, mouse; o, ovine; SVG, sauvagine; URO, urotensin I (picture adapted from (Dautzenberg and Hauger, 2002)).

1.3. CRH receptors

Since CRH was first characterized, a growing family of ligands and receptors have been indentified. The mammalian family members include CRH, UCN, UCN2, and UCN3, along with two receptors – the CRH type 1 receptor (CRH-R1) and the CRH type 2 receptor (CRH-R2), and a CRH binding protein (CRH-BP). In addition to HPA axis, CRH acting upon receptors at hypothalamic and extra-hypothalamic sites mediates behavioral and autonomic responses to stress (Holmes et al., 1987) (Vale et al., 1983a). Excessive or deficient production of CRH can result in psychiatric disorders, alterations of growth, reproductive and immune systems. It is hypothesized that CRH exerts its pathophysiological effects by acting upon receptors in the limbic system and autonomic areas of the brain, and also by causing inappropriate control of glucocorticoid secretion.

The two classes of CRH receptors were identified in 1993 (Chen et al., 1993) (Perrin et al., 1993) (Perrin et al., 1995) (Kishimoto et al., 1995) (Lovenberg et al., 1995) (Stenzel et al., 1995). They are the products of distinct genes which all contain seven trans-membrane helical domains and they show 70 % amino acid identity, but differ considerably in their pharmacology and tissue distribution. Several CRH-R1 and CRH-R2 splice variants have been described. The CRH-R1 gene expresses multiple subtypes (Ross et al., 1994) (Grammatopoulos et al., 1999) (Pisarchik and Slominski, 2001), which are produced by differential exon splicing. Similar exon rearrangements have been described for the calcitonin and parathyroid hormone/parathyroid hormone-related peptide receptors. Each CRH-R1 variant has a defect in its expression, binding or signalling characteristics. The CRH-R1α is a 415-amino acid protein, containing seven hydrophobic α-helices that are predicted to span the plasma membrane. CRH-R1α is widespread both within the CNS and periphery (Chen et al., 1993). The human CRH-R1β (hCRH-R1β) carries a 29 amino acid insertion in its first intracellular loop (Chang et al., 1993) and only weakly couples to the stimulatory G protein (Xiong et al., 1995). The hCRH-R1γ, in which exon 3 of the hCRH-R1 gene (Xiong et al., 1995) has been deleted by exon-skipping, is lacking 40 amino acids in its first intracellular loop domain (Ross et al., 1994). This variant is a low-affinity receptor. The hCRH-R1δ is lacking 14 amino acids in the C-terminal part of the seventh trans-membrane helices due to the deletion of exon 13 (Grammatopoulos et al., 1999). In addition, a CRH-R1 variant from sheep (oCRH-

R1var) has been described (Myers et al., 1998). This receptor differs from the normal oCRH-R1 in the C-terminal part of the seventh trans-membrane helices and the fourth intracellular loop. Both hCRH-R1δ and oCRH-R1var bind CRH with high affinity but are unable to stimulate the accumulation of intracellular cyclic AMP. Finally, a CRH-R1 mutant was cloned from the rat (Chen et al., 1993), the receptor is a truncated 224 amino acid receptor protein lacking all sequences C-terminal to the third trans-membrane helices. No data on the pharmacology of this receptor is available yet. The CRH-R1 splice variants seem to be expressed at low levels and may not be of physiological relevance. While CRH-R2 gene expresses three known subtypes 2α, 2β and 2γ and one truncation mutant (Liaw et al., 1996) (Valdenaire et al., 1997) (Kostich et al., 1998) that differ in their N-termini due to alternative splicing. The splice variants have a common 377 amino acid C-terminal part, whereas the extreme N-termini, which are encoded by one or two exons (Dautzenberg and Hauger, 2002), differ substantially from one another. The CRH 2α receptor, a 411-413 amino acid protein, has been isolated from mammalian and amphibian species (Dautzenberg et al., 1997) (Liaw et al., 1996) (Liaw et al., 1996) (Palchaudhuri et al., 1999). In contrast, CRH 2β receptors, which are 430-438 amino acids in size, have only been reported in mammals (Kishimoto et al., 1995) (Lovenberg et al., 1995) (Palchaudhuri et al., 1999) (Valdenaire et al., 1997). Finally, the 397 amino acid CRH 2γ receptor is exclusively found in humans (Kostich et al., 1998) (Palchaudhuri et al., 1999). In addition to the functional splice variants, a truncated CRH 2α mutant (CRH 2avar) has been identified in the rat (Miyata et al., 1999). This receptor is lacking all sequences C-terminal to the third transmembrane helices. CRH 2avar binds CRH but not sauvagine or urocortin, and is unable to stimulate cAMP production. The physiological role of this receptor variant needs to be determined. Because of its existence in mammalian and amphibian species and its higher degree of sequence conservation between the species (Palchaudhuri et al., 1999), it is suggestive that the CRH 2α receptor arose very early during evolution. The CRH 2β receptor, which is conserved to a lower extent, has developed in mammalian species only and thus probably arose later in evolution than the CRH 2α receptor. Finally, the CRH 2γ receptor because of its restriction to humans has probably occurred very late during evolution.

The CRH-R1 is expressed in anterior pituitary corticotropes and the intermediate lobe of the pituitary, and is thus thought to be the primary mediator of CRH-induced

ACTH release. The many CNS sites of CRH-R1 expression include the neocortex, olfactory and hippocampal cortices, cerebellum, septum, amygdala, and brainstem sensory relay structures (Potter et al., 1994). The CRH-R2, exhibits an mRNA expression profile which is distinct from CRH-R1. CRH-R 2β in rodents is found largely in the periphery (i.e. heart skeletal muscle, intestine, lung, kidney, and epididymis, but also choroid plexus (CP) and cerebral arterioles). In the rat, CRH-R 2α is expressed primarily in the CNS, including olfactory bulb, lateral septum, medial amygdala, dorsal and median raphe, and ventromedial and medial preoptic nuclei of the hypothalamus (Van Pett et al., 2000) (Chalmers et al., 1995). However, in primate species, CRH-R2 is more widely distributed and occurs in higher densities in cortical regions. None of the forms of CRH-R2 are expressed in the pituitary, suggesting that CRH-R1 is the key receptor for CRH in the pituitary (Chalmers et al., 1995). The different distribution is complementary to that of CRH-R1 and has been suggested to underline a putative function dissociation between the CRH receptor subtypes.

More recently, in the diploid catfish species, a third CRH receptor (CRH-R3), encoding a 428-amino-acid protein, has been identified (Arai et al., 2001). This novel CRH receptor is structurally closer to catfish CRH-R1 (85 %) than CRH-R2 (80 %), and binds CRH with a 5-fold higher affinity than urotensin I and sauvagine. CRH-R3 in the catfish is expressed in the pituitary gland, urophysis and brain. This multiplicity of receptor subtypes and ligands provides for diversity of receptor expression and signaling.

In addition to the distinct anatomic profiles of CRH-R1 and CRH-R2, the receptors also differ in their pharmacological properties. CRH-R1 has high affinity for UCN and CRH, binding both peptides with similar affinity, while not showing any affinity for UCN2 or UCN3 (Vaughan et al., 1995). In contrast, CRH-R2 has a significantly higher affinity for UCN, UCN2 and UCN3 than for CRH (Vaughan et al., 1995). The higher affinity of CRH-R2 for UCN coupled with anatomic studies that identified several potential sites of UCN/CRH-R2 interactions suggested that UCN is the endogenous mammalian ligand for CRH-R2 (Vaughan et al., 1995). However, CRH-R1 also has a very high affinity for UCN, suggesting that UCN could also mediate effects through CRH-R1 at sites of UCN/CRH-R1 co-localization (Bittencourt et al., 1999) (Vaughan et al., 1995). Therefore, some of the functions originally attributed to CRH may in fact be mediated, at least in part, by UCN or other CRH-like ligands.

The specific tissue distribution and pharmacological properties of these receptors suggests that distinct CRH pathways exist, thus providing separate routes to direct adaptive changes. CRH-R1 deficiency in mice leads to a decrease in CRH and UCN signaling, resulting in a significantly impaired HPA axis and decreased anxiety-like behavior (Smith et al., 1998) (Timpl et al., 1998). CRH-R1 deficient mice showed increased exploration of the open arms on an elevated plus maze and they spent more time in the brightly lit compartment of a light-dark transition box than did wild-type (WT) control mice. This pattern of behavior has been suggested to reflect a reduced level of anxiety in CRH-R1 knock out (KO) mice (Contarino et al., 1999) (Smith et al., 1998) (Timpl et al., 1998). One study found that AVP mRNA and protein levels in the PVN were elevated in the CRH-R1 deficient mice, thus suggesting that in the absence of CRH-R1, AVP V1a receptors maintained a compensatory activation of basal HPA axis hormone levels (Muller et al., 2000). Other studies examining these mice, however, have detected no increases in AVP protein or mRNA expression in the PVN in the absence of CRH-R1 (Smith et al., 1998) (Bale et al., 2002). CRH mRNA and protein levels were elevated in the absence of CRH-R1, which was partially due to decreased endogenous corticosterone negative feedback, as glucocorticoid replacement diminished the increased CRH expression in these mice (Smith et al., 1998) (Bale et al., 2002). This observation also suggests an additional regulatory mechanism for CRH expression that might involve CRH-R2. CRH-R1 KO mice had normal baseline levels of plasma ACTH, probably due to a maintenance of ACTH levels by AVP through AVP receptors (Turnbull et al., 1999). Taken together, these results support the notion that CRH-R1 plays an important role in the expression of stress-like behavioral responses and that blockade of this receptor may lead to reduced baseline anxiety-like states.

The conditional CRH-R1 (exon 9-13) KO mice (*Crhr1*$^{loxp/loxp}$*Camk2α-cre*) was generated in 2003 (Muller et al., 2003), in which CRH-R1 function is inactivated postnatal in anterior forebrain and limbic brain structures, but not in the pituitary. *Crhr1*$^{loxp/loxp}$*Camk2α-cre* mutants showed reduced anxiety, and the normal basal activity of their HPA system. Moreover, conditional mutants were hypersensitive to stress, corticotropin and corticosterone levels remained significantly elevated after stress. All those results suggested that limbic CRH-R1 modulates anxiety-related behavior and that this effect is independent of HPA system function.

Lack of CRH-R2 also resulted in reduced CRH and UCN signaling in the CNS and periphery. CRH-R2 deficient mice exhibited impaired cardiovascular function and altered feeding after food deprivation or UCN administration (Bale et al., 2000) (Coste et al., 2000) (Kishimoto et al., 2000). Compared to WT mice, none of CRH-R2 KO mice showed any alteration in baseline locomotor activity levels, but exhibited significantly reduced grooming behavior in a novel open-field suggesting that CRH-R2 is involved in adaptations to stress and supporting a role for this receptor in maintaining allostasis. An increase in stress-induced ACTH and corticosterone levels was observed in the mutant mice (Bale et al., 2002) (Coste et al., 2000), and basal levels of these hormones did not appear to be affected by the gene deletion. Interestingly, no differences were found in basal CRH mRNA or protein levels in the PVN in CRH-R2 KO mice (Bale et al., 2000) (Coste et al., 2000). However, Vale and colleagues have found increased expression levels of AVP in the PVN, which may be augmenting the CRH response in these mice and thus resulting in the increased sensitivity and hormone levels detected (Bale et al., 2002).

The response to stress in mice deficient for both CRH receptors (CRH-R1/2 KO) has also been examined. In the absence of either known receptor, mice displayed remarkably little HPA axis response to a restraint stress (Bale et al., 2002) (Preil et al., 2001). ACTH and corticosterone levels following restraint stress were significantly lower in the CRH-R1/2 KO mice compared to CRH-R1 KO mice suggesting a possible role of CRH-R2 in mediation of HPA-axis sensitivity (Bale et al., 2002).

The response to stress involves initiation, maintenance and recovery processes, both CRH and CRH-R1 are critical to initiate HPA activation in response to stress (Smith et al., 1998) (Timpl et al., 1998). It is possible that CRH-R2 also modifies the stress response following initial activation of the HPA axis.

The development of possible therapeutically relevant CRH receptor antagonists has become a current focus in drug discovery for treatment of stress-related disorders, such as depression and anxiety. Several small-molecular CRH-R1 antagonists have been produced and studied in recent years. CRH-R1 antagonists NBI-27914 (Chen et al., 1996) and NBI-30775 (formerly R-121919) (Oshima et al., 2003) have shown promising results in treatment of stress-related behaviors in rodents. Chronic treatment of mice with NBI-30775 caused complex changes in hippocampal serotonergic neurotransmission that may reveal molecular mechanisms of CRH-R1 involvement in the development of depression.

1.4. CRH-binding protein (CRH-BP)

The CRH-BP is a 37-kDa secreted glycoprotein that binds human CRH with an equal or greater affinity than the CRH receptor, and is expressed in rodent and primate brain and pituitary (Potter et al., 1991) (Potter et al., 1992) (Potter et al., 1994) (Chen et al., 1993) (Cortright et al., 1995). In humans, CRH-BP was found in plasma, amniotic fluid, placenta, pituitary and brain, where it inactivates CRH and has been proposed to prevent inappropriate pituitary-adrenal stimulation during pregnancy (Potter et al., 1991). In contrast, CRH-BP was not found in rodent plasma, rat and mouse CRH-BP have been detected only in the brain and pituitary.

The association of CRH with its binding protein forms a dimer complex and is thought to modulate the endocrine activity of CRH (Lowry et al., 1996). Recombinant CRH-BP has been shown to block CRH-induced adrenal ACTH secretion from rat anterior pituitary cells (Potter et al., 1991). CRH-BP has also been detected in brain regions not associated with CRH activity, suggesting that it may also have CRH-independent actions.

1.5. Arginine vassopressin (AVP)

CRH neurons are the principal regulators of the HPA axis and are present in the parvocellular subdivisions of the PVN. The decapeptide AVP is primarily a neurohypophysial hormone and is coexpressed in some of these CRH neurons. AVP is released into the portal circulation with CRH in response to stress and potentiates CRH-induced ACTH secretion. Thus, AVP and CRH are both endogenous releasing peptides for ACTH with AVP acting as a second ‘releasing factor’ for ACTH along with CRH. However, most vasopressinergic neurones are present in the magnocellular subdivisions of the PVN and the supraoptic nucleus. In certain conditions, increases in vasopressin of magnocellular origin could facilitate pituitary ACTH secretion (Holmes et al., 1986) (Irvine et al., 1989). However, prolonged activation of the magnocellular vasopressinergic system during chronic osmotic stimulation decreases rather than increases pituitary ACTH responsiveness (Dohanics et al., 1990) (Chowdrey et al., 1991).

Acute stress stimulates release of CRH and AVP from the median eminence into the pituitary portal circulation and increases expression of both peptides in parvocellular neurons of the PVN (Plotsky et al., 1991) (Plotsky, 1991). In addition,

in vivo and *in vitro* studies have shown that the rate of release of immunoreactive AVP from median eminence terminals increases in response to repeated or chronic stress (de Goeij et al., 1991) (Aguilera et al., 1993). Even a transient activation of hypothalamic CRH neurons by a single stressor can cause long lasting increases in AVP coexpression, irrespective of the nature of the stressor, which in most cases is not accompanied by changes of CRH (Schmidt et al., 1997). Basal levels of CRH and AVP expression are under feedback inhibition by glucocorticoids, and AVP transcription is highly sensitive to glucocorticoid inhibition.

1.6. Corticosteroid receptors

Glucocorticoids (corticosterone in rodents, cortisol in primates) are synthesized in the adrenal cortex and secreted from the gland into the circulation, which allows them to act on many different cell types throughout the body (Bartlett and Miller, 1956) (Tronche et al., 1998). Two closely related corticosteroid receptors have been identified so far, the type I mineralocorticoid receptor (MR) and the type II glucocorticoid receptor (GR) (Arriza et al., 1987) (Hollenberg et al., 1985).

In general, GR has a much broader expression pattern than does MR, the GR is found throughout the brain, with high density in the limbic system (hippocampus, septum) and in the parvocellular neurons of the PVN, and is also found in relatively high concentrations in the ascending monoaminergic neurons of the brain stem, prefrontal cortex, nuleaus accumbens, bed nucleus of the stria terminals, and central nucleus of the amygdala. During stress, corticosterone levels may increase about 100-fold and GRs get occupied by corticosterone. Their main function in brain is to suppress stress-induced hyperactivity of the HPA axis at the level of the PVN, anterior pituitary, but also at the hippocampal level (Joels and De Kloet, 1992). Thus, it has been suggested that the adaptive function of the HPA axis is critically dependent on glucocorticoid feedback mechanisms to dampen the stressor-induced activation of the HPA axis and to shut off further glucocorticoid secretion (Jacobson and Sapolsky, 1991). The MR is found only in the brain and in epithelial cells of the kidney, the colon, and exocrine glands (Kretz et al., 2001) (Reul et al., 2000). Moreover, MR and GR appeare to be co-localized in abundance in limbic neurons including hippocampal CA1 and dentate gyrus (DG) (van Steensel et al., 1996). Co-localization occurres also in the nuclei of amygdala and medial prefrontal cortex areas

that have an important function in emotion and cognition (Helm et al., 2002). In the hippocampus, the glucocorticoid concentration determines which receptor is activated. Low concentrations are believed to activate only MR, and higher concentrations are believed to activate both MR and GR (Reul and de Kloet, 1985). Pharmacological and antisense-RNA studies confirmed the hypothesis that MR in the hippocampus inhibits basal HPA axis activity by influencing CRH and AVP secretion from the PVN, whereas hippocampal GR acts the opposite (Dittmar et al., 1998) (Ratka et al., 1989). GR exerts inhibitory action in the PVN and the pituitary, which prevents an overreaction of the HPA axis under stress conditions and during the diurnal peak.

In behavior, central MR activation stimulates autonomic outflow (Van den Berg et al., 1994) (van den Buuse et al., 2002), in the spatial learning tests MR affects interpretation of environmental information and selection of the appropriate behavioral response to deal with the stressor. Dysregulation of GR function has been associated with human depression and anxiety disorder. Blockade of brain GRs impairs the storage of new information (Oitzl and de Kloet, 1992) (Sandi et al., 1997). Likewise, mutant mice with a point mutation in GR, which obliterates binding to DNA, were unable to store learned information (Oitzl et al., 2001). Transgenic mice with down-regulated GR (knock down) showed also cognitive defects and elevated plasma ACTH and corticosterone concentrations in response to stress. GR overexpression in the forebrain resulted in increased anxiety- and depressant-like behavior. No changes in basal plasma ACTH and corticosterone levels in GR overexpression mice were detected either in the morning nor in the evening. Moreover, GR overexpressing mice showed no differences from WT mice in corticosterone levels immediately and 10 min after the elevated plus maze test, a mild stressor. The cellular data in various limbic regions suggest that MR prevents disturbance of homeostasis, while GR promotes its recovery.

Glucocorticoid levels undergo a diurnal rhythm reaching peak levels before the onset of the active phase of the organism (in the morning for humans and in the evening for rodents).

1.7. CRH gene targeting mice

Gene targeting in mouse embryonic stem cells and mice has been used widely to study *in vivo* functions of genes during delevopment and in adult life. The efforts to

identify and characterize genetic/environment interactions in the regulation of stress have followed two general directions in rodent studies: the evaluation of stress-related behaviors in animals that have gained by classic transgenesis or lost one particular gene function by knock out or antisense approaches.

CRH knock out (KO) mice were generated in 1995 by Muglia and colleagues (Muglia et al., 1995). In terms of behavioral effects, CRH KO mice appeared to be nearly indistinguishable from genetically unaltered WT control mice. Baseline locomotor, exploratory, stereotypic, startle, and operant learning behaviors were unaffected by CRH gene deletion (Dunn and Swiergiel, 1999) (Weninger et al., 1999). Interestingly, deletion of the CRH gene seemed to alter certain endocrine measures, normal levels of ACTH were found in the pituitary and plasma, but the circadian pattern for this hormone was disrupted in CRH knockouts (Muglia et al., 1997) (Muglia et al., 2000). Stress-induced activation of the HPA axis was preserved in mice without the CRH gene, albeit the level of activation was significantly decreased (Jacobson et al., 2000). It is possible that this relative sparing of HPA axis function is related to increases in PVN AVP mRNA that were seen in CRH KO mice (Muglia et al., 2000).

To gain more insight into the relationship between central CRH hyperactivity and neuroendocrine, automonic, physiological, and behavioral changes associated with chronic stress, two CRH-overexpressing mouse lines have been developed. The first one was generated in 1992 by Stenzel-Poore and colleagues (Stenzel-Poore et al., 1992). In these mice, a metallothionine (MT) promoter, which drives wide-spread gene expression throughout the brain and the peripheral organs, was used; gene overexpression was thus achieved not only in brain regions where CRH is endogenously found, but also in other CNS regions and in peripheral organs (testis, heart, and lung) where CRH is not endogenously found. These mice have been found not only to display the behavioral effects associated with acute CRH administration, but also to show marked long-term alterations in endocrine and immune function that are associated with disease states involving hypercortisolemia. Thus, over-production of CRH in the CNS clearly led to elevated ACTH release and increased circulating glucocorticoid levels. Compared to WT mice, basal ACTH levels were elevated 3-fold and corticosterone levels were increased 10-fold. The high circulating corticosterone levels of CRH transgenic (CRH-Tg) mice resulted in a striking, physical phenotype that resembles Cushing's syndrome: thin skin, hair loss, brittle

bones, truncal obesity and a characteristic buffalo hump (Stenzel-Poore et al., 1992). CRH-Tg mice reverted to a normal phenotype upon adrenalectomy and subsequently survived for months without corticosterone replacement. These mice also showed a number of physiologic and behavioral features that parallel those seen in stress, and thus are particularly well suited to explore the consequences of altered CRH system activation as occurs during chronic stress. In addition, overexpression of the CRH gene also resulted in a marked decrease in immune functioning, as evidenced by overall reductions in cell numbers and tissue weight in immune system organs such as the spleen and thymus gland and a failure to mount immunoglobulin antibody responses to immune challenge (Bremner et al., 1997). Behaviorally, CRH-Tg mice exhibited a profile that was consistent with increased levels of stress, such as reduced baseline and stress-induced exploration of a novel environment, and decreased activity and time spent in the open arms of an elevated plus maze (Stenzel-Poore et al., 1994). These effects were potently blocked by administration of the CRH receptor antagonist α-helical CRH. CRH-Tg mice also showed a profound decrease in sexual behaviors and significant deficits in learning (Heinrichs et al., 1996) (Heinrichs et al., 1997).

The second CRH overexpressing mouse line (CRH-OE2122) was generated in 2002 by Olivier and colleagues (Dirks et al., 2002). In CRH-OE2122 mice, CRH was expressed under the control of the Thy-1 promotor, which drives constitutive transgene expression in neurons in postnatal and adult brain (Morris and Grosveld, 1989) (Vidal et al., 1990) (Moechars et al., 1996) (Luthi et al., 1997) (Wiessner et al., 1999) resulting in central CRH overexpression only. Increased amounts of CRH peptide and CRH mRNA were observed throughout the CNS in transgenic mice. CRH immunoreactivity, especially in the PVN and CeA, was increased significantly compared with WT mice, increased amounts of CRH were also present in the BNST. These changes in hypothalamic CRH content were accompanied by increased plasma corticosterone levels (4.4-fold) and adrenal gland hypertrophy, whereas basal plasma ACTH concentrations were not increased significantly in CRH-overexpressing mice (Groenink et al., 2002). This could suggest that the adrenal cortex of the CRH-OE2122 mice was hyper-responsive to ACTH. Moreover, CRH-OE12122 mice clearly exhibited significant changes including increased body temperature and heart rate as well as food and water intake. However, the CRH-OE2122 mice had no Cushing-like phenotype despite elevated plasma corticosterone levels. Only after 6

months did increase fat deposition and hair loss become apparent. In CRH-OE2122 mice, chronic CRH hyperactivity was associated with reductions in startle reactivity, disturbed habituation of the acoustic startle response, and impaired prepulse inhibition. The latter two abnormalities are also observed in schizophrenia patients.

1.8. Conditional mutagenesis in the mouse

The ability to modify the mouse genome by random integration of transgenes or at predetermined sites by homologous recombination in embryonic stem (ES) cells has greatly advanced our understanding of mammalian gene function in health and disease (Jaenisch, 1988) (Capecchi, 1989). These techniques allow us to introduce a permanent genetic alterations into the mouse germ line, but not to manipulate the genome in a spatio-temporally controlled manner *in vivo* and therefore there are some of the problems inherent to these technologies, such as embryonic lethality, possible compensatory effects during development, and difficulties in interpreting phenotypes caused by interactions of multiple tissues carrying the mutation. In case of random integration, it is difficult to know how many copies and where the transgene is integrated in the genome.

To circumvent these limitations of conventional gene targeting, conditional mutagenesis has become an important tool for investigating the function of a gene in a specific tissue/cell at a defined period of time (Metzger and Feil, 1999) (Nagy, 2000) (Hadjantonakis et al., 1999). Gain-of-function as well as loss-of-function can be engineered with the Cre-loxP system. The system allows for the inactivation or overproduction of a selected gene in a spatially and temporally controlled manner (Gu et al., 1994). There are two components in the system which permit an evaluation of gene function within genetically modified animals in a tissue-specific manner: one is the site-specific recombinase, such as *Cre* recombinase, which is a 38-kDa bacteriophage P1 protein, binds to the 34-bp DNA sequences loxP target sequences and catalyzes the recombination between the two loxP sites in the same orientation without the need for any cofactors (Abremski et al., 1983). A “floxed” allele, a gene in which two loxP sites in the same orientation introduced by homologous recombination in ES cells (Thomas and Capecchi, 1987) flank one or more important exons of the gene of interest, which permits subsequent recombination and gene disruption coincident with expression of the appropriate recombinase.

Recombination occurs only in cells expressing *Cre* recombinase. Expression of a site-specific/cell-type-restricted or inducible recombinase is achieved either through a tissue or cell-restricted transgene or by inserting the recombinase cDNA into a genetic locus with a known tissue expression profile. For example, in transgenic *CamKIIα-Cre* mice (Minichiello et al., 1999), the *Cre* recombinase expression was driven by the *calcium /calmodulin-dependent kinase IIα (CamKIIα)* promoter. In this mouse line, the pattern of *Cre* recombinase expression matches the expression of the endogenous *CamKIIα* gene. At or after P20, *Cre* recombination is specifically observed in the forebrain including pyramidal neurons of the hippocampus (strong staining in CA1) and anterior forebrain neocortex, striatum, and amygdala. To another *Cre*-expressing transgenic mouse line, *Cre* recombinase expression is driven by the rat *nestin* (*Nes*) promoter and enhancer. In these mice, *Cre* recombinase is expressed in neuronal and glia cells from embryonic day 7.75 (Tronche et al., 1999) (Dahlstrand et al., 1995).

In such experimental outlines, it is necessary to monitor if *Cre* activity at desired time points or if the recombination takes place in the desired region, this can be achieved by crossing *Cre* transgenic mice to a *Cre* recombinase dependent reporter mouse line. For example the ROSA26 (R26) reporter mouse line, which has been generated using the previously trapped ROSA26 locus (Friedrich and Soriano, 1991). It is known that the proviral ROSA26 allele transcribes the βgeo gene as early as the morula/blastocyst stage (Mao et al., 1999). This reporter line has been used to monitor *Cre* expression in transgenic mice since it was first shown to be successful in the Soriano laboratory (Soriano, 1999). In this line, the native ROSA26 gene drives expression of the LacZ enzyme upon *Cre* recombination. The resulting transgenic mice consequently express LacZ only in the cells that express *Cre* recombinase (Soriano, 1999). The recombination efficiency will depend on the level of *Cre* expression and thus might vary between different mouse strains.

Tissue- or cell-specific gene disruption or overproduction is often used to circumvent embryonic or fetal lethality associated with complete somatic disruption, thus permitting an examination of gene function in the tissue of interest or at later developmental stages. However, one significant limitation of these present approaches is the liability to control the timing of *Cre*-mediated recombination, because gene disruption closely parallels the earliest expression profile of the chosen promoter. As a result, some groups developed inducible *Cre* mouse line mice, for example, Mantamadiotis and colleagues (Casanova et al., 2002) generated tamoxifen inducible

CamKIIα ERiCreER transgenic mice. They were able to induce the *Cre* recombinase expression by intraperitoneal injection or oral admistration of tamoxifen at the time they liked.

1.9. Aim of the thesis

The neuropeptide CRH functions as a key mediator of the responses to stress (Vale et al., 1981). In addition to its neuroendocrine effects, CRH acts as a neuromodulator in stress-associated limbic regions to propagate and integrate stress-induced behaviors, including anxiety-like behavior, arousal and motor function (Dunn and Berridge, 1990a) (Steckler and Holsboer, 1999). Chronic CRH hyperactivity is implicated in human stress-related and affective disorders, including major depression. To examine the effects of chronic CRH excess, two CRH transgenic mice were generated previously (Stenzel-Poore et al., 1992) (Dirks et al., 2002a). Due to their wide-spread expression pattern – whole body or entire CNS respectively, CRH overexpressing mice showed elevated ACTH and glucocorticoids levels resulting in symptoms of Cushing-like syndrome (Stenzel-Poore et al., 1992) (Groenink et al., 2002). Therefore, it can not be excluded that part of the CRH effects e.g. on anxiety-related behavior observed in these mice may be mediated via its chronic activation of the HPA-system. In order to circumvent these problems, we utilized the Cre/LoxP system – a highly flexible, reliable and reproducible system to generate a mouse model of central CRH hyperactivity that allows the in depth analysis of effects of CRH overexpression in different brain regions.

2. Materials and Methods

2.1. Materials

2.1.1. Buffers and Solutions

Buffers and solutions were prepared using Milipore Q-destilled water. Chemicals were purchased from Sigma, Roth, Merck unless indicated otherwise.

2.1.1.1. Buffers for electrophoresis

2.1.1.1.1. Buffers for DNA agrose Gel

0.5 M EDTA: 181.6 g EDTA-Na.2 H_2O
800 ml aqua dest H_2O
→ adjust pH to 8.0 with 1 M NaOH
→ adjust volume to 1 liter with aqua dest H_2O

2 M Tris/HCl: 242.2 g Tris
800 ml aqua dest H_2O
→ adjust pH to 7.5 with HCl
→ adjust volume to 1 liter with aqua dest H_2O

TAE buffer: 4.84 g Tris
1.142 ml Acetate acid
20 ml 0.5 M EDTA, pH 8.0
800 ml aqua dest H_2O
→ adjust pH to 8.3 with acetate acid
→ adjust volume to 1 liter with aqua dest H_2O

TBE buffer: 10.8 g Tris
5.5 g Boric acid
4.0 ml 0.5 M EDTA, pH 8.0
800 ml aqua dest H_2O
→ adjust pH to 8.3 with acetate acid
→ adjust volume to 1 liter with aqua dest H_2O

Loading buffer:

6 x Loading buffer:	1 g	Orange G
(Orange)	10 ml	2 M Tris/HCl, pH 7.5
	150 ml	Glycerol
	→ adjust volume to 1 liter with aqua dest H_2O	

6 x Loading buffer:	0.25 g	Bromophenol Blue
(Blue)	600 ml	Glycerol
	10 ml	2 M Tris/HCl, pH 7.5
	→ adjust volume to 1 liter with aqua dest H_2O	

2.1.1.1.2. Buffers for RNA Gel

10 x running buffer:	41.94 g	MOPS
	4.1025 g	NaAceticum
	20 ml	0.5 M EDTA, pH 8.0
	800 ml	DEPC-H_2O
	→ adjust pH to 7.4 with 2 N NaOH	
	→ adjust volume to 1 liter with DEPC-H_2O	

Loading buffer:	0.0025 g	Bromphenoblue
	4 ml	Formamid
	2 ml	Formaldehyd
	2 ml	10 x running Buffer
	→ adjust volume to 10 ml with DEPC-H_2O	

2.1.1.2. Buffers for Southern blot

Denaturation buffer:	100 ml	5 M NaOH
	300 ml	5 M NaCl
	600 ml	H_2O

Neutralization buffer:	250 ml	2 M Tris/HCl, pH 7.5
	300 ml	5 M NaCl
	10 ml	0.5 M EDTA, pH 8.0
	440 ml	H_2O

Transfer buffer:

20 x SSC	175.3 g	NaCl
	88.2 g	Na-citrate.2H_2O
	800 ml	aqua dest H_2O
	→ adjust pH to 7.4 with 1 M HCl	
	→ adjust volume to 1 liter with aqua dest H_2O	

Washing buffer:

2 x SSC, 0.1 % SDS:	100 ml	20 x SSC
	10 ml	10 % SDS
	→ adjust volume to 1 liter with aqua dest H_2O	

0.2 x SSC, 0.1 % SDS:	10 ml	20 x SSC
	10 ml	10 % SDS
	→ adjust volume to 1 liter with aqua dest H_2O	

2.1.1.3. Buffers for Mini-Southern blot in 96-Well-Tissue-Culture-Plates

Lysis buffer:	5 ml	1 M Tris HCl, pH 8.0
	10 ml	0.5 M EDTA, pH 8.0
	1 ml	5 M NaCl
	12.5 ml	20 % Sarcosyl

→ adjust volume to 500 ml with aqua dest H_2O

→ add 0.6 ml/12 ml (20 mg/ml stock) Proteinase K to lysis solution prior to use

Precipitation buffer: 0.15 ml of 5 M NaCl to 10 ml cold 100 % Ethanol

Restriction Digest Mix:	23.5 µl	H_2O
	3.0 µl	Restriction buffer
	1.2 µl	Spermidine (0.1 M)
	0.3 µl	RNase (10 mg/ml)

mix well, add 2 µl Enzyme (*EcoRV* or *ApaI*, 40 u/µl) to each well, final volume is 30 µl

2.1.1.4. Buffers for preparation of genomic DNA from tissues

SDS/Proteinase K buffer:	0.44 g	NaCl
	1 ml	1 M Tris/HCl, pH 8.0
	5 ml	0.5 M EDTA, pH 8.0
	10 ml	10 % SDS

→ adjust volume to 100 ml with aqua dest H_2O and store at room temperature (RT)

→ add 0.4 mg/ml proteinase K just prior to use

2.1.1.5. Buffers for X-Gal staining of cells and sections

10 x PBS:	80 g	NaCl
	2 g	KCl
	26.28 g	$Na_2HPO_4.12H_2O$
	2.4 g	KH_2PO_4
	800 ml	aqua dest H_2O

→ adjust pH to 7.4 with 1 M HCl

→ adjust volume to 1 liter with aqua dest H_2O

20 % PFA:	20 g	PFA

→ adjust volume to 100 ml with 1 x PBS, incubate at 65 ^{o}C overnight

20 % sucrose:	20 g	sucrose

→ adjust volume to 100 ml with 1 x PBS (containing 0.005 M EGTA, 0.001 M $MgCl_{2)}$

LacZ-Fix buffer:	20 ml	20 % PFA / PBS, pH 7.4
	0.5 ml	1 M EGTA
	0.1 ml	1 M $MgCl_2$

→ adjust volume to 100 ml with 1 x PBS

LacZ-Wash buffer:	0.2 ml	1 M $MgCl_2$
	0.2 ml	5 % Deoxycholate
	0.2 ml	10 % NP40

→ adjust volume to 100 ml with 1 x PBS

LacZ-Stain buffer:	2 ml	4 % X-Gal (in DEMF)
(prepare fresh)	10 ml	0.05 M potassium-ferrocyanide
	10 ml	0.05 M potassium-ferricyanide

→ adjust volume to 100 ml with LacZ-Wash buffer

2.1.1.6. Buffers for *in situ* hybridization (ISH)

Hybridization-mix: 50 ml formamide, 1 ml 2 M Tris/HCl, pH 8.0, 1.775 g NaCl, 1 ml 0.5 M EDTA, pH 8.0, 10 g dextransulphate, 0.02 g Ficoll 400, 0.02 g PVP40, 0.02 g BSA, 5 ml 10 mg/ml tRNA, 1 ml 10 mg/ml carrier DNA, 4 ml 5 M DTT.

This buffer can not be autoclaved and thus should be handled with care. Use DEPC-treated buffers. Store hybridization mix as 1 to 5 ml aliquots at –80 °C. It can be kept for at least one year.

5 M DTT/DEPC: 7.715 g DTT + 4 ml DEPC-H_2O

→ shake the FALCON tube until the powder is nearly solved

→ adjust volume to 10 ml with DEPC-H_2O

10 x TEA/DEPC-stock solution:	84 ml	TEA (1 M triethanolamine (Sigma T1377))
	300 ml	DEPC-H_2O

→ adjust pH to 8.0 with 32 % HCl

→ adjust volume to 500 ml with DEPC-H_2O, autoclave once

10 x Proteinase K buffer/DEPC:	500 ml	1 M Tris/HCl, pH 7.5
	100 ml	0.5 M EDTA, pH 8.0

→ adjust volume to 1 liter with DEPC-H_2O, autoclave once

Hybridization Chamber Fluid:	250 ml	formamide
	50 ml	20 x SSC
	200 ml	aqua dest H_2O

5 x NTE stock solution:	146.1 g	NaCl
	50 ml	1 M Tris/HCl, pH 8.0
	50 ml	0.5 M EDTA, pH 8.0

→ adjust volume to 1 liter with DEPC-H_2O, autoclave once

2.1.1.7. Buffers for transfection of embryonic stem (ES) cell

2 x BBS: 1.1 g BES
1.6 g NaCl
0.02 g Na_2HPO_4
→ adjust pH to 6.95 with 5 N NaOH
→ adjust volume to 100 ml with aqua dest H_2O, pass through 0.22 µM filter, store 1 ml aliquots at –20 °C.

2 M $CaCl_2$: 24.8 g $CaCl_2$
→ adjust volume to 100 ml with aqua dest H_2O, pass through 0.22 µM filter, store 1 ml aliquots at –20 °C.

10 mM Tris: 0.121 g Tris
80 ml aqua dest H_2O
→ adjust pH to 7.6 with 1 M HCl
→ adjust volume to 100 ml with aqua dest H_2O, pass through 0.22 µM filter, store 1 ml aliquots at –20 °C.

2.1.1.8. Buffers for Radioimmunoassay (RIA)

Lysis Buffer: 26.7 g NaH_2PO_4 (Merck 6346)
0.2 g NaN_3
2.0 g Calfalbumin (Roth 8076)
1.86 g EDTA (Triplex III) (Merck 8418)
1 ml TritonX100 (Sigma X-100)
400.000 KIE trasylol (Bayer)
800 ml aqua dest H_2O
→ adjust pH to 7.5 with 5 N NaOH
→ adjust volume to 1 liter with aqua dest H_2O

TEAF Buffer: 1 % formic acid with triethylamine, pH 3.0

2.1.2. Media

2.1.2.1. Media for *E.coli*

LB-medium:	10 g	Bacto-Trypton
	5 g	Bacto Yeast-extract
	10 g	NaCl
	→ adjust volume to 1 liter with aqua dest H_2O, autoclave once	

TY broth medium:	20 mg	Bacto-Trypton
	5 g	Bacto Yeast-extract
	6 g	NaCl
	800 ml	aqua dest H_2O
	→ adjust pH to 7.0 with NaOH	
	→ adjust volume to 1 liter with aqua dest H_2O, autoclave once	

TFB1:	2.94 g	postassium acetate
	4.75 g	manganese chloride
	7.4 g	potassium chloride
	→ adjust pH to 5.8 with 0.2 M acetic acide	
	→ adjust volume to 1 liter with aqua dest H_2O, filtrate to sterilize	

TFB2:	4.194 g	sodium-MOPS
	8.325 g	calcium chloride
	0.74 g	potassium chloride
	200 ml	Glycerol
	→ adjust volume to 1 liter with aqua dest H_2O, filtrate to sterilize	

2.1.2.2. Media for ES and Feeder cells

Growth Medium for ES cells:

500 ml	Dulbeccos' Modified Eagles Medium, high glucose, + Na-Pyruvate
75 ml	Gibco-ES Fetal Calf Serum (FCS) (inactivated for 30 min at 56 °C)
1 ml	β-Mercaptoethanol 500 x (Gibco) (aliquots –20 °C)
5 ml	Glutamine (aliquots at –20 °C)
90 µl	Leucocyte Inhibitor Factor (LIF) from Gibco for TBV2 cells

Wash Medium:

contains already all supplements, but only 10 % FCS and no LIF

Growth Medium for feeder cells:

500 ml	DMEM high glucose
57 ml	FCS from PAA
5.7 ml	Glutamine
5.7 ml	non-essential Amino Acids

2 x Freezing Medium for ES and feeder cells:

5 ml	FCS
3 ml	DMEM complete
2 ml	DMSO

2.1.3. Kit systems

QIAquick Gel extraction kit (250) (QIAgen)

QIApre spin midiprep kit (250) (QIAgen)

QIAfilter Plasmid Maxi kit (25) (QIAgen)

QIAprep spin Midiprep kit (250) (QIAgen)

RNeasy Clean up kit (QIAgen)

pCRII-TOPO cloning kit (Invitrogen)

Radioimmune assay kits (ICN Biomedicals)

Quick Ligation kit (NEB)

Wizard® Genomic DNA Purification Kit (Promega)

Megaprime DNA labeling system (Amersham Bioscience)

2.1.4. Primers for cloning and sequencing

Oligonucleotides were purchased from MWG (Ebersberg, Germany) and applied as primers for the following cloning PCR or Sequencing reactions.

Primers for cloning into pCR II-TOPO vector

Primer name	Sequence (5-prime to 3-prime)	Length of insert [bps]
Notch-5	TTAATTAACTCGACGGTATCG	282 bp
Notch-3	TTCGGCCGGCAGCTTGGACTT	
CRH-5	TATAAGCGGCCGCCATGCGGCTGCGGCTGCT	620 bp
CRH-3	TTAATTAATTGTGTGCTAAATGCAGAATC	
ROSA26-5 F	TTCCTGCTACCAGG	584 bp
ROSA26-5 R	ATAAGTGTGGGGCGGTGG	
ROSA26-3 F	GTTGAGCCACTGAGAATGG	610 bp
ROSA26-3 R	ACAAACACTTCTACATGTCAG	

Primers for genotyping

Primer name	Sequence (5-prime to 3-prime)	Length of PCR product [bps]
LacZ1	CGCCATTTGACCACTACC	650 bp
LacZ2	GGTGGCGCTGGATGGTAA	
CreF	GATCGCTGCCAGGATATACG	500 bp
CreR	CATCGCCATCTTCCAGCAG	
ROSA26-1	AAAGTCGCTCTGAGTTGTTAT	300 bp
ROSA26-2	GCGAAGAGTTTGTCCTCAACC	
ROSA26-1	AAAGTCGCTCTGAGTTGTTAT	600 bp
ROSA26-4	GGAGCGGGAGAAATGGATATG	

Primers for sequencing

Primer name	Sequence (5-prime to 3-prime)
Rs-Con3	GGAATGCAAGGTCTGTTGAA
Rs-Con4	ATCAAGCTGATCCGGAACC

2.1.5. Probes for Southern blot, Nothern blot and *In situ*

Probes for Southern blot

Probe	Restriction enzyme to isolate probe from plasmid	Length of probe [bps]
ROSA26-5	XhoI	180 bp
ROSA26-3	EcoRI	700 bp
Cre	XbaI/MluI	1500 bp

Probes for Nothern blot

Probe	Restriction enzyme to isolate probe from plasmid	Length of probe [bps]
CRH	EcoRI	700 bp
LacZ	EcoRI	800 bp

Probes for *in situ*

Probe	Orientation	Restriction enzyme for linearization	Vector	RNA polymerase
CRH	Antisense	SpeI	pCRII-TOPO	T7
CRH	Sense	EcoRV	pCRII-TOPO	Sp6
AVP	Antisense	HindIII	pCRII-TOPO	T7
AVP	Sense	XhoI	pCRII-TOPO	Sp6
CRH-R1	Antisense	XbaI	pCRII-TOPO	T7
CRH-R1	Sense	XhoI	pCRII-TOPO	Sp6
CRH-R2	Antisense	XbaI	pCRII-TOPO	SP6
CRH-R2	Sense	BamHI	pCRII-TOPO	T7
MR	Antisense	XhoI	pCRII-TOPO	SP6
MR	Sense	BamHI	pCRII-TOPO	T7
GR	Antisense	NotI	pCRII-TOPO	SP6
GR	Sense	HindIII	pCRII-TOPO	T7

2.1.6. Cell lines

Neomycin resistant embryonic mouse fibroblasts (EMFI) feeder cell (provided by S. Bourier, IDG/GSF).

TBV2 (129 SvP) ES cell (provided by S. Bourier, IDG/GSF).

2.1.7. Animals

All animals were housed with a 12 h/12 h light-dark cycle and allowed to access food and water *ad libitu*m. *CamKIIα-Cre* mouse line and *Nestin-Cre* mouse line were obtained from R. Kuehn of IDG/GSF and R. Klein of MPI of Neurobiology, Martinstried (Tronche et al., 1999) respectively.

2.2. Methods

2.2.1. DNA preparation

2.2.1.1. Preparation of plasmid DNA

E.coli cells containing plasmid DNA were usually grown, if not differently specified, in autoclave sterilized LB-medium with a selective specific antibiotic, ampicillin (100 µg/ml) or kanamycin (30 µg/ml), for overnight at 37 °C. Mini- (5 ml culture), medium- (25 ml culture) and large- (100 ml culture) scale preparations of plasmid DNA were carried out by means of the respectively Plasmid Mini-, Midi- and Plasmid Maxi-Kit from QIAgen, according to the provided enclosed protocol. Elution from the column was performed with water.

To elute large DNA fragments (>15 kb) from the QIAgen column, pre-warmed (70 °C) QE buffer was applied.

2.2.1.2. Preparation of genomic DNA

2.2.1.2.1. Mouse genomic DNA preparation from tail

1-1.5 cm of mouse tail tips from at least 3-week old mice were cut. They were used directly or were frozen at –20 °C until used. DNA was prepared with the Wizard Genomic DNA Purification kit from Promega according to the enclosed protocol (see 2.1.3).

2.2.1.2.2. Mouse genomic DNA preparation from tissue

Tissue was dissected, chopped into pieces and placed it in 1 ml of SDS/proteinase K buffer (see 2.1.1.4.) per 100 mg of tissue. The mixture was pipetted up and down several times. Lysed tissue was shaked for 30-45 min gently at 50 °C and pipetted up and down from time to time, then 25 µg/ml RNAase was added for 15-20 min at 37 °C. 1 vol of phenol /chloroform (1:1, v/v) was added to the clear lysate, vortexed for 1 min and centrifuged for 5 min at 8,000 g. The supernatant was transfered to a new tube and this step was repeated once more. The step once was repeated with chloroform/isoamylalcohol (24:1, v/v) alone. The supernatant was transfered to a new tube. 0.1 vol of 3 M sodium acetate and 2.5 vol of pre-cooled pure ethanol were added to the aqueous phase, mixed gently but thoroughly and centrifuged at 8,000 g for 15 min at 4 °C. The supernatant was removed carefully and washed the pellet with 75 % Ethanol, centrifuged again for 2 min, aspirated off the ethanol and repeated this step

four times. The pellet was dried in air and resuspended the pellet thoroughly in TE buffer. To facilitate solubilization, DNA was shaken gently at 65 °C for several hours.

2.2.2. RNA preparation

Total RNA was isolated from ES cells using TRIZOL (Chomczynski and Sacchi, 1987). In brief, cells were lysed directly in a culture dish by adding 3 ml of TRIZOL reagent to a 10 cm diameter dish, and the cell lysate was passed several times through a pipette. The homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml TRIZOL Reagent was added. Sample tubes were capped securely. Tubes were shaked vigorously by hand for 15 s and were incubated at room temperature for 2-3 min. Then samples were centrifuged at 4,000 rpm for 25 min at 4 °C. Following centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The aqueous phase was transfered to a fresh tube, 0.5 ml of isopropanol per 1 ml of TRIZOL Reagent used for the initial homogenization to precipation RNA was added. Samples were incubated at room temperature for 10 min and centrifuged at 4,000 rpm for 10 min at 4 °C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube. The supernate was removed, the RNA pellet was washed once with 75 % Ethanol, adding at least 1 ml of 75 % Ethanol per 1 ml of TRIZOL Reagent used for the initial homogenization. The samples were mixed by vortexing and centrifuged at 4,000 rpm for 5 min at 4 °C. The RNA pellet was left in air for 20-30 min, then dissolved in RNase-free water by passing the solution a few times through a pipette tips, and incubated for 10 min at 60 °C.

2.2.3. Restrict digestion of DNA samples

Restriction digestion of DNA was performed for screening of plasmid clones and related orientation analysis, for Southern blot analysis of genomic DNA, and for isolation and preparation of DNA fragments in cloning procedures. Restriction enzymes from the following suppliers were used, Gibco BRL, Roche, New England Biolabs (NEB). Enzyme units to use were empirically determined for each reaction and working buffers were chosen in accordance to the information provided by the

suppliers. Incubations took place, if not differently specified, for a minimum time of 30 min up to overnight at 37 °C or at temperature recommended by the supplier.

2.2.4. DNA Gel Electrophoresis

Conventional gel electrophoresis for separation of DNA molecules in the range of 100 bp-20 kb was usually performed on 1 % agarose gels (Ultra Pure Agarose, Invitrogen BRL) in 1 x TAE or 1 x TBE buffer (see 2.1.1.1.1.). The agarose percentage was adjusted between 0.8 % and 2 % according to the desired separation range for specific purposes as described in the single cases. For separation of DNA molecules between 80 bp and 200 bp, 1.5-2 % agarose gels were performed. The Gel run was performed with variable time and voltage conditions according to the separation range and agarose percentage using an electrophoresis power supply (Pharmacia Biotech). For size comparison, a DNA molecular weight marker was loaded on the gel next to the samples (SmartLadder, Eurogentec, or 100 bp Ladder, Gibco BRL). DNA was stained with the intercalating fluorescent reagent ethidium bromide (EtBr), which was added in the gel before solidification at the concentration of 0.5 µg/ml. Stained DNA was visualized on a UV-transilluminator at a wavelength of 254 nm and photographed with a gel documentation apparatus.

2.2.5. Cloning and Transformation

2.2.5.1. Competent cells preparation and transformation

Chemically competent *E.coli* cells (DH5α) were prepared as following. One single colony from an LB-Agar plate was inoculated into 2 ml LB and grew overnight at 37 °C. On the next day, 500 µl of starter culture was transferred into 100 ml LB medium and shaked at 37 °C until cell density reached an optical density (OD600) between 0.4 and 0.6. Growth was stopped by placing the culture on ice for 15 min. The cells were subsequently centrifuged at 2,500 rpm for 5 min at 4 °C and the pellet was resuspended in 25 ml TFB1 (see 2.1.2.1.), left on ice for 1 h, cells were spun down at 2,000 rpm for 5 min at 4 ^{o}C, then were resuspended in 4 ml TFB2 (see 2.1.2.1.) and left on ice for 1 h before use. Finally, cells were split in 100 µl aliquots, snap frozen in liquid nitrogen and stored at –80 °C. For transformation, 100 µl cell aliquots were thawed on ice and incubated for 30 min on ice with up to 10 µl of a 20 µl ligation mix or 1 to 10 ng of plasmid DNA for retransformation.

For uptake of plasmid DNA, the cells were heat-shocked at 42 °C for 45 s and subsequently put on ice for 5 min, 950 µl SOC medium was added and cells were incubated shaking at 37 °C for 30 min-1 h. 100 µl of cells were plated on LB plates of supplemented with 15 % agar and the appropriate antibiotic for selection. Plates were then incubated overnight at 37 °C, if not differently required. Single colonies were picked and inoculated in LB medium with appropriate antibiotic and grown overnight at 37 °C for DNA mini-prep analysis (see 2.1.3.).

In the case of utilizing pCRII-TOPO TA cloning (Invitrogen), blue-white selection of the colonies was possible. For this purpose, LB-Agar plates were previously added with 40 µl of 40 mg/ml X-Gal in dimethylformamide (DEMF) solution. White colonies after 37 °C overnight incubation were picked for screening. Bacterial clones were stored as glycerol stocks at –80 °C (250 µl volume bacterial culture + 750 µl volume autoclaved 80 % glycerol). Blue-white selection is also possible for other vectors, for example, pBluescript II KS(+) and pBluescript II KS(-).

2.2.5.2. Cloning

PCR products with A-overhangs, obtained from amplification with Taq DNA polymerase, were cloned into the pCRII-TOPO vectors (Invitrogen) according to the provided protocol. Cloning of DNA fragments obtained from restriction digestion was accomplished into linearized plasmid vectors with compatible ends. If vector ends were compatible to each other, appropriate 5'-end dephosphorylation was executed by incubation with 1 unit of Alkaline Phosphatase (Calf Intestinal Phosphatase (CIP) (NEB)) for 1 h at 37 °C, with subsequent purification by means of the QIAquick Gel Extraction Kit (QIAgen) (see 2.1.3.) according to the provided protocol. Ligations were performed in a molecular ratio of insert and vector 6:1 using the quick ligation Kit (NEB).

2.2.6. Polymerase Chain Reaction (PCR)

This technique for DNA amplification has several different applications: e.g. production of DNA fragments for subcloning, checking of correctness of clone constructs, probe synthesis, gene expression analysis and genotyping of mice. According to the specific purpose, PCR amplifications are realized from various types of template: genomic DNA, first strand cDNA and cloned DNA, like plasmids.

Moreover, different types of DNA polymerases were chosen. In general, when no subcloning of the PCR product was required, a normal Taq polymerase was employed from Roche. In general, if not differently specified, PCR reaction mixes were set up as following:

10 µl	10 x PCR buffer,
2 µl	10 mM dNTP,
2 µl	10 pmol/µl each primer
50 ng	template DNA
0.5 µl	5 u/µl of Taq polymerase (Roche)
x µl	H_2O
100 µl	total volume

PCR reaction were performed with the following standard program

PCR-cycling:

1.	95 °C	5 min
2.	95 °C	30 sec
3.	57 °C	30 sec
4.	72 °C	1 min/kb
Repeated steps 2 to 4 for 30 to 35 times		
5.	72 °C	7 min
6.	4 °C	forever

If the PCR product was cloned, the proofreading (Pfu) DNA polymerase was used (Gibco, BRL), mixed with Taq polymerase (3:7).

2.2.7. Southern blot

2.2.7.1. Blotting of gels onto nylon membranes

This method was used for transferring DNA from agarose gel onto nylon membranes for subsequent hybridization with specific probes. Electrophoresis was executed at low voltage overnight (40 v for 19-24 h) to ensure a better separation of the bands. DNA was denatured by bathing the gel 2 x 15 min in denaturation buffer (not longer) (see 2.1.1.2.), which was bathed for 2 x 15 min in neutralization buffer (see 2.1.1.2.), equilibrated for at least 10 min in 20 x SSC (see 2.1.1.2.). Finally the right-up corner of the gel was cut and the nylon membrane was marked with necessary information with a pencil and also the right-up corner of the membrane was

cut. The nylon membrane (Hybond N+, Amersham) and Whatman 3 MM filter paper were prewetted in fresh 20 x SSC (see 2.1.1.2.). The gel (facing down) was blotted onto Whatman 3 MM filter paper, then the nylon membrane was put onto the Gel with alignment of the corners of Gel and membrane, then 2 x Whatman 3 MM paper were put onto the nylon membrane. Any air bubbles between the gel, the membrane and Whatman 3 MM filter paper were removed. The gel was blotted as depicted in figure 4 for overnight (12-16 h, not more than 24 h), and immobilized by UV cross-linking (UV Stratalinker® 2400; Stratagene).

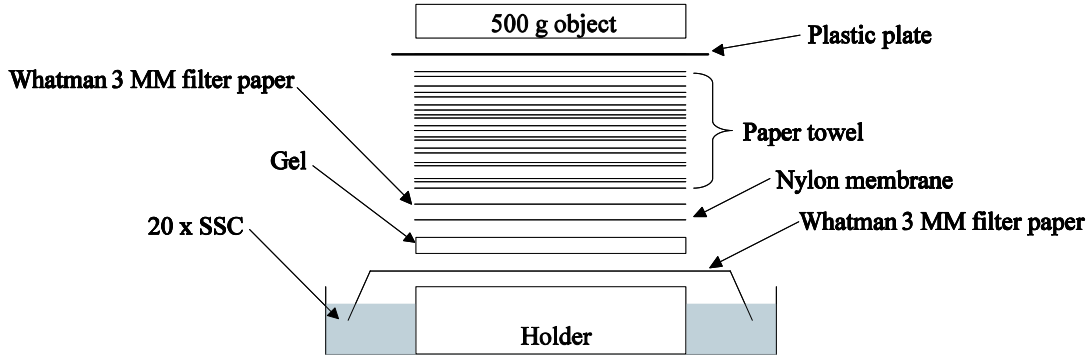

Figure 4 . Transfering DNA from agrose gel to Nylon membrane.

2.2.7.2. Probe labeling

25-50 ng of DNA probe were labeled with 50 µCi α -^{32}P-dCTP (Amersham) by means of the Megaprime DNA Labeling Kit (Amersham) (see 2.1.3.) according to the manufacture's instructions. The labeled probe was purified from free nucleotides through MicroSpin S-300 HR Columns (Pharmacia). The efficiency of labeling was checked by liquid scintillation counting of Ersicount 400.

2.2.7.3. Hybridization and washing

Prehybridization was carried out at 65 °C for 30 min in rapid-hyb buffer (Amersham). Radiolabeled linearized DNA probes were denatured for 5 min at 95 °C. Hybridization was carried out for 2-3 h at 65 °C in rapid-hyb buffer (Amersham). Blots were washed once in 2 x SSC, 0.1 % SDS for 20 min, in 0.2 x SSC, 0.1 % SDS for 2 x 15 min at 65 °C. Blots were exposed to a x-ray Kodak Biomax film with intensifying screens for 1-2 days at –80 °C. If the membrane was to be reused for hybridization with a different probe, the old probe was removed by bringing the

membrane to boil in 0.1 % SDS for 10 min and let it cool down to room temperature. After rinsing with 2 x SSC, the membrane was ready to be used again.

2.2.8. Colony Hybridization

Hybond N+ (Amersham) membranes were laid for 1 min onto agar plates with *E.coli* colonies for transfer. In the meanwhile they were marked with reference ink dots. Subsequently the membranes were laid twice facing up on denaturalization buffer (see 2.1.1.2.) for 10 min and similarly twice on neutralization buffer (see 2.1.1.2.) for 10 min, once on 2 x SSC for 5 min (the excessive solution was each time drained with Whatman paper to avoid washing off the colonies). Then they were let dry, immobilized by UV cross-linking (UV Stratalinker® 2400; Stratagene) and used for hybridization.

The following procedure was the same as for Southern blot (see 2.2.7.), but exposure time to the x-ray Kodak Biomax film was for only 30 min to 2 h.

2.2.9. Northern blot

Formaldehyde denaturing gels were prepared with a 0.8-1.0 % agarose concentration. Agarose was dissolved in 1 x MOPS buffer and cooled down to about 50 °C before addition of formaldehyde to a final concentration of 2.2 M. 5-10 µg RNA samples were diluted in 22 µl RNA loading buffer (see 2.1.1.1.2.) and heated up to 65 °C for 15 min in order to release RNA secondary structures. After running 4-6 h at 60-80 V in 1 x MOPS buffer, the gel was photographed, equilibrated in 20 x SSC for 15 min and blotted overnight (12-16 h, not more than 24 h) onto nylon membranes (Hybond N+, Amersham) as described for Southern blot (Figure 4). The RNA was immobilized by UV cross-linking (UV Stratalinker® 2400; Stratagene).

Hybridization and washing was carried out as described above for Southern blotting (see 2.2.7.), but hybridization time was at least 4 h or overnight.

2.2.10. *In situ* hybridization

2.2.10.1. Tissue preparation

All animal experiments were conducted in accordance with the guide for the care and use of laboratory animals of the government of Bavaria, Germany. 3-5 months old mice were anesthetized with Isoluran and brains were transcardially perfused with 4 % PFA.

To prepare cryosections, the brains were shock frozen on dry ice directly after removal, and used directly or stored at –80 °C prior to sectioning. Frozen brains were mounted on Tissue Tek (Polysciences, PA, USA), and 20 µM-thick consecutive sections were cut from brain regions of interest on a cryostat (Microtrome HM 560, Microm). Sections were mounted onto frozen SuperFrost/plus slides (Fisher Scientific), dried on a 37 °C warming plate and stored at –20 °C until used (for longer storage, sections were frozen into –80 °C).

To prepare paraffin sections, the brains were post-fixed in 4 % PFA for 4-5 h, then dehydrated by immersing the brain into series of Ethanol: 2 x 1 h in 70 % Ethanol, 2 x 1 h in 90 % Ethanol, 2 x 1.5 h in 95 % Ethanol, 2 x 1.5 h in 100 % Ethanol, 2-3 x 1.5 h in Xylol until the brains were clear. Then the brain was embedded with paraffin. Paraffin embedded brains were cut into 8 µm-thick sections on a microtome. Sections were mounted onto frozen SuperFrost/plus slides (Fisher Scientific), dried on a 37 °C warming plate and then the slides were put in 37 °C incubator overnight. Sections were stored at 4 °C until used.

2.2.10.2. Probe synthesis

Radioactive (^{35}S) labeled riboprobes were used. The majority of probes were generated from DNA as templates cloned into pCRII-TOPO vector with the T3, T7 or Sp6 promotor. The identity of all clones used for ISH was checked by sequencing. Linearized template DNA was precipitated by 1/10 vol 3 M NaAc and 2 vol 100 % Ethanol and resuspended in DEPC-H_2O at a concentration of 1 µg/µl. The template DNA was stored at –20 °C until used. For ^{35}S-labeled riboprobes, *in vitro* transcription was carried out for 3 h at 37 °C in a total volume of 42 µl mix as following:

x µl	linearized plasmid DNA template (1.5 µg DNA)
y µl	DEPC-H_2O
30 µl	total volume
3 µl	10 x transcriptions buffer
3 µl	NTP-mix (rATP/rCTP/rGTP 10 mM each)
1 µl	0.5 M DTT
1 µl	RNasin (40 u/µl; Promega)
3 µl	^{35}S-thio-rUTP (Amersham)
1 µl	T7, T3 or SP6 RNA polymerase (20 u/µl)
42 µl	total volume

Reactions were treated with 20 units of RNase free DNaseI (Roche Molecular Diagnostics) for 15 min at 37 °C, and labeled probes were purified by column exchange (Nucleotide removal kit, QIAgen). Suitable restriction enzymes (NEB, Roche) were used for linearization and the corresponding RNA polymerases T3, T7 or Sp6 (Roche Molecular Diagnostics) were used in order to get labeled antisense and sense riboprobes, respectively, of the cloned insert. In ISH experiments, sense controls did not give any detectable signals (data not shown), and antisense probes gave distribution patterns identical to those already published in rat or mouse.

2.2.10.3. *In situ* hybridization

For paraffin sections, slides were dewaxed in Rotihistol for 2 x 15 min, rinsed in 100 % Ethanol for 2 x 5 min, in 70 % Ethanol for 5 min, in DEPC-H_2O for 3 min, in PBS/DEPC for 3 min. The slides were postfixed in 4 % PFA/PBS (on ice) for 20 min, and rinsed in PBS/DEPC for 2 x 5 min, followed by proteinase K treatment in proteinase-K buffer (20 µg/ml) for 7 min. Slides were rinsed in PBS/DEPC again for 5 min, and fixed in 4 % PFA/PBS for 20 min.

For cryosections, slides were warmed up for at least 30 min at room temperature, fixed in ice-cold 4 % PFA/PBS for 10 min, washed them for 3 x 5 min in 1 x PBS.

Both kinds of slides were incubated for 10 min in 0.1 M triethanolamine-HCl (pH 8.0) to which 0.6 ml of acetic anhydride was added dropwise. Slides were rinsed in 2 x SSC for 2 x 5 min, dehydrated in graded series of Ethanol: in 60 %, 75 %, 95 % and 100 % Ethanol for 1 min respectively, delipidized in chloroform for 1 min, rinsed in 100 % and 95 % Ethanol for 1 min respectively again, and air-dried. Hybridization

was carried out overnight (12-16 h) at 57 °C in 90-100 µl of hybridization buffer (see 2.1.1.6.) containing the respective ^{35}S-labeled riboprobe (35,000-70,000 c.p.m./µl). After incubation in a humid chamber, slides were rinsed 4 x 5 min in 4 x SSC at room temperature, incubated for 20 min at 37 °C in 20 µl/ml of RNaseA in NTE buffer (see 2.1.1.6.), rinsed at room temperature in decreasing concentrations of SSC (2 x 5 min in 2 x SSC, 10 min in 1 x SSC, 10 min in 0.5 x SSC) containing 1 mM DTT, washed for 2 x 30 min at high stringency in 0.1 x SSC/1 mM DTT at 64 °C, 2 x 10 min at room temperature in 0.1 x SSC. At this point, ^{35}S-labeled slides were dehydrated in graded ethanol series: 30 %, 50 %, 70 %, 95 % and 2 x 100 % Ethanol in 300 mM NH_4OAC for 1 min respectively, air-dried and exposed to x-ray Kodak Biomax film (Kodak, Germany). On the next day, slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1:1 in distilled water). After exposure for 4-5 weeks at 4 °C, slides were developed for 3 min (D-19, Kodak) in developing solution, fixed for 6 min in fixing solution (Kodak fixer), rinsed for 25 min in tap water and air-dried. Slides were mounted in histofluid (Marienfeld, Lauda-Königshofen).

Semi-quantitative analysis of mRNA levels was performed blind to genotype using NIH Image (http://rsb.info.nih.gov/nih-image/). At least three serial tissue sections per animal and region were analyzed. In the case of CRH-R1, CRH-R2 and GR mRNA, CA1, CA3 and DG were analyzed.

2.2.11. ES and Feeder cells culture

2.2.11.1. Preparation of EMFI feeder cells plate

A frozen vial of EMFI cells was thawed quickly at 37 °C. 10 ml DMEM complete was added and centrifuged at 1,200 rpm for 5 min. The cell pellet was resuspended gently in 10 ml DMEM complete and cells were split onto 3 x 15 cm plates each containing a total of 25 ml DMEM complete. The cells were incubated at 37 °C, 5 % CO_2 for 3 days. Medium was removed from the plates and 15 ml DMEM complete containing 150 µl Mitomycin C (MMC) (1 mg/ml) was added, plates were swirled to ensure an even distribution of the medium, the cells were incubated at 37 °C, 5 % CO_2 for 2.5 h (not longer). The monolayer was washed twice with about 10 ml PBS and 7.5 ml trypsin/ EDTA was added to each plate, plates were incubated for about 5 min at 37 °C until the cells came off the plate, cell suspension was pipetted up and down for 3 times to break any cell aggregates, the cell solution was transfered to

complete medium in a FALCON tubes, containing at least the same amount medium as Trypsin volume. The cells were centrifuged and the pellet was resuspended in DMEM complete, 7.5 ml per 15 cm diameter plate, a cell suspension of 2.0 x 10^5 cells/ml was made. The cells were plated immediately on new dishes, containing DMEM complete, the best concentration was 1.0 x 10^4 cells/cm^2 for TBV2 cells. The feeder cells were allowed to attach overnight for best results or used at least 3 h later. The medium was changed to ES cell medium before adding ES cells. 1.5 x concentrated cell suspension was used for the Multi-well plates.

2.2.11.2. ES cell culture

The TBV2 ES cells were applied for electroporation of the targeting vector and functional analysis. One vial frozen ES cells was thawed and transfered to a FALCON tube containing 10 ml ES cell medium without LIF for washing, cells suspension was centrifuged at 1,200 rpm for 5 min, cells pellet was resuspended in 5 ml ES cell medium containing LIF and plated onto one 6 cm dish with EMFI cells, medium was changed next day. The cells was washed with PBS on the third day and 1.5 ml Trypsin/EDTA was added for 10 min until the cells began to get detached (37 °C, 5 % CO_2). The cells were gently pipetted up and down to get a single cell suspension, 10 ml of ES cell medium without LIF was added and the cells was centrifuged for 5 min at 1,200 rpm, supernatant was aspirated and cells were resuspended in ES cell medium with LIF. About 2 x 10^6 cells per 10 cm diameter dish were plated to a fresh 10-cm diameter feeder plate or on gelatinized plates with 0.1 % gelatin containing 9 ml ES cell medium (dilution 1:3) for freezing or further electroporation respectively.

2.2.12. Electroporation

ES cells were counted and prepared according to the calculation of 1 x 10^8 cells /electroporation/500 µl cold PBS. 120 µl of the linearized targeted ROSA26 construct DNA (120 µg) was added to cell suspension, not more than 900 µl totally (Vector linearization: digested with *SwaI,* concentration: 1 µg/µl). Cell suspension was transfered to the electroporation cuvette (BioRad cuvette). The cuvette was transfered into the cuvette holder with electrodes facing the output leads and electric pulse was delivered on 0.8 kV, 3 µF for the Bio Rad gene pulser about 0.1 msec (push both red pulse buttons). Cuvette was removed from the cuvette holder and left on ice for 10 to

20 min. The cell suspension was transfered from one cuvette very carefully into 12 ml ES cell medium containing LIF and distributed onto 12 x gelatinized with 0.1 % gelatin 10-cm diameter plates containing already 9 ml medium with LIF. Medium was changed the next day (supplemented with LIF). Two days after electroporation, the drugs for selection (G418 200 µg (inactive)/ml) (in addition to LIF) was added, one plate was prepared for control that would receive only G418 for selection. After about 6-8 days of selection, drug resistant colonies ($G418^r$) appeared and were ready for analysis. Colonies were picked into 96-well feeder cell coated plates and then resistant colonies were passaged until onto 2 x gelatinized and 2 x feeder cell coated 96-well plates, freezed 2 x feeder cell coated 96-well plates in –80 °C.

2.2.13. Screening for positive (homologously recombined) clones from Electroporated ES cells

When the cells were ready for DNA extraction procedure, the cell plates were rinsed twice with PBS and 50 µl lysis buffer per well was added (see 2.1.1.3.), the plates were incubated overnight at 50 °C in a humid atmosphere. The next day, the plates were spun down for 2 min at 2,500 rpm, then 100 µl per well of a mixture of NaCl and ethanol (150 µl of 5 M NaCl to 10 ml of cold absolute Ethanol) was added for precipitating DNA using a multichannel pipettor. The 96-well plate was being shaked for 30 min at room temperature, spun down again. The plate was inverted carefully to discard the solution, the nucleic acids was remained attached to the plate. The excess liquid was blotted on paper towels. The nucleic acids was rinsed 3 times by dripping 150 µl of 75 % Ethanol per well using the multichannel pipettor. The cell plates were spun down and the alcohol was discarded carefully by inverting of the plate each time. After the final washing, the plates were inverted and allowed to dry on the bench. The DNA was ready to be cut with restriction enzymes. 30 µl of restriction digest mix was added per well with a multichannel pipettor, the contents of the well was mixed using the pipette tip and the reaction was incubated at 37 °C for overnight in a humid atmosphere. Gel electrophoresis loading buffer was added to the samples and proceeded to conventional electrophoresis. DNA was transfered to Nylon membranes as described for Southern blot (Figure 4), immobilized by UV cross-linking (UV Stratalinker® 2400; Stratagene) and did Southern blot.

2.2.14. Transient transfection with pCre-Pac plasmid

The functionality of the strategy was tested in ES cells. In order to remove the transcriptional terminator sequence, targeted ES cell clones were transfected transiently with a *Cre*-recombinase expression plasmid – pCre-Pac plasmid (Taniguchi et al., 1998). Transfected clones were analyzed for LacZ reporter gene expression by X-Gal staining (see 2.2.17.). Cells were seeded the day before transfection onto 10-cm feeder cell dishes with 2 x 10^5 cells/dish. DNA mixture was prepared as following, distributed carefully and incubated it for 30 min at room temperature. DNA mixture was mixed under sterile conditions:

20 µg	DNA
x µl	10 mM Tris, pH 7.6
y µl	H_2O
450 µl	total volume
50 µl	$CaCl_2$ (2.5 M)
500 µl	2 x BBS
1000 µl	total volume

This DNA mixture was added drop-wise to the cells. The dish was tilt to ensure homogenous distribution of the precipitates and cultured on. In the following morning, cells were washed with PBS and medium was exchanged, from the third day, transfected clones were positively selected in puromycin (2 µg/ml) supplemented medium for 3-4 days. In the course of selection, specifically transfected cells were able to grow and formed dense islands. These clonal cell aggregates were picked in 96-well feeder cell coated plate and then the clones were passaged until onto 1 x 96-well plate coated with feeder cell and 1 x 96-well plate gelatinized with 0.1 % gelatin. 96-well feeder cell coated plate was frozen into –80°C, the gelatinized plate was used for X-Gal staining (see 2.2.17.).

2.2.15. Generation of conditional mutants

Mutant ES cells were used to generate chimeric mice by blastocyst injection. Chimeras were bred with C57BL/6J mice to obtain F1 offspring, and germ-line transmission of the mutant allele was determined by Southern blot analysis using the ROSA26 3' external probe after *ApaI* digestion. For the conditional overexpression of CRH in the brain, we used transgenic mice carrying the gene encoding *Cre*

recombinase under the control of the *CamKIIa* promoter (Minichiello et al., 1999) or under the control of the rat *Nestin* (*Nes*) promoter and enhancer (Tronche et al., 1999). To generate site specific CRH overexpression mice from the ROSA26 allel (COR) in the brain, we crossed mice harboring only one $R26^{neoCRH}$ allele with transgenic *CamKIIα-Cre* or *Nestin-Cre* mice to obtain COR-CamKIIα Cre (COR-Cam Cre) and COR-Nestin Cre (COR-Nes Cre) mouse lines respectively. Mice used for this study were kept on a mixed 129/Svp x C57BL/6J background. Genotyping was performed by PCR with ROSA26 specific primers, LacZ primers and *Cre* primers, and/or by Southern blot analysis of *EcoRV*- or *ApaI*-digested tail DNA using external probes and a *Cre* recombinase-specific probe.

2.2.16. X-Gal staining of mouse tissues

2.2.16.1. Tissues preparation

3-5 months old mice were anesthetized with Isofluran and perfused via intracardial perfusion using the LacZ-Fix buffer (see 2.1.1.5.). The brains were removed and incubated in 20 % cryoprotection sucrose for overnight.

Brains were frozen on dry ice for at least 15 min and mounted one brain on Tissue Tek (Polysciences, PA, USA), 100-µM thick consecutive sections were cut from brain regions of interest on a cryostat (Microtrome HM 560, Microm). Sections were put into 1 x PBS, which were used directly or were stored at 4 °C for at most two weeks until used.

2.2.16.2. X-Gal staining

Sections (or cell culture dishes) were immersed for 5 min in LacZ-Wash buffer (see 2.1.1.5.), and were then put in LacZ-Stain buffer (see 2.1.1.5.) at 37 ºC for up to 12 h (staining was checked from time to time), subsequently sections were washed in 1 x PBS for 6 x 10 min, fixed at least for 1 h in 4 % PFA/PBS (here storage possible at 4 ºC). Sections were dehydrated through series of Ethanol: 70 %, 80 %, 95 % and 2 x 100 % Ethanol for 30 min respectively, then the sections were transferred into BABB for about 20 min. Pictures were then taken directly.

2.2.17. Radioimmunoassay (RIA)

2.2.17.1. Extraction and Chromatographic Methods

Half brains and different brain regions were extracted with Sep-Pak C18 (Waters) by modification of Vale and colleagues (Vale et al., 1983b). For this reason cartridges were preconditioned with 6 ml TEAF (1 % formic acid with triethylamine, pH 3.0)/ acetonitrile (4/6, v/v) and 6 ml TEAF alone. Briefly, 2 ml lysis buffer (see 2.1.1.8.) was added for each sample, the samples were homogenated and then freezed at –80 ºC for overnight. Next day, 1 ml C18 Pulver (300 mg/ml) was added into new tube and then centrifuged for 5 min at 4,000 rpm, the supernatant was discarded and 3 ml TEAF (see 2.1.1.8.) was added, the supernatant was vortexed shortly and centrifuged in the same way as the above. The supernatant was discarded, 2 ml TEAF and 1 ml sample supernatant was added, the tubes were shaked it for 10 min, centrifuged again. Supernatant was discarded again, and this step was repeated with 2 ml TEAF for washing, then 3 ml TEAF/propanol (1:1, v/v) was added, tubes were shaked for 10 min and then centrifuged again, supernatant was taken to new tubes, stored in –80 ºC for overnight.

Next day, the TEAF/propanol solution was evaporated and extracted samples were suspended in assay buffer and measured by using a buffer standard curve.

2.2.17.2. Preparation of ^{125}I-CRH tracer

Rat CRH (rCRH) was labeled with 350 µl Ci ^{125}I within 3 s using 3 µg chloramines T resulting in a specific activity of the probe ranging from 40 to 70 µCi/µg. The iodine was separated from the labeled peptide by Sephadex G 10 column chromatography with 0.1 g /dl BSA in 0.1 N acetic acid.

Further purification was achieved by Sephadex G50 rechromagraphy (60 x 1 cm) which resulted in 2 peaks. The second peak was used as tracer in the radio-immunoassay. ^{125}I-Histidine-labeled CRH was used only for chromatographic studies and to follow extraction procedure. Since the ^{125}I-tyrosine tracer had no longer stability and was therefore more suitable for radioimmunoassay.

2.2.17.3. RIA protocol

The incubation volume consisted of 0.1 ml antibody in 0.05 M PO_4 buffer (pH7.4) with 5 mM EDTA, 400 kIU/ml Trasylol, 2 g/dl BSA. For direct measurement, CRH-

free serum was used for a standard curve (dose range 40-2,500 pg/ml), which was obtained by adsorption on Sep-Pak C18 cartilages. Preincubation was performed over 2 days. After 1 day of tracer incubation, bound/free separation was performed by the double antibody-PEG method. For direct measurement EDTA-plasma specimens with trasylol were used.

2.2.18. Endocrine analysis

Two weeks before the experiments, 3-7 months old animals were separated and housed singly to avoid uncontrolled stress reactions. All experiments and data analyses were performed only with male animals. To determine the basal hormone plasma levels, mice (n=10-12 per genotype) were left undisturbed throughout the night before the experiment. Blood sampling was collected in the early morning (07:00–09:00 am) by collecting trunk blood from rapidly decapitated animals, with the time from first handling of the animal to completion of bleeding not exceeding 1 min. Blood was collected in ice-cooled Eppendorf cups containing Potassium-EDTA.

Plasma was separated by centrifugation (10,000 g for 15 min at 4 °C). Supernatants were stored at –20 °C until assayed. Plasma corticosterone and ACTH concentrations were measured in duplicate by commercially available Radioimmunoassay kits (ICN Biomedicals).

2.2.19. Behavioral studies

2.2.19.1. Acoustic startle response (ASR)

Animals: 6-8 weeks old male animals were housed singly in a temperature-controlled (21-22 °C) room under a 12 h light/dark cycle (light on at 6:00 am). All testing occurred from 10:00 am to 1:00 pm and was conducted in accordance with the principles of laboratory animal care.

Apparatus: Startle chambers (SR-LAB; San Diego Instruments, San Diego, CA) consisted of nonrestrictive 5 cm in diameter Plexiglas cylinders resting on a Plexiglas platform in a ventilated chamber. High-frequency speakers mounted 33 cm above the cylinders produced all acoustic stimuli, which were controlled by SR-LAB software. Piezoelectric accelerometers mounted under the cylinders transduced movements of the animal, which were digitized and stored by an interface and computer assembly. Beginning at startling stimulus onset, 65 consecutive 1 msec readings were recorded

to obtain the peak amplitude of the animals' startle response. A dynamic calibration system was used to ensure comparable sensitivities across chambers. Sound levels were measured as described previously using the A weighting scale in units of decibels sound pressure level (Dulawa and Geyer, 2000). The house light remained on throughout all testing sessions.

Peptide infusions: The selective CRH-R1 antagonist NBI-30775 (also known as R-121919) was diluted in 5 % cremophor-sterile water and 2 mg/kg and 20 mg/kg dose were administered (icv) using a free-hand method in lightly anesthetized mice as described previously (Pelleymounter et al., 2002) (Risbrough et al., 2003).

Acoustic startle: For all acoustic startle sessions, the intertrial interval between stimulus presentations averaged 15 sec (range of 7-23 sec). A 65 dB background was presented continuously throughout the session. After placement into the startle chambers, a 5 min acclimation period preceded testing. Then 80 startle stimuli of varying intensities were presented, with the duration of 40 msec and variable inter-stimulus intervals (ISI) of 20-30 sec. The following stimulus intensities were used: 65, 80, 90, 100, 110, 120 dB noise.

Habituation: After an 5 min acclimation period, 100 startle stimuli of 110 dB noise were presented (duration 40 msec), with an interval of 15 seconds.

Prepulse inhibition: Two different procedures were used, one examined the effects of prepulse with varying intensities on PPI, and the other was designed to study the effects of different intervals between the onset of the prepulse and that of the startle stimulus on PPI. In the first procedure, the first block tested acoustic startle response only and included three different acoustic stimulus intensities: 90, 105 and 120 dB. After a 5 min acclimation period, startle stimuli (120 dB, 40 msec) were preceded by noise prepulse (20 msec) of 2, 4, 8 and 16 dB above background, with the inter-stimulus interval (100 msec) from onset of the prepulse to onset of the pulse. In the second procedure, after a 5 min acclimatization, prepulses (120 dB noise, 20 msec) preceded the startle stimuli (110 dB noise, 40 msec), with intervals (onset-onset) varying from 0 to 1080 msec (0, 20, 70, 120, 360 and 1080 msec). The trial with no interval between prepulse and startle stimulus was considered as a stimulus-alone trial.

Four to ten days before drug testing, mice were tested briefly to measure baseline acoustic startle response and PPI. The mice were then assigned to drug groups (i.e. counterbalanced) so that all drug groups averaged similar startle response levels and PPI performance before drug testing. Startle pulses were 40 msec in duration,

prepulses were 20 msec in duration, and prepulses preceded the pulse by 100 msec (onset-onset).

Data analysis: In all experiments, the average startle magnitude over the record window (i.e., 65 msec) was used for all data analysis. Either a two-way ANOVA as the between-subject factor, or prepulse-pulse intensity as within-subject factor, was used. Post hoc analysis followed significant main or interaction effects as appropriate. Unless specified otherwise, PPI data presented were collapsed across prepulse intensities, and startle reactivity data were shown at the 120 dB startle intensity.

2.2.19.2. Fear conditioning

Experiments were approved by the Committee on Animal Health and Care of the local governmental body of Bavaria.

Animals: All animals (n=10-12 mice per genotype) were housed singly under standard laboratory conditions and were maintained on an inversed 12 h light-dark cycle (lights off between 8:00 am and 8:00 pm) with food and water ad *libitum*. Mice were separated at least 2 weeks before starting the experiments.

Fear conditioning: animals were placed into conditioning chambers (ENV-307A; MED Associates, St. Albans, VT, USA) that was equipped with a shock floor and placed into a sound-attenuated isolation cubicle. Context- and tone-dependent fear conditioning were performed as described previously (Kamprath and Wotjak, 2004). Briefly, training consisted of exposure of the mice to a conditioning context (3 min) followed by a 20-s tone (sine wave, 9 kHz, 80 dB) that co-terminated with a 2-s electric foot shock (0.70 mA). Animals were returned to their home cages 60 s after shock application. The test for contextual memory was performed 24 h later by re-exposing the mice to the conditioning chamber for 3 min without tone presentation. Two hours later, mice were placed in a novel context (Plexiglas cylinder) for 7 min with a re-exposure to the tone for 3 min starting 3 min after insertion into the context. The test of tone-dependent freezing was repeated 5 and 20 days later. All sessions were videotaped. Freezing behavior (characterized as immobility except for respiratory movements) was analyzed off-line by trained observers that were blind to the genotype (Kamprath and Wotjak, 2004). The duration of freezing, which served as a measure of fear-related memory, was normalized to the respective observation periods.

Data analysis: data were analyzed by a one-way ANOVA using Graphpad software. Significance between groups was further analyzed using one of the following post hoc tests (Tukey, Bonferroni, Newman Keul). $P<0.05$ was considered as statistically significant.

2.2.19.3. Forced swimming test (FST)

Experiments were approved by the Committee on Animal Health and Care of the local governmental body of Bavaria.

All animals (n=10-12 mice per genotype) were housed singly under standard laboratory conditions and were maintained on an inversed 12 h light-dark cycle (lights off between 8:00 am and 8:00 pm) with food and water ad *libitum*. All experiment testing sessions were conducted between 1:00 pm and 7:00 pm and were performed with the experimenter unaware of the animals' genotype.

To facilitate adaptation to novel surroundings, 2-3 months old male mice were transported to the testing room at least 12 h before testing. Briefly, swim sessions were conducted by placing mice in glass cylinders (16 cm in diameter, 30 cm high) containing 24-26 °C or 32 °C warm water at a depth of 15 cm. A 6-min test duration was used, and the water was changed between subjects. All test sessions were recorded by a video camera and the behavior was scored by a trained observer who was blinded to genotype. Immobility was defined as the state in which mice were judged to be making only the movements necessary to keep their heads above water. Struggling was defined as any vertical movement in which mice were breaking the water surface with their front paws.

Data (from forced swimming test and tail suspension test) were analyzed by one-way ANOVA using Graphpad software (Graphpad Software Inc). Significance between groups was further analyzed using one of the following post hoc tests (Tukey, Bonferroni, Newman Keul). $P<0.05$ was considered as statistically significant.

2.2.19.4. Tail suspension test (TST)

Experiments were approved by the Committee on Animal Health and Care of the local governmental body of Bavaria.

All animals (n=10-12 mice per genotype) were housed singly under standard laboratory conditions and were maintained on an inversed 12 h light-dark cycle (lights

off between 8:00 am and 8:00 pm) with food and water ad *libitum*. All experiment testing sessions were conducted between 1:00 pm and 7:00 pm and were performed with the experimenter unaware of the animals' genotype.

The TST for antidepressant activity was conducted in a slightly modified version of the one previously described (Holmes et al., 2002) (Cryan and Mombereau, 2004). All mice were adapted to novel surroundings as described for the FST. Mice were securely fastened with medical adhesive tape by the tip (1.0-1.5 cm) of the tail to a round metallic bar and suspended 30 cm above the ground. The presence or absence of immobility, defined as the absence of limb movement, was scored over a 6-min test session by a highly experienced observer who was blinded to genotype.

3. Results

3.1. Generation of mice conditionally overexpressing CRH

3.1.1. Generation of targeting construct

To investigate potential effects of site-specific CRH overexpression in the brain, we utilized the properties of the retrovial-trapped ROSA26 (R26) locus (Figure 5A). The ROSA26 locus has proven as a useful tool to achieve ubiquitous expression during development and in the adult (Zambrowicz et al., 1997). We introduced a single copy of the murine CRH (mCRH) cDNA (0.6 kb fragment) into the ROSA26 locus (Soriano, 1999) (Figure 5B) generating a R26 knock in (Ki) allele ($R26^{neoCRH}$) (Figure 5C).

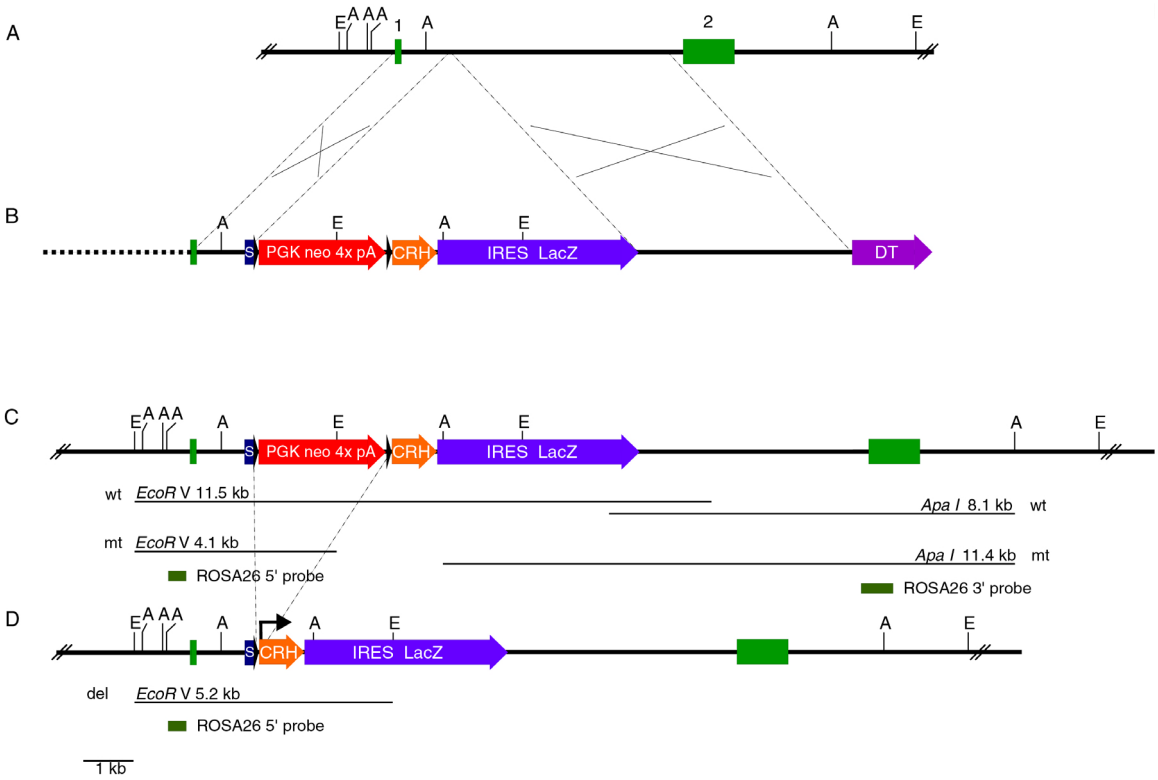

Figure 5. Strategy for CRH knock-in by homologous recombination in ES cells. (A) Genomic structure and partial restriction map of the wild type ROSA26 locus. Exons were numbered. A, *ApaI*; E, *EcoRV*. (B) Targeting vector with about 5 kb homology arm to the ROSA26 gene locus. DT, diphteria toxin. (C) ROSA26 gene locus after homologous recombination in ES cells ($R26^{neoCRH}$). Informative restriction digests and external probes were indicated below. (D) Removal of transcriptional terminator cassette after *Cre*-recombination (del, deletion fragment following Cre mediated recombination; mt, mutant fragment; wt, wild-type fragment).

3.1.2. Screening of Electroporated ES cell

The ROSA26 targeting vector (Figure 5B) carries a 5-kb genomic fragment subcloned in a plasmid vector along with a diphtheria toxin (DT) expression cassette for negative selection, a splice acceptor sequence (SA), and a neomycin expression cassette flanked by two loxP sites. A triple polyadenylation sequence (pA) (Maxwell et al., 1989) was added to the 3' end of the neomycin expression cassette to prevent transcriptional read-through. A IRES-LacZ gene was inserted at a unique *XbaI* site approximately 300-bp 5' of the original gene-trap integration site. The mCRH cDNA was inserted in front of the IRES-LacZ gene by a unique *PacI* site. This targeted construct was linearized with *SwaI* and electroporated into TBV2 ES cells. Following G418 selection, G418 resistant ($G418^r$) clones were analysed for homologious recombination by Southern blot. 2 of 298 $G418^r$ colonies were found to have correctly undergone homologous recombination and were further verified by Southern blot analysis with external ROSA26 5' and 3' probes (Figure 6A and B).

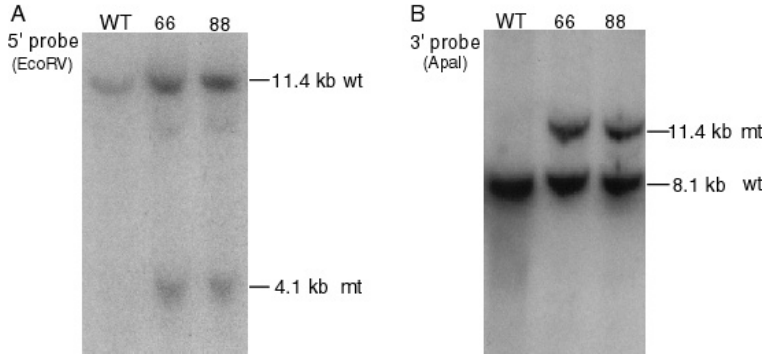

Figure 6. Southern blot analysis of wild-type (WT) and targeted ES cell clones. (A) Southern blot analysis of WT and targeted ES cell clones with ROSA26 5' external probe hybridized to *EcoRV*-digested tail genomic DNA. The targeted allele was indicated by the presence of an additional mutant 4.1 kb fragment. (B) ROSA26 3' external probe was hybridized to *ApaI*-digested DNA from the same WT and targeted ES cell clones confirming homologous recombination, a additional mutant fragment was detected at 11.4 kb (WT, wild-type cells; 66, 88, targeted cells; mt, mutant fragment; wt, wild-type fragment).

3.1.3. *In vitro* characterization of the overexpression strategy

In parallel with the generation of mCRH knock-in mice, the functionality of the strategy was tested in ES cells. In order to remove the transcriptional terminator sequence (Figure 5D), we transfected targeted ES cell clones transiently with the *Cre*-recombinase expression plasmid pCre-Pac (Taniguchi et al., 1998). Transfected

clones were analysed for LacZ reporter gene expression by X-Gal staining (Figure 7A), and excision of the termination sequence was analysed by Southern blot with ROSA26 5' probe (Figure 7B). Finally, the expression of CRH and LacZ was analysed by Northern blot using CRH-, LacZ- and Neo-probe, simultaneously with a β-actin control probe (Figure 7C and D).

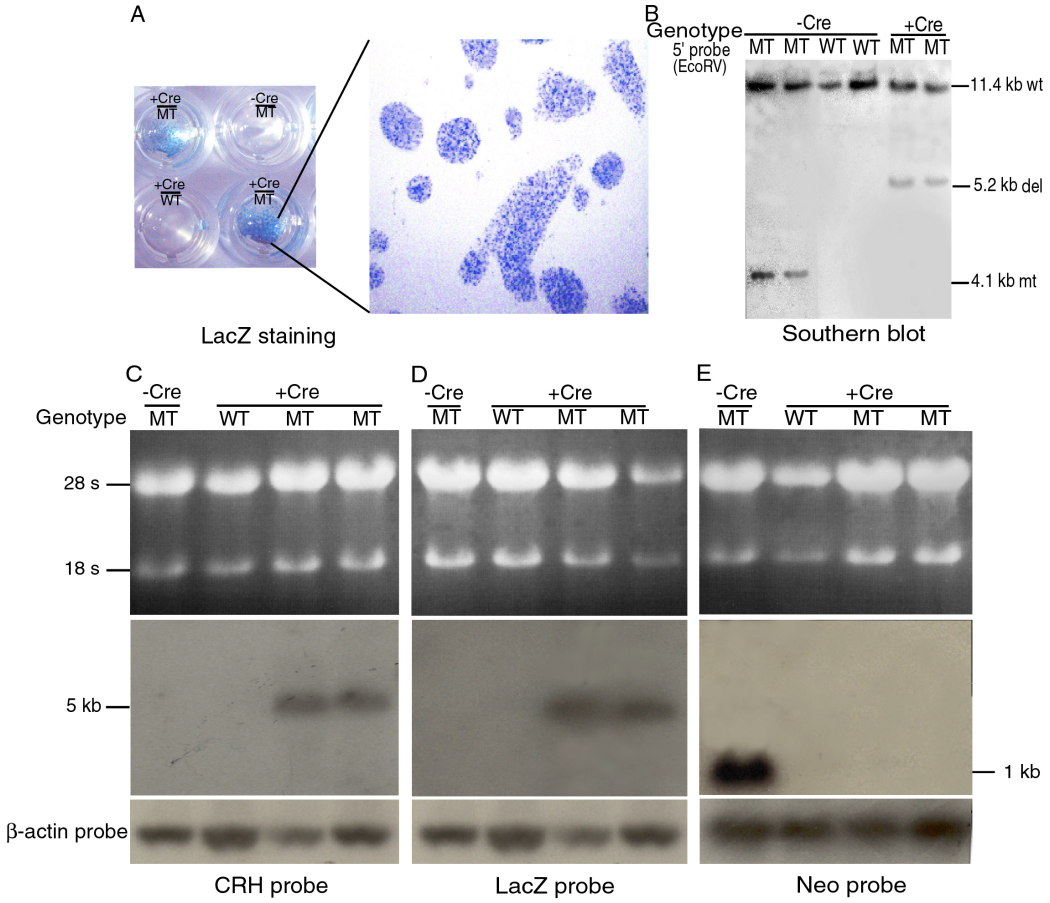

Figure 7. Functional analysis of the mCRH knock-in strategy in targeted ES cell clones. (A) X-Gal staining of targeted ES cell clones transiently transfected with *Cre*-recombinase expression plasmid pCre-Pac. (B) Southern blot analysis of targeted ES cell clones following transient *Cre* transfection. Removal of the transcriptional terminator sequence was indicated by the presence of a 5.2 kb fragment. (C, D and E) Northern blot analysis of targeted ES cell clones following *Cre*-mediated recombination. The removal of the terminator sequence and transcription of a CRH-IRES- LacZ fusion transcript was indicated by the presence of a 5 kb fragment detected by CRH- and LacZ-probe respectively. The Neomycin cassette was present in targeted ES cells without *Cre*-mediated recombination, which was indicated by the presence of a 1 kb transcript by Neomycin probe. The blots were hybridized simultaneously with a β-actin specific probe for comparison of loaded RNA amount for CRH-, LacZ- and Neo-probes (WT, wild-type cells; MT, mutant cells; del, deletion fragment following Cre mediated recombination; mt, mutant fragment; wt, wild-type fragment).

3.1.4. Generation of conditional mutant mice

Targeted ES cell clones were used to produce chimeric mice and germline transmission of the modified ROSA26 allele was confirmed in offspring from male chimeras bred to wild-type C57BL/6J mice. The obtained $R26^{+/neoCRH}$ mice were intercrossed with each other to obtain homozygous ($R26^{neoCRH/neoCRH}$) COR mice (CRH overexpression from the ROSA26 locus) (Figure 8). As previously reported (Soriano, 1999), these mice were indistinguishable from wild-type littermates in terms of development, fertility, endocrine parameters and behavior (data not shown).

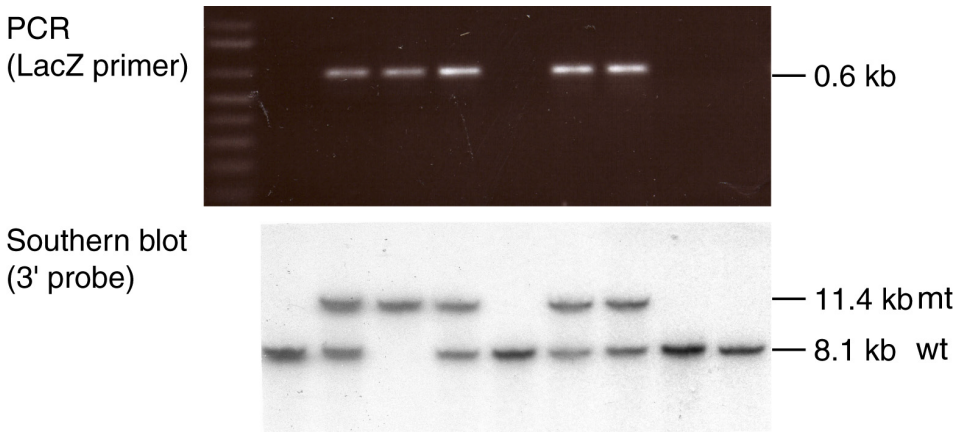

Figure 8. Generation of COR mouse line. PCR analysis of tail DNA by LacZ primers and Southern blot analysis of *ApaI*-digested tail DNA by ROSA26 3' external probe from F2 offspring mice (wt, wild-type fragment; mt, mutant fragment).

The $R26^{neoCRH}$ allele is sensitive to *Cre* recombinase, which catalyses site-specific recombination between two loxP sites, removing the transcriptional terminator sequence and thus activating CRH expression. Therefore, the COR mouse line was crossed either to transgenic CamKIIα-Cre mice (Minichiello et al., 1999), or to transgenic Nestin-Cre mice (Tronche et al., 1999) to obtain COR-Cam mice (Figure 9A) or COR-Nes mice (Figure 9B).

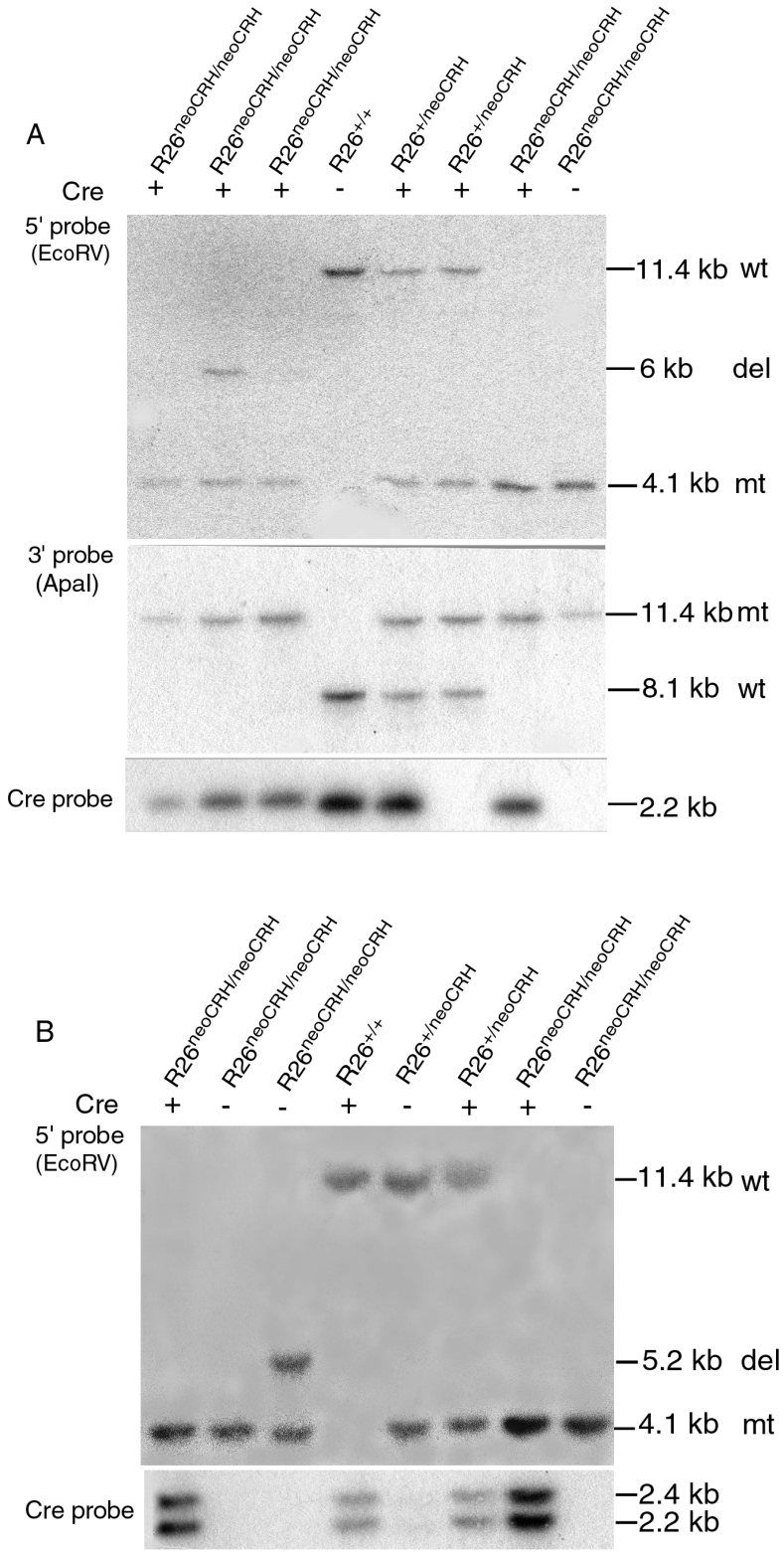

Figure 9. Generation of COR-Cam and COR-Nes mouse lines. Southern blot analysis of *EcoRV*- or *ApaI*-digested tail DNA of COR-Cam (A) and COR-Nes mice (B) with ROSA26 5' or ROSA26 3' external probe, hybridized simultaneously with a *Cre* recombinase-specific probe (del, deletion fragment caused by occasional/transient Cre activity in the genomes; mt, mutant fragment; wt, wild-type fragment).

3.2. Analysis of the region-specific overexpression of CRH

3.2.1. Analysis of LacZ report gene expression by X-Gal staining

To assess the regional pattern of *Cre* activity *in vivo*, saggital and coronal sections of mouse brains from control ($R26^{neoCRH/neoCRH}$), heterozygous ($R26^{+/neoCRH}$ Cre) and homozygous ($R26^{neoCRH/neoCRH}$Cre) mice of both conditional mouse lines (COR-Cam and COR-Nes) were analysed for *Cre* recombination by X-Gal staining. No *Cre* recombination and LacZ reporter gene expression to be observed in control mice (data not shown). Homozygous COR-Cam mice (Figure 10A and C) showed stronger LacZ gene expression than heterozygous COR-Cam mice (Figure 10B), in which *Cre* recombination and LacZ reporter gene expression was specifically observed in the anterior forebrain, including the olfactory bulb (OB), hippocampus (HC) (strong staining in CA1), neocortex (Ctx), striatum (Str), the central nucleus of amygdala (CeA) and lateral hypothalamus area (LHA) (Figure 10A and C), indicating *CamKIIα*-driven *Cre* expression in these selected brain regions. In heterozygous COR-Nes mice, *Cre* recombination and LacZ reporter gene expression was also specifically observed in the olfactory bulb, hippocampus, neocortex and the central nucleus of amygdala, but additional strong staining was detected in the cerebellum (Cb), brainstem and spinal cord (Sp) (Figure 10E). *Cre* recombination and LacZ reporter gene expression was observed in all most the entire brain in homozygous COR-Nes mice (Figure 10D and F). No *Cre* recombination was observed in the pituitary and adrenal gland of both CRH overexpressing mouse lines (data not shown).

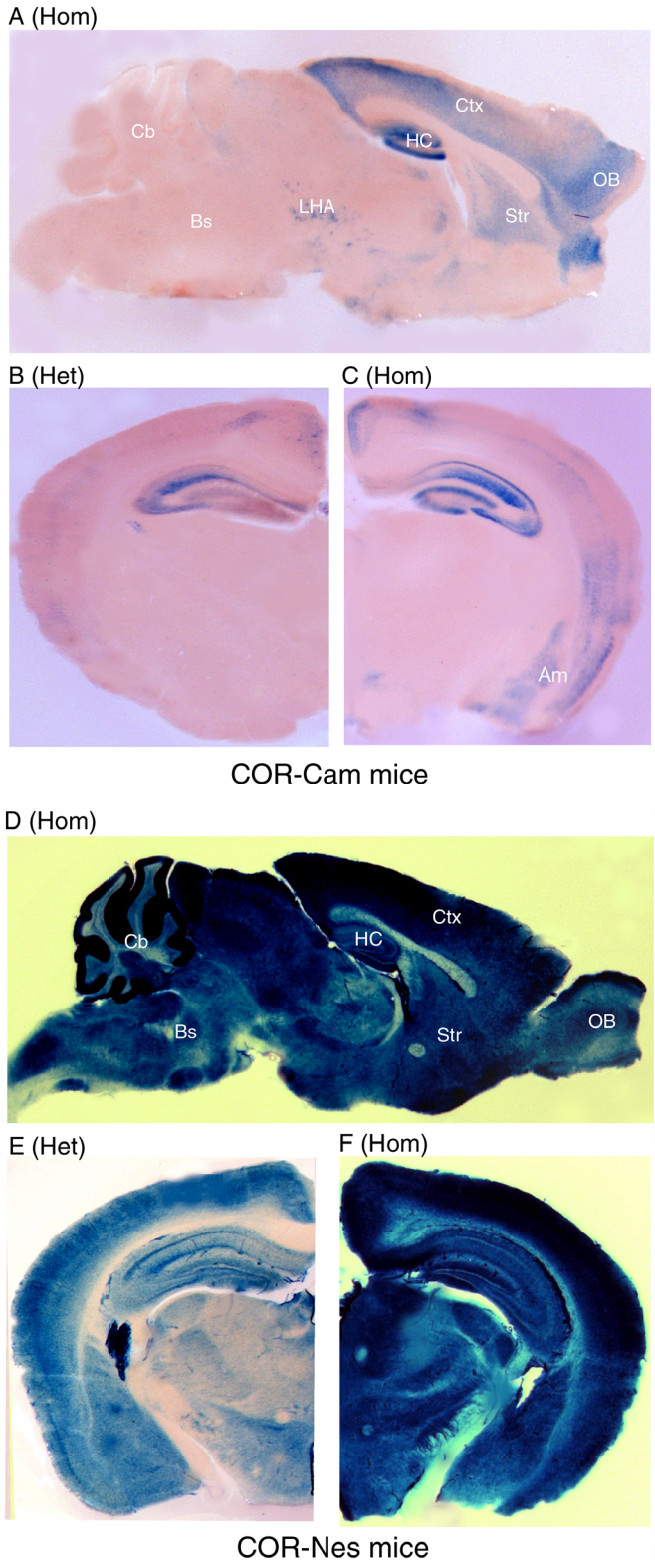

Figure 10. Analysis of *Cre*-dependent LacZ expression patterns by X-Gal staining in COR-Cam mice (A and C: homozygous mice; B: heterozygous mice), and in COR-Nes mice (D and F: homozygous mice, E: heterozygous mice). Abbreviations: Am, amygdala; Bs, brainstem; Cb, cerebellum; Ctx, cortex; HC, hippocampus; LHA, lateral hypothalamus area; OB, olfactory bulb; Str, striatum (Het, heterozygous mice; Hom, homozygous mice)

3.2.2. Analysis of CRH mRNA overexpression by *in situ* hybridization

Conditional CRH overexpression was verified by means of *in situ* hybridization with a CRH-specific probe on sagittal and coronal sections of mouse brains of different genotypes from both conditional mouse lines. In control mice, endogenous CRH expression was detected heterogeneously throughout the central nervous system (CNS). The highest density of CRH expression was found in the PVN, the piriform cortex (Pir) and noradrenergic neurons in the brainstem including nucleus ambiguus, locus coerulus, lateral dorsal tegmental and parabrachial nucleus. Its localization also included the hypothalamus, central nucleus of amygdala (CeA), bed nucleus of the stria terminalis (BNST), septum, cerebellum, supraoptic nucleus and cerebral cortex as previously described (Cummings et al., 1983) (Imaki et al., 1991) (Mezey and Palkovits, 1991) (Vamvakopoulos et al., 1990) (Figure 11A and D). For both CRH overexpressing mouse lines, the CRH overexpression patterns were similar to that of *Cre* activity as demonstrated by X-Gal staining (Figure 10). According to the expression of CRH from both or one R26 allele, CRH expression was stronger in homozygous mice than heterozygous mice. Moreover, high and specific CRH expression was observed in the cortex, hippocampus and amygdala in COR-Cam mice (Figure 11B, C, E, F). COR-Nes mice displayed high intensities of overexpressed CRH in olfactory bulb (OB), brainstem and cerebellum besides in the cortex, hippocampus and amygdala (Figure 12B,C, E).

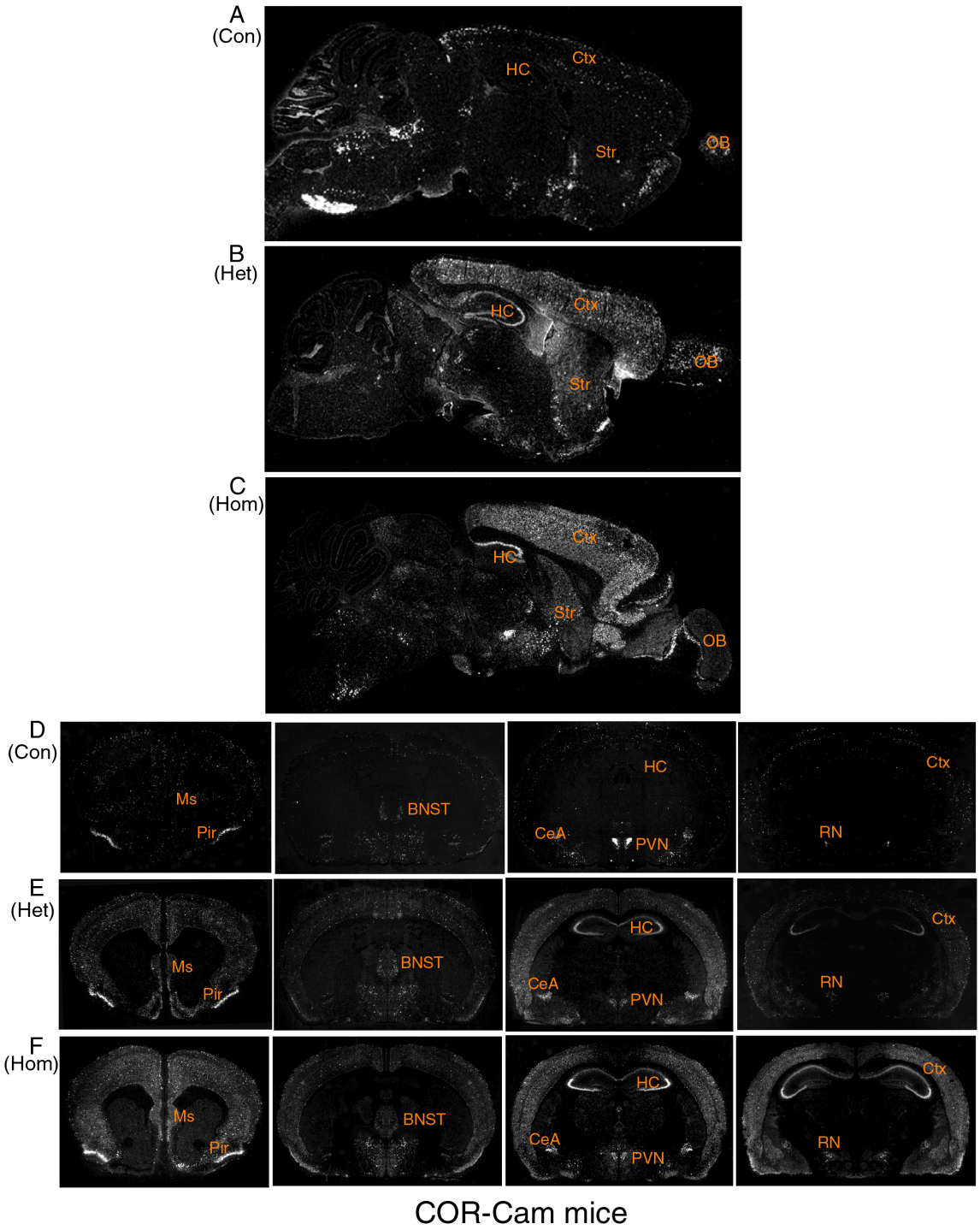

Figure 11. Representative overview of CRH mRNA expression in the brain of control, heterozygous and homozygous COR-Cam mice (A and D, control mice; B and E, heterozygous mice; C and F, homozygous mice). In COR-Cam mice, CRH was overexpressed selectively in the anterior forebrain, not in the cerebellum and brainstem. Abbreviations: BNST, bed nucleus of striatum terminalis; Cb, cerebellum; Ctx, cortex; CeA, central nucleus of amygdala; Pir, piriform cortex; Str, striatum; MS, medial septal nucleus; HC, hippocampus; RN, Retical nucleus; OB, olfactory bulb; PVN, paraventricular nucleus.

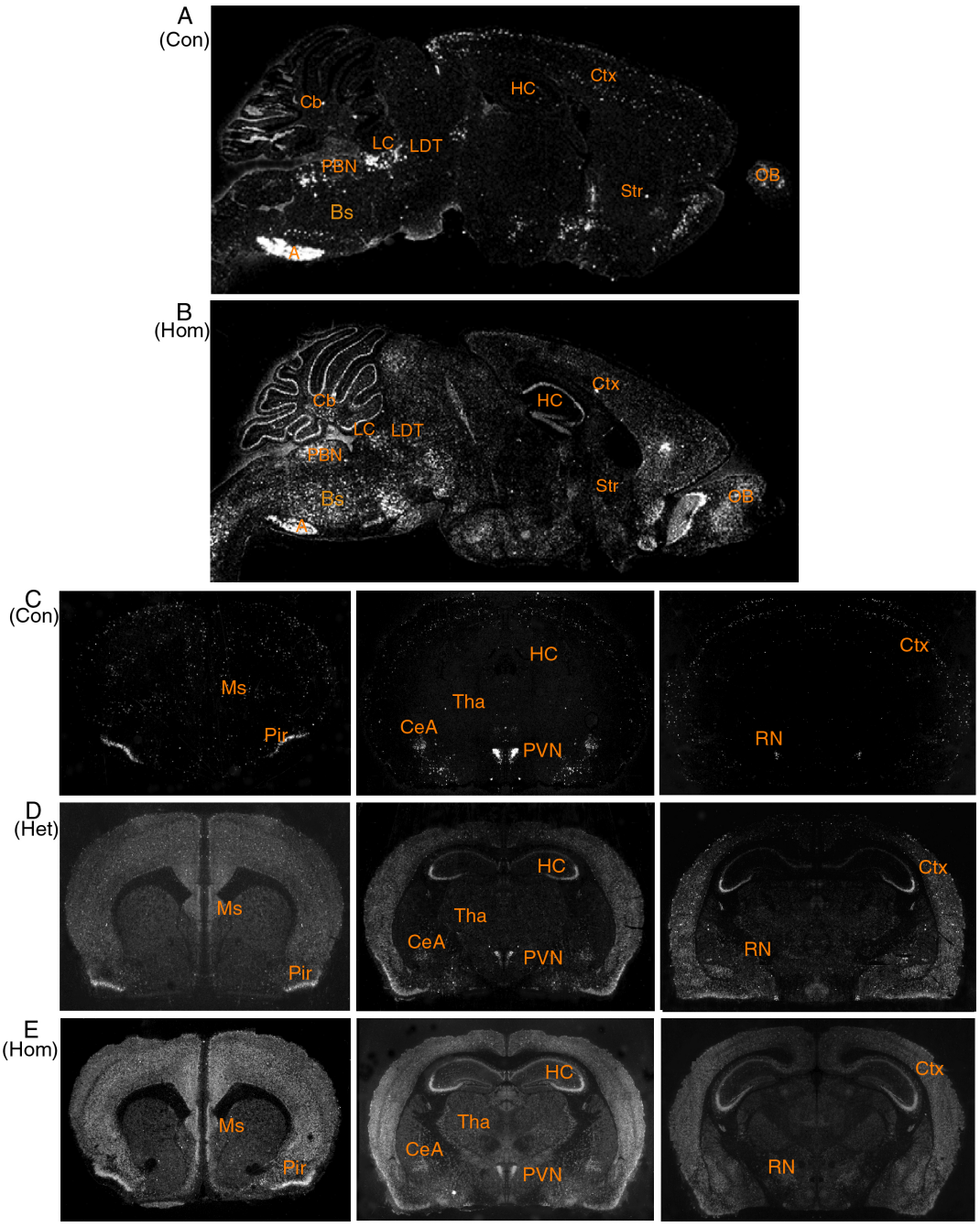

Figure 12. Representative overview of CRH mRNA expression in the brains of control, heterozygous and homozygous COR-Nes mice (A and C, control mice; B and D, heterozygous mice; E, homozygous mice). In COR-Nes mice, CRH was overexpressed almost entire brain, strongly in the olfactory bulb, cortex, hippocampus, cerebellum and spinal cord. Abbreviations: A, nucleus ambiguus; Bs, brain stem; Cb, cerebellum; Ctx, cortex; CeA, central nucleus of amygdala; Pir, piriform cortex; Str, striatum; Tha, thalamus; MS, medial septal nucleus; HC, hippocampus; RN, Retical nucleus; OB, olfactory bulb; LC, locus coerulus; LDT, lateral dorsal tegmental; PBN, parabrachial nucleus; PVN, paraventricular nucleus.

3.2.3. Analysis of CRH protein expression by Radioimmunoassay

We quantified CRH protein expression in the total brain of control and CRH overexpressing mice by radioimmunoassay (RIA). Compared to control mice, CRH content was increased about 1.2- and 1.6-fold in heterozygous and homozygous COR-Cam mice respectively (Figure 13A), and 1.4- and 2.1-fold in heterozygous and homozygous COR-Nes mice respectively (Figure 13B).

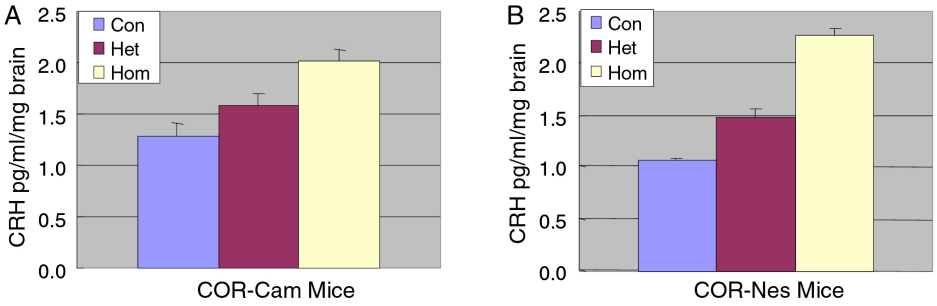

Figure 13. CRH protein expression in the total brain of COR-Cam (A) and COR-Nes (B) mice (Con: control mice; Het: heterozygous mice; Hom: homozygous mice).

CRH protein expression was also quantified in different brain regions by RIA. In control mice, CRH was strongly expressed in thalamus, moderately in hippocampus, cortex and weakly in cerebellum. CRH was increased by 1.9-, 4.6- and 1.6-fold and by 5.6-, 2.4- and 1.8-fold in heterozygous and homozygous COR-Cam mice in hippocampus, cortex and thalamus respectively. No change was observed in cerebellum (Figure 14A). CRH was increased by 2.9-, 5.1-, 2.2- and 5.8-fold and by 7.7-, 4.3-, 3.5- and 6.3-fold in heterozygous and homozygous COR-Nes mice in hippocampus, cortex, thalamus and cerebellum respectively (Figure 14B).

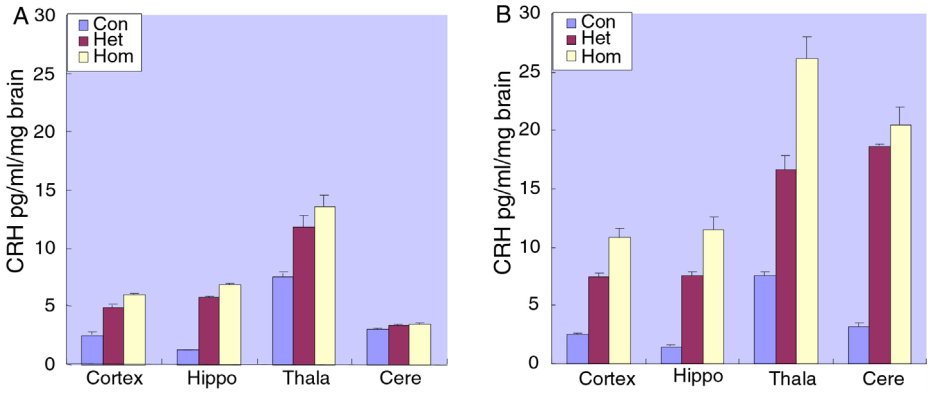

Figure 14. Analysis of CRH expression in various brain regions (cortex, hippocampus, thalamus and cerebellum) of COR-Cam (A) and COR-Nes (B) mice with rat CRH serum, showing the difference in CRH overexpression and reflecting the spatial expression pattern of *CamKIIα-Cre* and *Nestin-Cre*.

3.2.4. Analysis of *Cre*-recombinase mediated excision of the transcriptional terminator sequence by Southern blot

The *Cre*-recombinase mediated excision of the transcriptional terminator sequence on the gemonic levels was verified by Southern blot with the ROSA26 5' external probe in different tissues of both CRH overexpressing mouse lines. Cre mediated excision of the transcriptional terminator sequences resulted in expression of CRH as indicated by X-Gal staining and *in situ* (Figure 10 and Figure 11 and 12). In homozygous COR-Cam mice, *Cre*-mediated recombination took place in more than half of the cells in the cortex, in about half of cells in hippocampus and in less half of cells in the thalamus. No *Cre*-mediated recombination was observed in the cerebellum, tail and liver (Figure 15A). However, in most of cells in the cortex, hippocampus, cerebellum and in about half of cells in the thalamus of homozygous COR-Nes mice, *Cre*-mediated recombination was observed. Moreover, the same as COR-Cam homozygous mice, no *Cre*-mediated recombination was observed in the tail and liver of COR-Nes mice (Figure 15B).

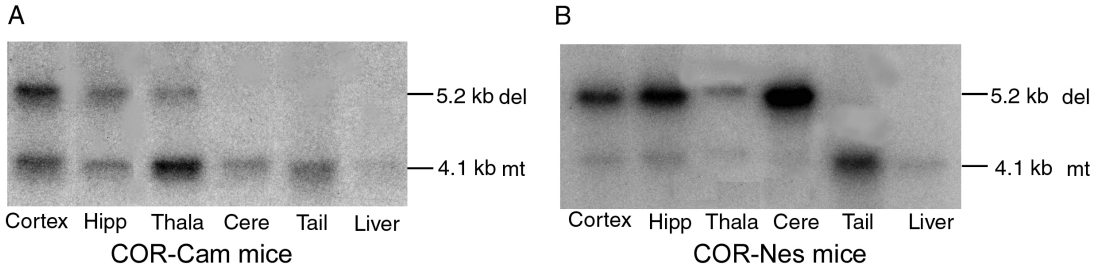

Figure 15. Southern blot analysis of *EcoRV*-digested DNA from various tissues of homozygous COR-Cam animals (A) and homozygous COR-Nes animals (B) by ROSA26 5' external probe, showing the extent of *Cre*-mediated deletion of the transcriptional terminator sequence in depicted tissues (cortex, hippocampus, thalamus, cerebellum, tail and liver). Removal of the transcriptional terminator sequence was indicated by the presence of an additional 5.2 kb fragment.

3.3. Altered expression of genes related to the CRH system

3.3.1. CRH expression in PVN and CeA

ISH experiments showed that CRH overexpression in the entire CNS (COR-Nes) decreased endogenous CRH expression in the PVN in a dose dependent manner. The reduction was also observed in mice expressing CRH in the forebrain (COR-Cam) but to a less extent (Figure 16A and B). Expression of CRH in the forebrain (COR-Cam) slightly increased endogenous CRH expression in the CeA (Figure 16C), however, overexpression of CRH in the entire CNS (COR-Nes) decreased endogenous CRH expression in this brain area (Figure 16D).

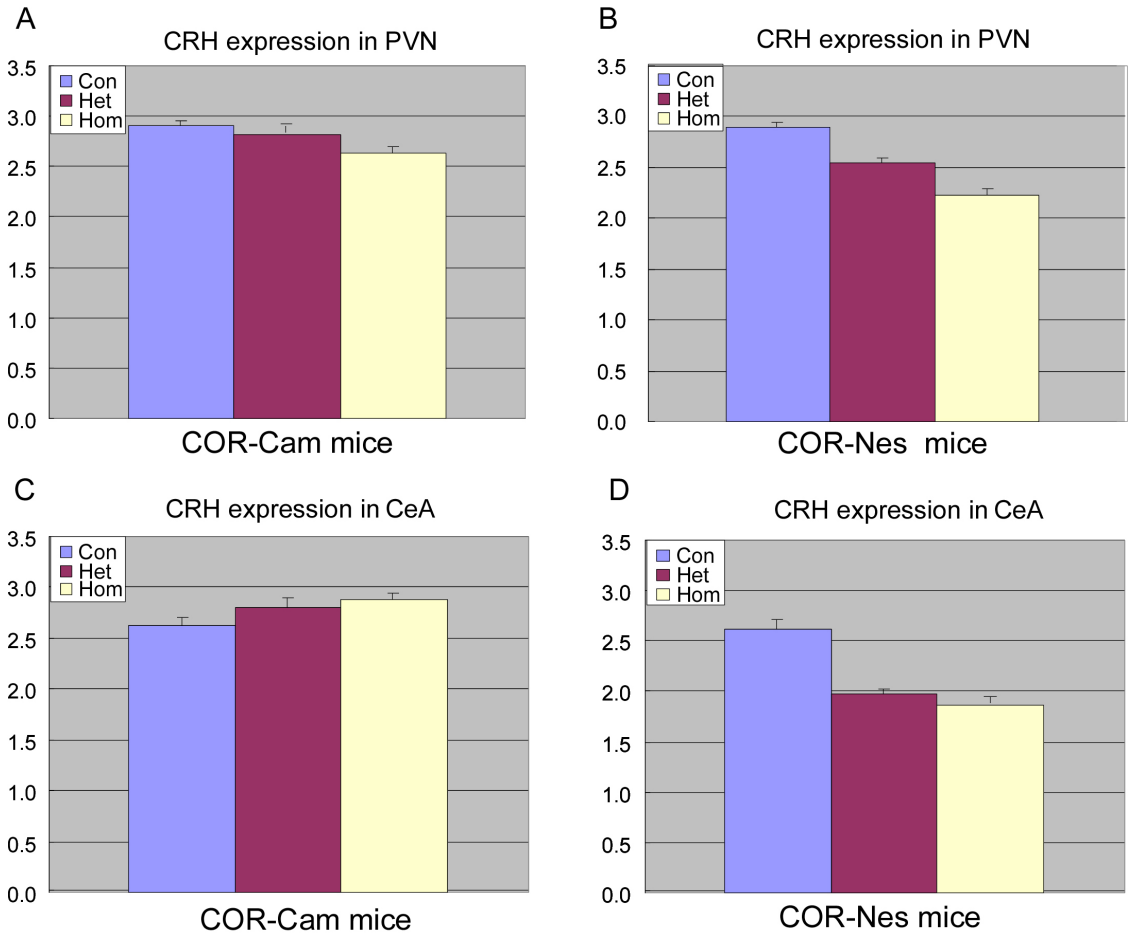

Figure 16. Quantification of CRH mRNA expression in PVN (A and B) and CeA (C and D) in brains of control and COR-Cam (A and B) and COR-Nes (C and D) mice.

3.3.2. Expression of CRH-R1

One intriguing question was whether the CRH overexpression in the forebrain of COR-Cam mice and in the entire brain of COR-Nes mice would lead to alterations in the expression of CRH system related genes that are involved in the endocrine, metabolic and behavioral response to stress.

CRH initiates its biological effects through two receptors which are coupled to G proteins and activate adenylate cyclase. In control mice, ISH revealed that the neocortex comprised the dominant site of CRH-R1 mRNA expression in the mouse forebrain. CRH-R1 mRNA expression in the hippocampus displayed a moderate signal continuously over the pyramidal cell layers of Ammon's Horn and the subicular complex. In addition, a moderate number of hybridizing cells were localized principally to the hilar region of the dentate gyrus and a low density of labeled cells seen in the medial nucleus of amygdala (MeA). The dominant site of CRH-R1 expression in the mouse thalamus was the reticular nucleus (RT). In the brainstem regions, CRH-R1 mRNA was intensively expressed in pontine gray (pG) (Figure 17A and D). In both CRH overexpressing mouse lines, CRH-R1 mRNA was decreased in the neocortex, CA1, CA2 and CA3 of hippocampus and the reticular nucleus of the thalamus (RT), whereas, CRH-R1 mRNA was increased in the dentate gray (DG). Interestingly, we observed CRH-R1 expression in the pontine gyrus was decreased in COR-Cam mice. When CRH overexpressed in the entire brain (COR-Nes), CRH-R1 mRNA in the pontine was decreased only in heterozygous mice, whereas, increased in homozygous mice (Figure 17B, C, E, F and Figure 18A and B).

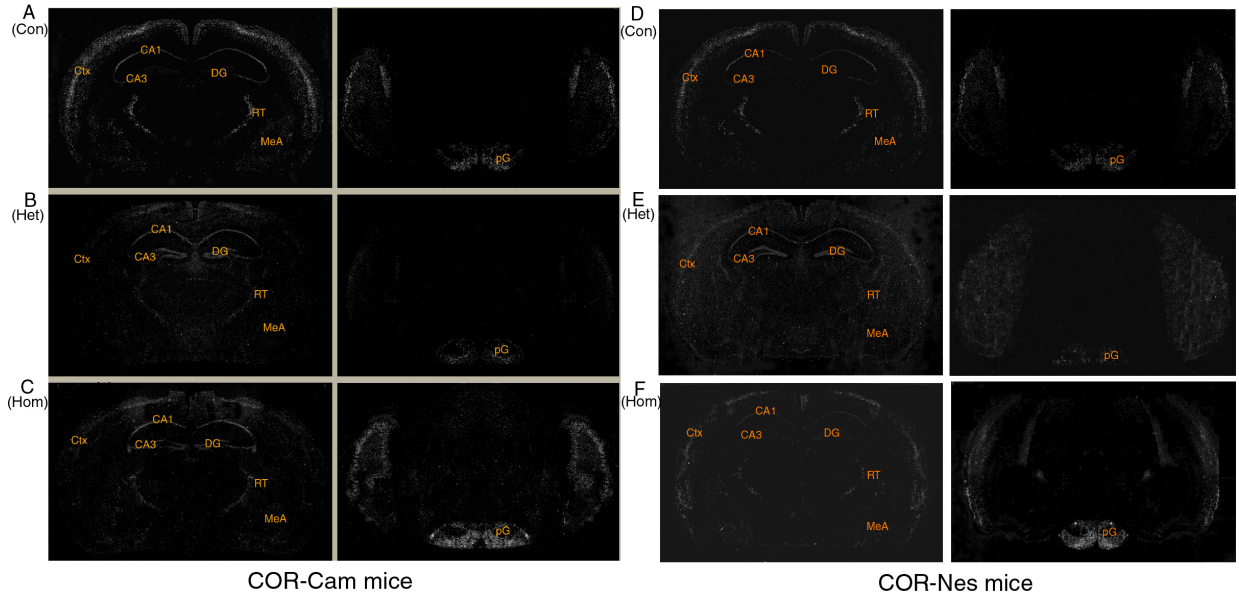

Figure 17. Expression of CRH-R1 mRNA in COR-Cam (A-C) and COR-Nes (D-F) mice (A and D: control mice; B and E: heterozygous mice; C and F: homozygous mice). Abbreviations: Ctx, cortex; DG, dentate gyrus; MeA, medial nucleus of amygdala; RT, reticular nucleus (thalamus); pG, pontine gray.

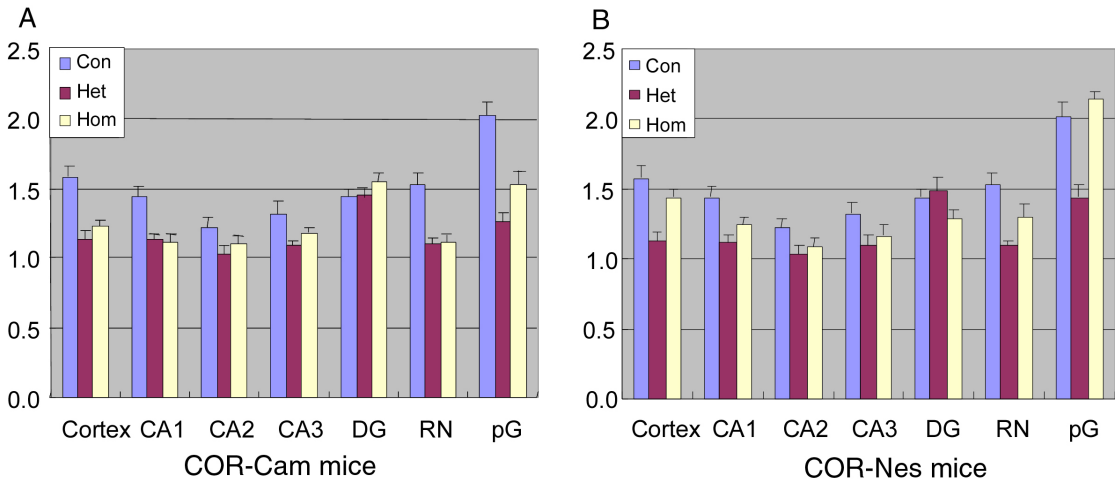

Figure 18. Quantification of CRH-R1 mRNA expression in indicated brain regions of COR-Cam (A) and COR-Nes (B) mice.

3.3.3. Expression of CRH-R2

CRH-R2 mRNA was also found to express in the cortex. In the cortex, this was manifested mainly as positively hybridizing neurons in deeper layers. Major sites of CRH-R2 expression in mouse brain were evident in the intermediate lateral septal nucleus (LSi) and ventral lateral septal nucleus (LSv). In the hippocampal formation, a sparse signal was seen over the principal cell layers of both the dentate gyrus and Ammon's horn. In addition, in the amygdala, CRH-R2 expression was focused in the medial nucleus, being particularly robust in its posterior aspect (MeAp) and in the basomedial nucleus. Non-neuronal elements of the choroid plexus (CP) comprised another major sites of CRH-R2 mRNA expression. We also found a moderate number of positively hybridizing cells which were localized in the lateraldorsal nucleus of thalamus (LD) and ventral lateral nucleus of thalamus (VL) (Figure 19A and D). CRH overexpression in the forebrain (COR-Cam) increased expression of CRH-R2 in the thalamus. Increased CRH-R2 mRNA expression was also found in the septum, dentate gyrus, choroids plexus only in heterozygous COR-Cam mice. However, CRH-R2 expression in the choroid plexus was decreased in homozygous COR-Cam mice (Figure 19B and C and Figure 20A). CRH overexpression in the entire brain (COR-Nes) decreased CRH-R2 mRNA expression in the CP. CRH-R2 mRNA was increased in lateral septum and thalamus in homozygous COR-Nes mice compared to control (Figure 19E and F and Figure 20B).

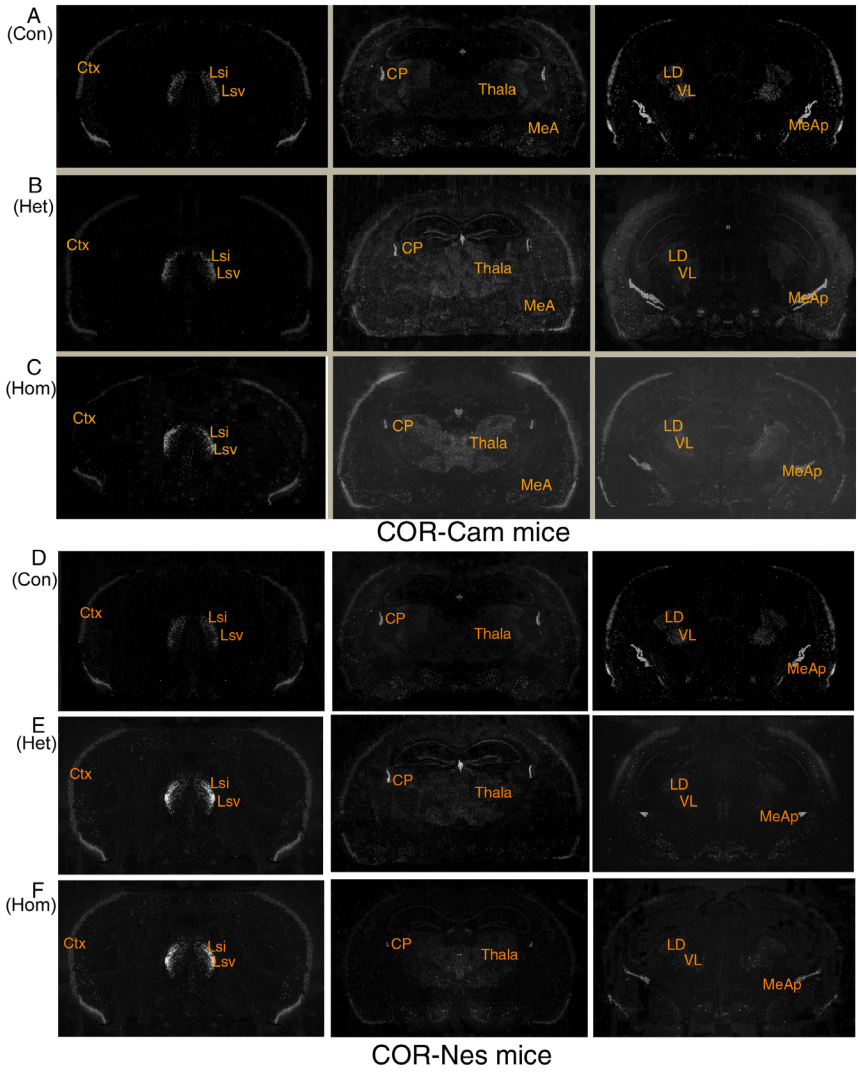

Figure 19. Expression of CRH-R2 mRNA in COR-Cam (A-C) and COR-Nes (D-F) mice (A and D: control mice; B and E: heterozygous mice; C and F: homozygous mice). Abbreviations: CP, choroid plexus; Ctx, cortex; LD, lateral dorsal nucleus of thalamus; Lsi, intermediate lateral septal nucleus; Lsv, ventral lateral septal nucleus; MeA, medial nucleus of amygdala; MeAp, medial nucleus of amygdala posterior part; Thala, thalamus; VL, ventral lateral nucleus of thalamus.

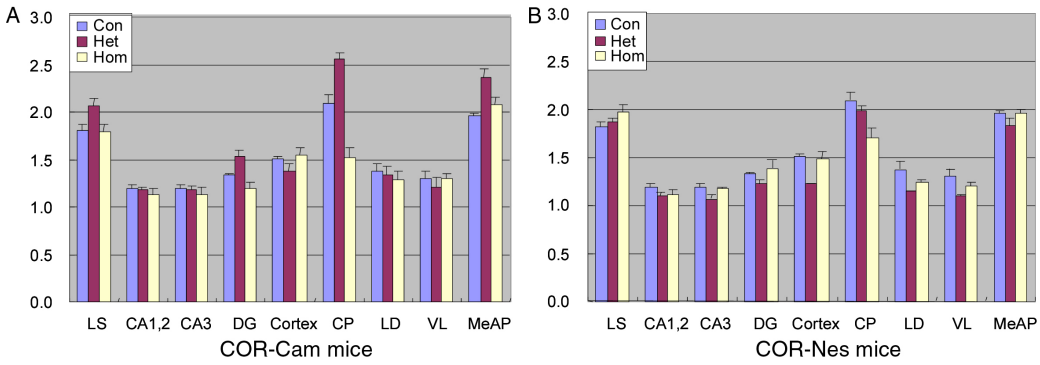

Figure 20. Quantification of CRH-R2 mRNA expression in the different brain regions of COR-Cam (A) and COR-Nes (B) mice.

3.3.4. Expression of AVP

ISH experiments showed AVP expression in the magnocellular subdivisions of the PVN and in the supraoptic nucleus (SON). There was no difference detectable between the different genotype of both CRH overexpressing mouse lines (Figure 21).

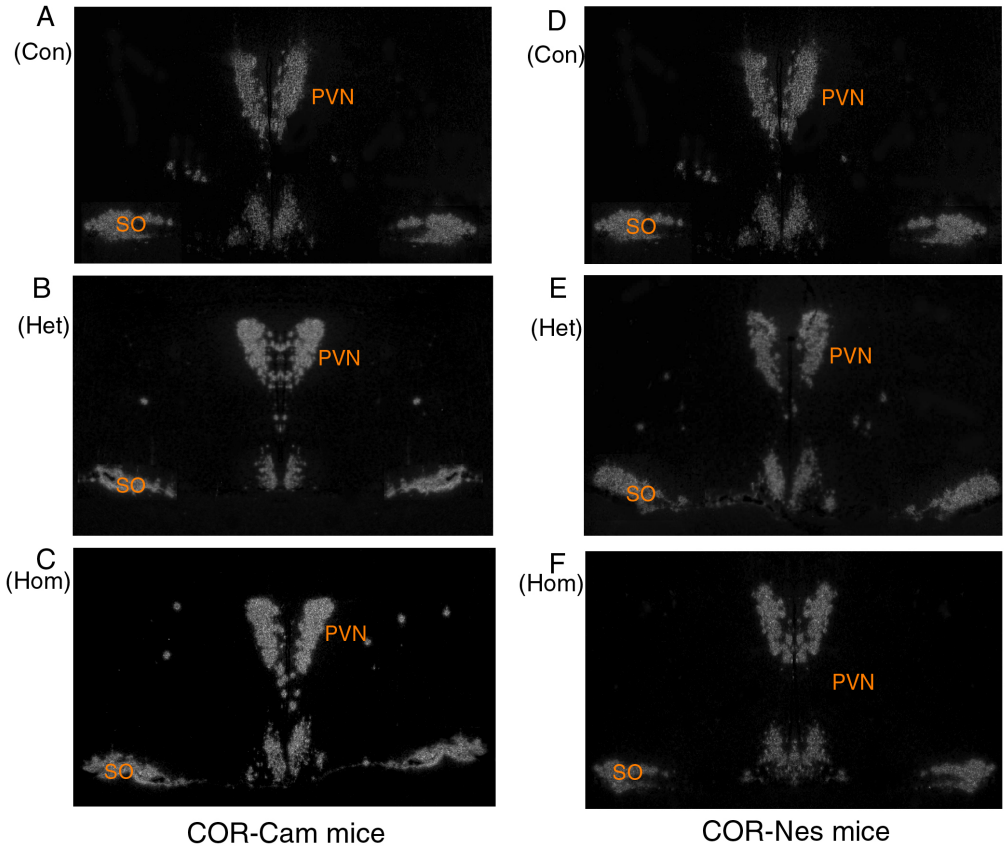

Figure 21. Expression of AVP mRNA in COR-Cam (A-C) and COR-Nes (D-F) mice (A and D: control mice; B and E: heterozygous mice; C and F: homozygous mice). Abbreviations: PVN, paraventricular nucleus of hypothalamus; SON, supraoptic nucleus.

3.3.5. Expression of GR

The glucocorticoid receptor (GR) is a widely expressed ligand-dependent transcription factor that belongs to the nuclear hormone receptor superfamily and modulates a broad range of neural functions, including stress responsiveness and cognitive function (Sapolsky et al., 1984) (McEwen and Sapolsky, 1995) (Meaney et al., 1996) (De Kloet et al., 1998) (Roozendaal et al., 2003). Dysregulation of GR function has been associated with depression and anxiety disorders. In control mice, layer 1 of neocortex possessed a small number of GR expressing cells, high densities of GR expressing cells were demonstrated in layers 2, 3 and layer 6 of neocortex. Layer 4 and 5 of neocortex showed a moderate number of hybridized cells. In the Ammon's horn, very high densities of GR expressing cells were present in the CA1 and CA2, in contrast, the CA3 had lower densities of GR expressing cells. A large number of hybridizing cells were recognized in the granule cell layer of the dentate gyrus (DG). The central nucleus of the amygdala (CeA) and medial nucleus of the amygdala (MeA) possessed a large number of GR mRNA-containing cells. The intensities of the GR mRNA-containing cells in the thalamus were weak to moderate. The paraventiricular nucleus of the thalamus (PVT) and the lateral posterior nucleus (LP) possessed moderate densities of GR labeled cells. The other areas of the thalamus showed only a few GR expressing cells. The intensities of GR mRNA observed in the hypothalamus were moderate to strong, the high density of GR mRNA-containing cells was noted in the ventralmedial part of hypothalamus (VMH), the moderate density of the GR mRNA in the arcuate nucleus (ARH) and in the cells of periaqueductal gray (PAG). The other zones of the hypothalamus showed low densities of GR expressing cells (Figure 22A and D). CRH overexpression decreased GR mRNA expression in both CRH overexpressing mouse lines (Figure 22B, C, E, F and Figure 23A and B).

These results suggested that overexpression of CRH in the forebrain or in the entire brain substantially altered basal mRNA expression of CRH-R1, CRH-R2 and GR in a highly region specific fashion.

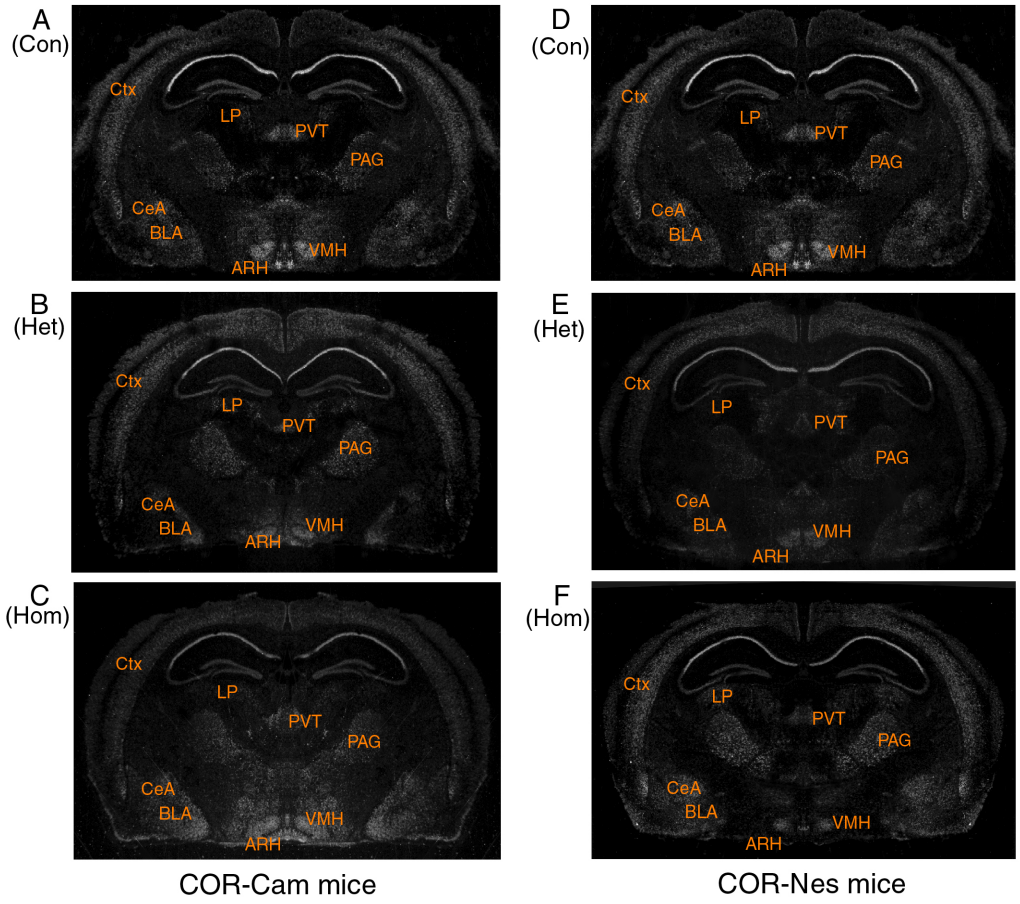

Figure 22. Basal expression of GR mRNA in COR-Cam (A-C) and COR-Nes (D-F) mice (A and D: control mice; B and E: heterozygous mice; C and F: homozygous mice). Abbreviations: ARH, arcuate nucleus; BLA, basolateral nucleus of amgdala; CeA, central nucleus of amygdala; LP, lateral posterior nucleus thalamus; PAG, periaqueductal gray; PVT, paraventricular nucleus thalamus; VMH, ventromedial nucleus hypothalamus.

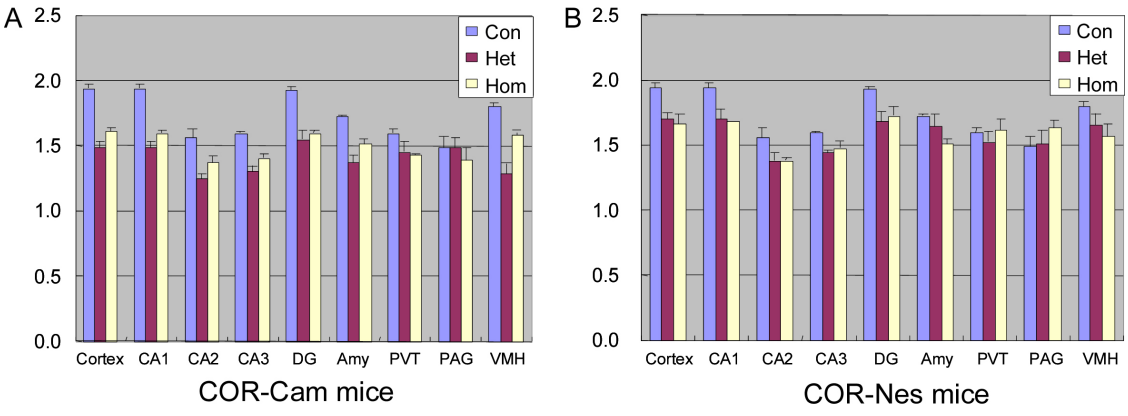

Figure 23. Quantification of GR mRNA expression in the different brain regions of COR-Cam (A) and COR-Nes (B) mice.

3.4. Hypothalamic-Pituitary-Adrenal axis activity

To investigate the effect of long-term central CRH overexpression on HPA axis regulation, basal plasma corticosterone concentrations were measured. In COR-Cam mice, corticosterone in the morning was slightly increased in heterozygous mice, and increased 1.6-fold in homozygous mice compared to control mice (Figure 24A). In COR-Nes mice, corticosterone in the morning was increased 1.8- and 2.4-fold in heterozygous and homozygous mice respectively compared to control mice (Figure 24B).

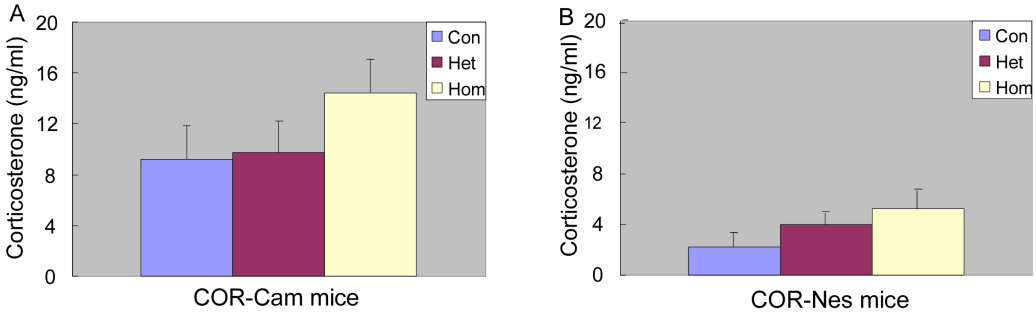

Figure 24. Plasma corticosterone concentrations in control and COR-Cam (A) and COR-Nes (B) mice under basal conditions in the morning (8:00 am).

3.5. Body weight

In addition to stress-related behaviors, CRH is also thought to have an important role in control of food intake and energy balance (Richard, 1993) (Rothwell, 1990). Administration of exogenous CRH (injected into third ventricular (i3vt)) reduced food intake and body weight (De Souza, 1987) and increased thermogenesis (Richard, 1993). The first described CRH overexpressing mouse line (Stenzel-Poore et al., 1992) displayed truncal obesity compared to control mice because of the increased corticosterone resembling a Cushing syndrome-like phenotype. However, the second CRH overexpressing mouse line generated revealed significantly lower body weights than control mice (Dirks et al., 2002b). To assess the body weight of our CRH overexpressing mice, we weighted 4-month old mice. Forebrain specific over-expression of CRH (COR-Cam) decreased body weight slightly compared to control mice (Figure 25A); CNS wide overexpression of CRH (COR-Nes) decreased body weight significantly compared to control mice (Figure 25B).

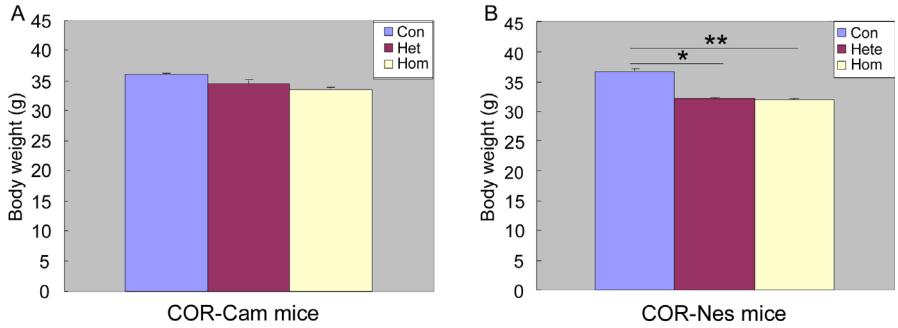

Figure 25. Body weight of COR-Cam (A) and COR-Nes (B) mice. *P<0.05, **P<0.01. n=15.

3.6. Acoustic startle response (ASR)

3.6.1. Startle reactivity

Startle is a fast twitch of facial and body muscles evoked by a sudden and intense tactile, visual or acoustic stimulus. The startle pattern consists of eye-lid closure and a contraction of facial, neck and skeletal muscles, as well as an arrest of ongoing behaviors and an acceleration of the heart rate (Koch, 1999). This response pattern is suggestive of a protective function of startle against injury from a predator or from a blow, and of the preparation of a flight/fight response. It serves as a valuable behavioral tool to assess mechanism of sensorimotor response plasticity (Koch, 1999). To investigate the effects of chronic CRH excess on sensory information processing of startling stimuli, both CRH overexpressing mouse lines were analyzed in the acoustic startling paradigm. Increased startle magnitudes to increasing stimulus intensities were observed in both CRH overexpressing mouse lines (Figure 26A and B). Post hoc indicated that only mice overexpressing CRH just from a single allele in the anterior forebrain (COR-Cam) showed significantly increased startle magnitude at high stimulus intensities (100-120 dB) (Figure 26A).

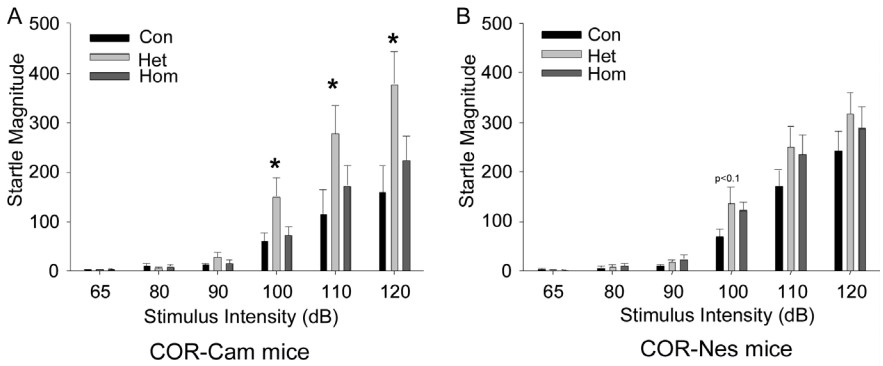

Figure 26. Startle reactivity of COR-Cam (A) and COR-Nes (B) mice. Mean startle magnitude as function of stimulus intensity (dB). *P<0.05 versus control, n=11.

3.6.2. Habituation

Habituation is a theoretical construct referring to the reduction in magnitude of the ASR after repeated presentation of the startling stimulus that is not due to muscle fatigue or blunting of sensory receptor responsiveness (Christoffersen, 1997). The response during the 5 trials reflects the response to the initial acoustic stimuli. COR-Cam mice and COR-Nes mice did not show any normal habituation (Figure 27), only homozygous COR-Nes mice showed a deficit habituation in the trials 3-5 (Figure 27B). Overall, the startle magnitude was higher in both CRH overexpressing mouse lines compared to control mice.

Post hoc tests showed that, startle magnitude was significantly increased in block 2-3 in heterozygous COR-Cam mice (Figure 27A), and in block 5 in homozygous COR-Nes mice (Figure 27B) compared to control mice. A slightly increased startle magnitude was observed in homozygous COR-Cam and heterozygous COR-Nes mice.

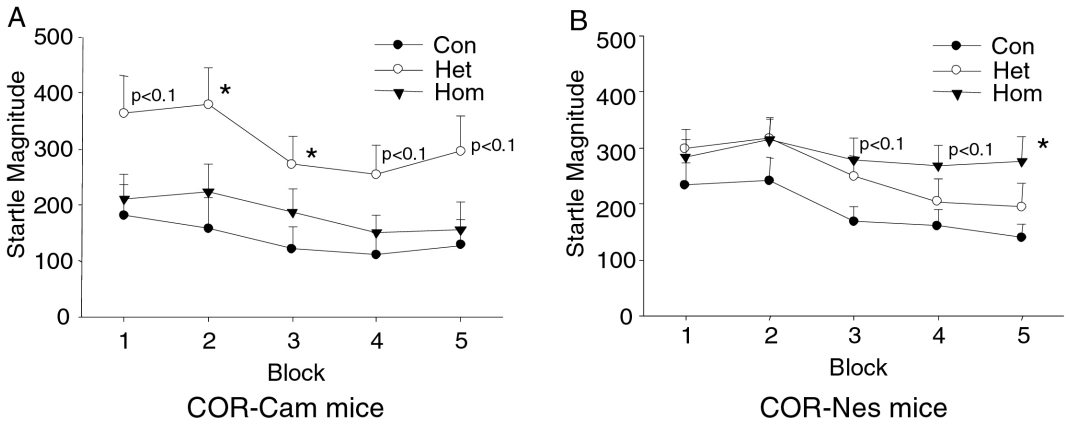

Figure 27. Habituation of the acoustic startle response in COR-Cam (A) and COR-Nes (B) mice. Mean startle magnitude (in arbitrary units, ± SEM) as function of blocks of control and CRH overexpressing mice, *P<0.05 versus control, n=11.

3.6.3 The CRH-R1 antagonist (NBI-30775) reversed CRH-induced startle response in COR-Nes but not in COR-Cam mice

Figure 28 showed the effects of the CRH-R1 specific antagonist (NBI-30775) on startle magnitude. NBI30775 was infused into the lateral ventricle at different concentration (2 mg/kg, 20 mg/kg) and animals were compared to vehicle treated mice. Any concentration of NBI-30775 did not reverse the increased startle magnitude

in COR-Cam mice (Figure 28A). However, the startle magnitude was reversed with increasing NBI-30775 concentration in COR-Nes mice (Figure 28B).

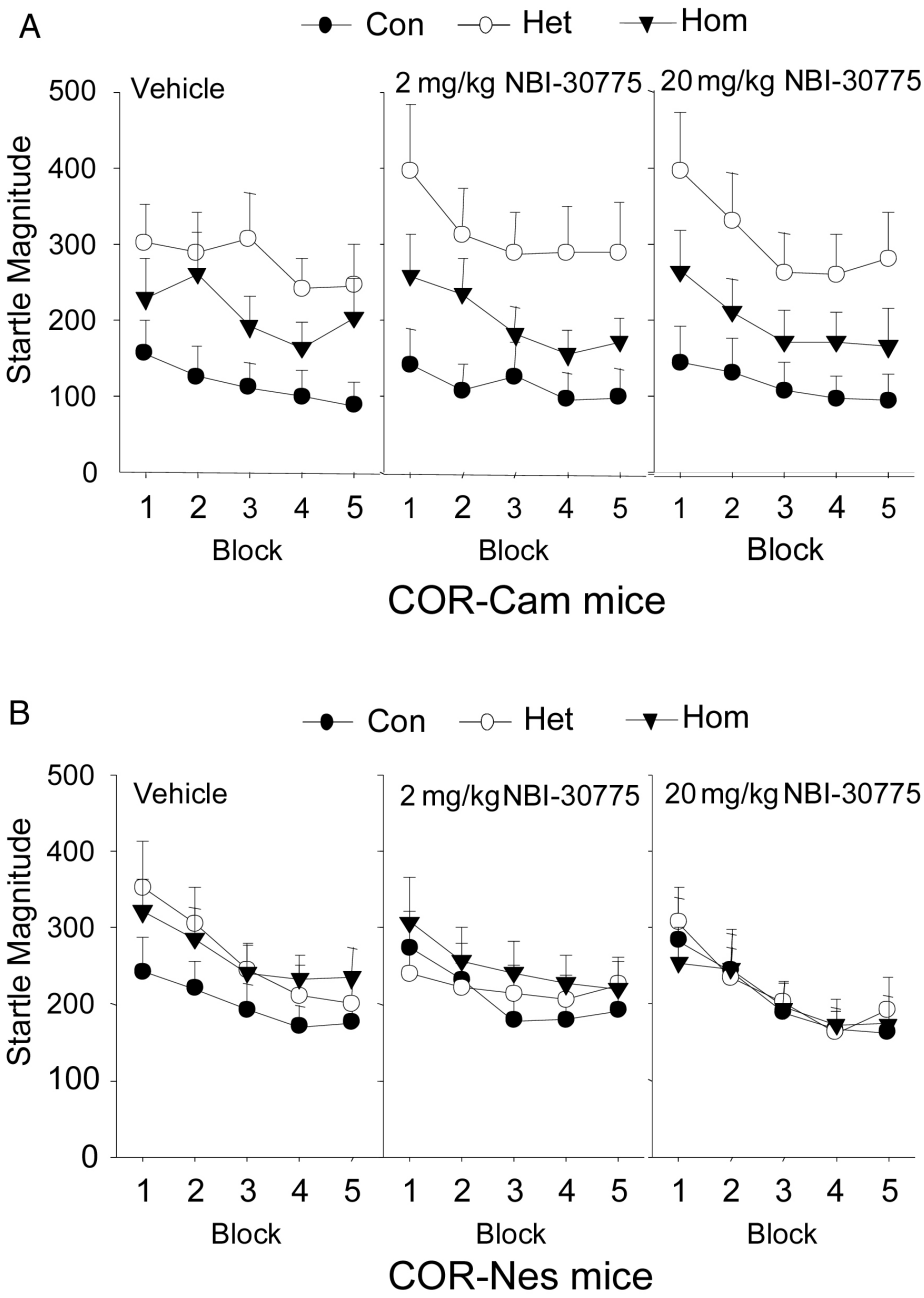

Figure 28. Effect of CRH-R1 antagonist (NBI-30775) on habituation and magnitude of startle response in COR-Cam (A) and COR-Nes (B) mice, n=11.

3.6.4. Reduced prepulse inhibition (PPI) in COR-Nes but not in COR-Cam mice

The ASR magnitude is reduced if a distinctive non-startling tactile, visual (Campeau and Davis, 1995) or acoustic (Hoffman and Ison, 1980) stimulus is presented 30-500 msec before the startling stimulus. This phenomenon is termed as PPI and is used as an operational measure for sensorimotor gating mechanisms. PPI of the ASR is reduced in a variety of neuropsychiatric disorders that are characterized by a general reduction of the ability to gate intrusive sensory, motor or cognitive information, for example in schizophrenia, schizotypal personality disorder, Huntington's disease, obsessive compulsive disorder, Tourette's syndrome and attention deficit disorder (Swerdlow and Geyer, 1998).

To investigate role of chronic CRH excess on sensorimotor gating mechanism, two different procedures were used in our study: one examined the effects of prepulse with varying intensities on PPI, and the other was designed to study the effects of different intervals between the onset of the prepulse and that of the startle stimulus on PPI.

With increasing prepulse intensities, the percent of PPI was increased in all genotypes of both CRH overexpressing mouse lines. The percent of PPI was increased slightly in heterozygous COR-Cam mice, and slightly decreased in homozygous COR-Cam (Figure 29A). Heterozygous the homozygous COR-Nes mice displayed lower PPI levels compared to control mice. At a prepulse intensity of 16 dB above background, this decrease is significant in homozygous COR-Nes mice (Figure 29B).

The percent PPI as a function of the interval between prepulse onset and startle stimulus onset could be described as a u-shaped curve in control and both CRH overexpressing mice. No big difference was observed between control, heterozygous and homozygous COR-Cam mice. Heterozygous and homozygous COR-Nes mice showed lower percent of PPI compared to control mice at different intervals between prepulse and stimulus (from 20-1080 msec) (Figure 29C and D).

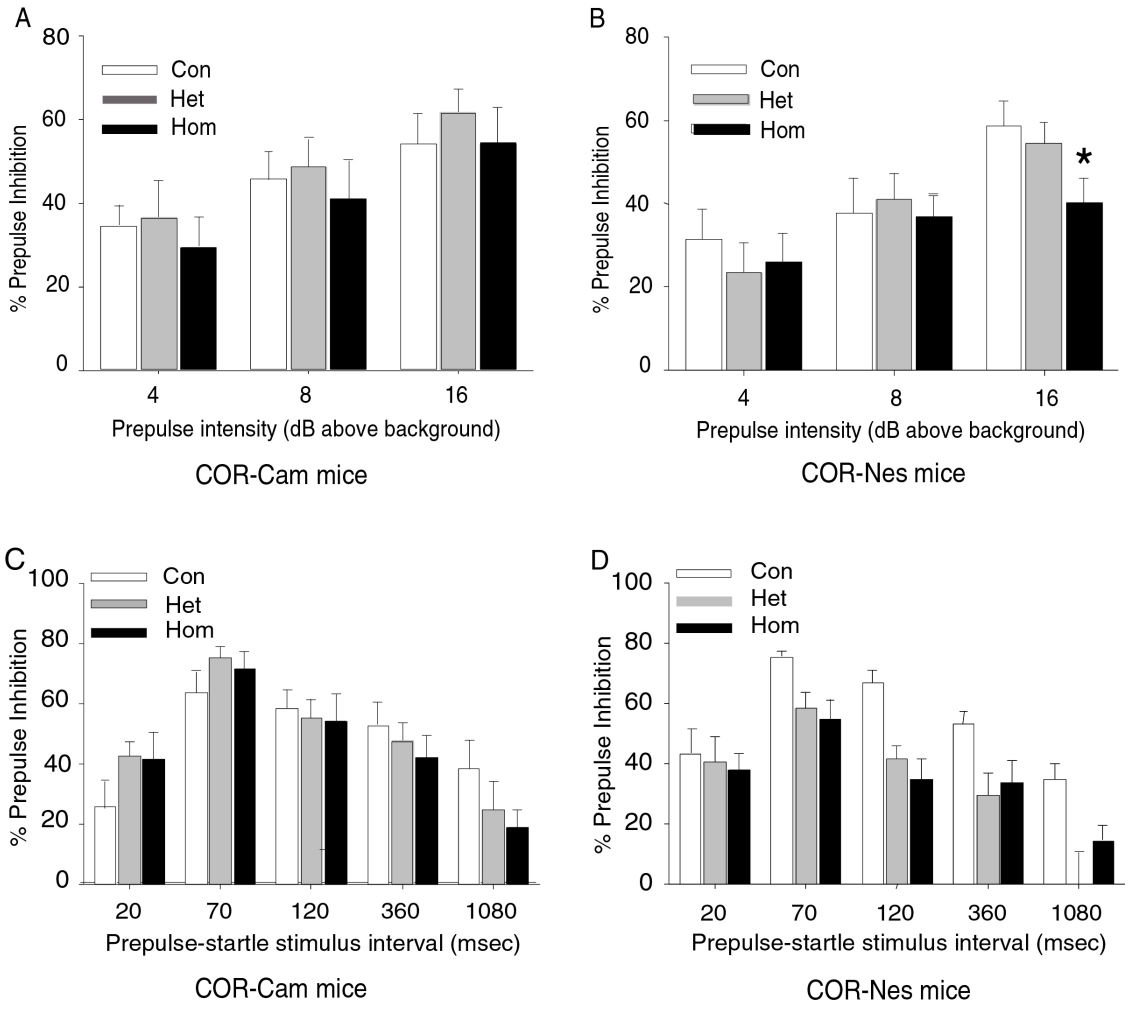

Figure 29. Prepulse inhibition of the acoustic startle response in COR-Cam (A and C) and COR-Nes (B and D) mice. A and B: Percent PPI as function of prepulse intensity (dB above background); C and D: Percent PPI as function of prepulse-startle stimulus interval (msec). *P<0.05 versus control, n=11.

3.6.5. CRH-R1 antagonist increased PPI in COR-Nes mice, but not in COR-Cam mice

COR-Nes mice displayed decreased levels of PPI (Figure 29B and D), therefore, we hypothesized that activation of CRH-R1 via CRH overexpression in the entire CNS was responsible for the CRH-induced decreases in PPI. We injected a CRH-R1 antagonist (NBI-30775) into the lateral ventricle with different concentration (2 mg/kg and 20 mg/kg), and found NBI-30775 did not have an effect on PPI in COR-Cam mice (Figure 30A). However, NBI-30775 reverted the decrease of PPI observed in heterozygous and homozygous COR-Nes mice dose-dependently in comparison with vehicle treated throughout all prepulse-startle stimulus intervals (Figure 30B).

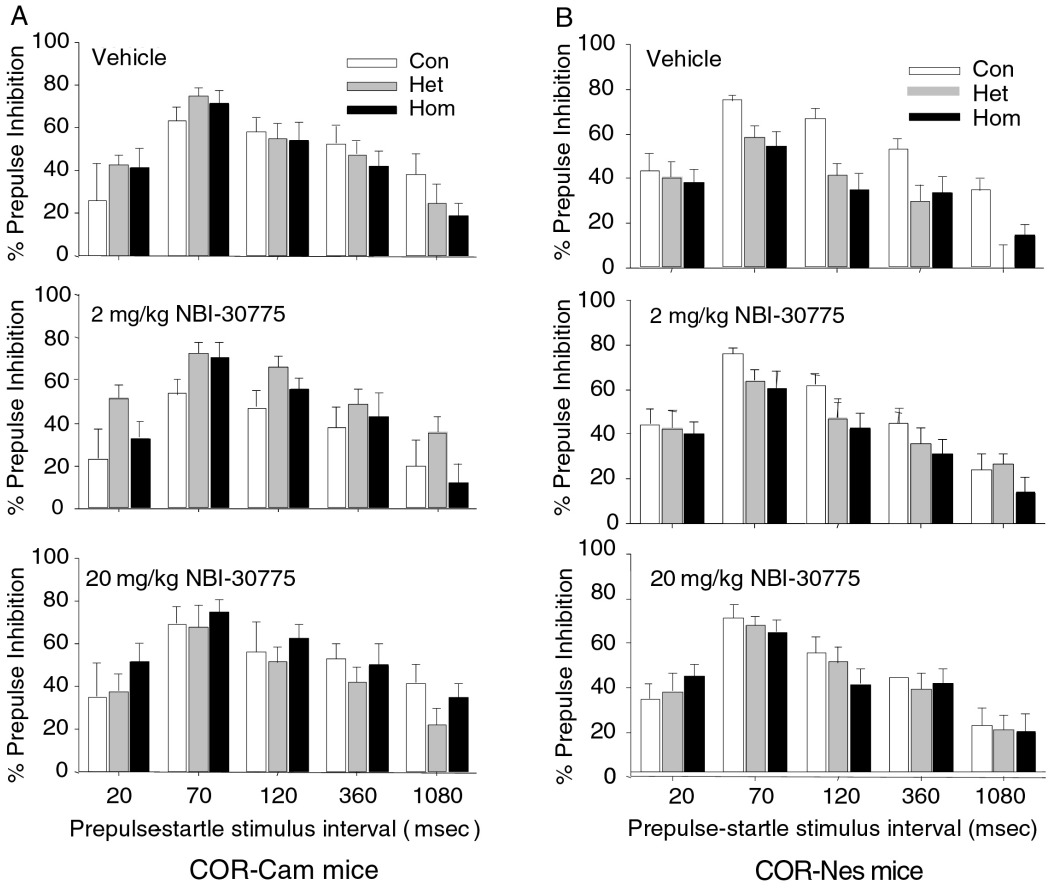

Figure 30. Effect of CRH-R1 antagonist (NBI-30775) at different concentration (2 mg/kg and 20 mg/kg) on prepulse inhibition of the acoustic startle response in COR-Cam (A) and COR-Nes (B) mice compared to control mice, n=11.

3.7. Assessment of conditioned fear in COR-Nes mice

The fear-potentiated startle paradigm was introduced in 1951 (Brown et al., 1991). In this model, the animals are trained to associate a neutral stimulus, for example, a light or a tone, with an aversive stimulus such as a mild electric footshock. As a result of this association, the condition stimulus will evoke a variety of behavioral, endocrine and autonomic responses that are typically elicited in dangerous situations (LeDoux, 2000). In the last decades, fear conditioning has emerged as a leading paradigm for studying cellular correlates of learning and memory (Tang et al., 2001).

To assess the involvement of CRH in modulation of learning and memory, COR-Nes mice were tested in auditory fear conditioning. Mice were trained to associate a tone with a foot shock (conditioning). The relative time not moving was analyzed at different time point after providing the tone. Heterozygous and homozygous COR-Nes mice showed less freezing compared to control mice at 1 day (d1) after conditioning. The result also revealed an effect of time, because heterozygous and homozygous COR-Nes mice did not differ in their freezing response at 6 (d6) and 21 day (d21) after conditioning compared to control littermates (Figure 31A). Moreover, heterozygous and homozygous COR-Nes mice also showed less freezing than control mice at 24 h later by re-exposing to the conditioning chamber for 3 min without tone presentation after conditioning (Figure 31B).

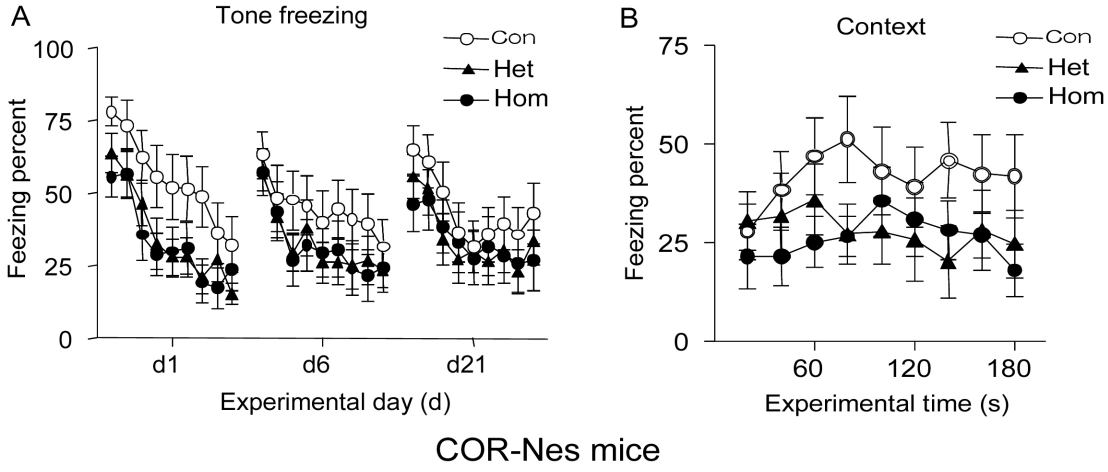

Figure 31. Response to tone at d1, d6 and d21 (A) and to contextual at d1 (B) after fear conditioning of COR-Nes mice.

3.8. Forced swimming test (FST) in COR-Nes mice

The FST is utilized to reproduce passive coping responses to stress that may model a relevant aspect of human depression in rodent species (Porsolt, 2000). Animals showing high levels of passive responses to the FST are assumed to model pathologically depressed individuals. Among various theoretical models, forced swimming is widely used for an antidepressant screening test (Porsolt et al., 1977).

In order to investigate the role of CRH in this depression model, COR-Nes mice were tested in 24-26 °C warm water (depth 15 cm, a 6-min test duration) firstly. A significant decrease of immobility (floating) time (Figure 32A) and increase of struggling time (Figure 32B) was observed in heterozygous and homozygous COR-Nes mice compared to control mice.

Water temperature influences the degree of immobility time in the FST (Petit-Demouliere et al., 2005). The influence of water temperature on immobility time of the mice was also studied in COR-Nes mice. Homozygous COR-Nes mice showed less immobility in compared to control mice as observed in the 24-26 °C warm water (Figure 32C). However, no difference in the struggling time was observed between different genotypes (Figure 32D) suggesting an effect of water temperature.

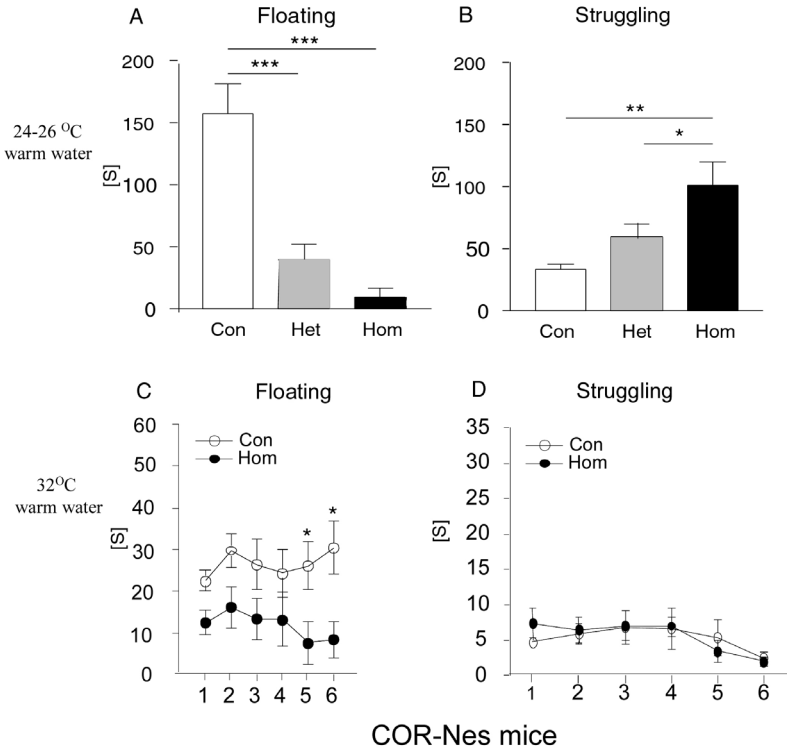

Figure 32. Immobility (A and C) and struggling (B and D) time during the last 3 min in 24-26 °C (A and B) and 32 °C (C and D) warm water in COR-Nes mice. Data were presented as mean ± SEMs, ***p <0.001, **p<0.01, *p<0.05, n=11.

3.9. Tail suspension test (TST) in COR-Nes mice

The tail suspension test (TST) shares a common theoretical basis and behavioral measure with the FST (Steru et al., 1985). In the TST, mice are suspended by the tail using adhesive tape to a horizontal bar. The presence or absence of immobility, defined (Steru et al., 1985) as the absence of limb movement. Typically, mice immediately engage in several "agitation- or escape-like" behaviors, followed temporally by increasing bouts of immobility that is reversed by antidepressant treatments. The TST has been shown to be sensitive to various antidepressants.

COR-Nes mice were analyzed. Mice were securely fastened with medical adhesive tape by the tip (1.0-1.5 cm) of the tail to a round metallic bar and suspended 30 cm above the ground, a 6-min test duration was used. A significant decrease of immobility was observed from 2-5 min during 6-min test period in homozygous COR-Nes mice compared to control mice (Figure 33).

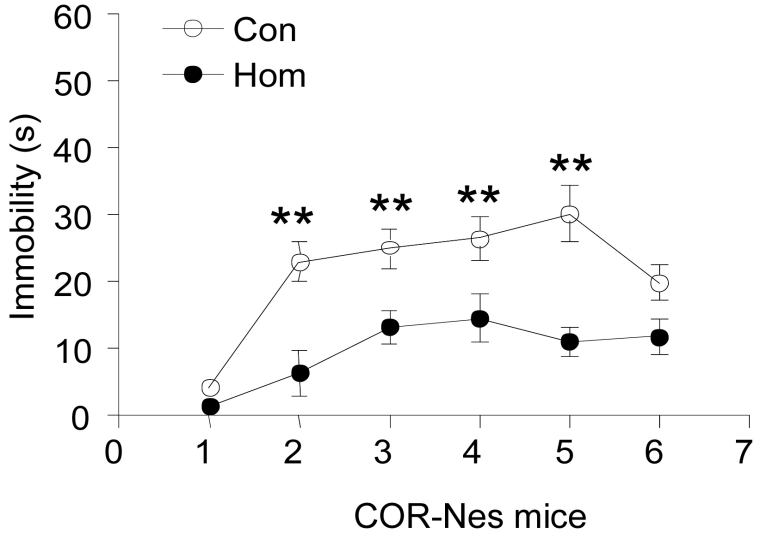

Figure 33. Immobility time during 6 min in tail suspension test in COR-Nes mice. Data were presented as mean ± SEMs, n=11, **P<0.01.

4. Discussion

4.1. Generation of site-specific CRH overexpressing mice

The CRH system is involved in mediating behavioral, autonomic, neuroendocrine, and immune responses to stress. Chronically elevated levels of CRH are implicated in human stress-related and affective disorders, including generalized anxiety disorder and major depression (Arborelius et al., 1999) (Mitchell, 1998). In previously established CRH transgenic mouse lines (Stenzel-Poore et al., 1992) (Dirks et al., 2002a). CRH overexpression results in elevated ACTH and glucocorticoids levels accompanied by symptoms of Cushing-like syndrome (Stenzel-Poore et al., 1992) (Groenink et al., 2002). These CRH overexpressing mice showed enhanced anxiety-like behavior, although it can not be excluded that part of the CRH effects on anxiety-related behavior may be mediated via its chronic activation of the HPA-system. Moreover, it has been shown that the number of copies integrated and the site of integration into the genome in classic transgenics can have an effect on the level and spatio-temporal pattern of transgene expression. To gain more insight into the relationship between hyperactivity of the CRH system and associated physiological changes in particular neuroendocrine, autonomic, and behavioral alterations/ consequences, we utilized the properties of the retroviral-trapped ROSA26 (R26) locus, which has proven as a useful tool to achieve ubiquitous expression during embryonic development and in the adult. In addition to the mCRH cDNA we introduced a IRES-LacZ reporter gene into the ROSA26 locus, the β-galactosidase (β-gal) activity constitutes an excellent marker for labeling of expressing cells (Friedrich and Soriano, 1991) (Zambrowicz et al., 1997). Homozygous ROSA26 mutant mice have no overt phenotype (Soriano, 1999).

Combining the knock-in of a single copy of the murine CRH cDNA into the ROSA26 locus with the Cre/loxP system enabled us to overexpress CRH in a spatially regulated fashion at different dosages. We developed two conditioned CRH over-expressing mouse lines: COR-Cam with CRH overexpression restricted in the anterior forebrain and COR-Nes with CRH overexpression in the entire central nervous system. In COR-Cam mice, CRH was not overexpressed in the PVN thus regulation of basal and stress-associated activation of the HPA system showed remain unaltered. These two CRH overexpressing mouse lines provide powerful genetic tools to study the site-specific and dose-dependent effect of chronic CRH hyperactivity.

4.2. Alterations in expression of CRH and CRH related genes

4.2.1. CRH mRNA and protein expression

Endogenously, the highest density of CRH-containing cell bodies was found in the nucleus ambiguus (A) and PVN, moderate density of CRH-containing cell bodies was found in piriform cortex and locus coerulus (Figure 11A and D). In COR-Cam mice, CRH was strongly overexpressed in the neocortex, hippocampus, medial septal nucleus, CeA and BNST (Figure 11B, C, E, F). In COR-Nes mice, CRH was strongly overexpressed in the neocortex, hippocampus, medial spetal nucleus, BNST, cerebellum, brainstem (Figure 12B, D, E). CRH-containing interneurons distributed in the neocortex are believed to be important in several behavioral actions of the peptide, including effects on cognitive processing (Lee and Davis, 1997); CRH was strongly overexpressed in the hippocampus, which contains different amounts of CRH-R1, CRH-R2, and CRH-BP. The hippocampus appears to be required for context-dependent (Kim and Fanselow, 1992) (Phillips and LeDoux, 1992) and tone-dependent fear conditioning (Maren et al., 1997). Hippocampal function is particularly influenced by glucocorticoids (de Kloet et al., 1999) (McEwen, 1999). By acute modulation of neuronal excitability, glucocorticoids affect hippocampus-dependent behavior such as spatial memory. Chronic changes in glucocorticoid levels impair hippocampal function and morphology, which ultimately leads to cognitive impairments (McEwen and Sapolsky, 1995). The BNST is another potential site of CRH action and potential CRH/norepinephrine interaction. CRH injected directly into the BNST enhances the startle response, and neurotoxin cell-body-specific lesions of the BNST or microinfusion of a CRH receptor antagonist blocked the effects of icv administered CRH on the startle response (Lee and Davis, 1997). Moreover, it has been proposed that CRH cell bodies in the BNST processed cue information to activates the CeA (Davis, 1998). The CeA in turn activates hypothalamic and brainstem target areas involved in conditioned fear or anxiety, respectively. In COR-Nes mice, CRH is strongly overexpressed in the cerebellum, spinal cord, and limbic parts of the telencephalon, thalamus, and autonomic-related cell groups in the brainstem. Cells and fibers in many of these sites are associated with systems that regulate the output of the autonomic nervous system. CRH projections to the brainstem (specifically LC, A1 catecholamine cell groups, and dorsal vagal complex)

and spinal cord stimulate sympathetic tone to the adrenal medulla resulting in epinephrine secretion, stimulation of sympathetic noradrenergic outflow, and inhibit parasympathetic nervous activity (Fisher, 1989).

Gold and colleagues indicated that stress initially activates the hypothalamic CRH system (i.e. CRH in the PVN), resulting in the hypersecretion of glucocorticoids from the adrenal gland. In addition, the psychological component of the stressor stimulates the amygdaloid CRH system (i.e. CRH in the central nucleus of the amygdala). Glucocorticoids exert GR-mediated negative feedback effects on the biosynthesis and release of CRH in the PVN and ACTH in the anterior pituitary (AP) directly or indirectly through the brainstem catecholaminergic nuclei such as the LC, resulting in the termination of stress-induced HPA axis activation. In the chronic phase of stress, down-regulation of GR in the PVN and other brain structures such as the LC fails to restrain hyperfunction of the HPA axis. The persistent activation of the HPA axis further up-regulates the amygdaloid CRH system involved in the expression of fear and anxiety. Additionally the amygdala may have stimulatory effects on the HPA axis. Thus, the hypothalamic and the amygdaloid CRH systems cooperatively constitute a stress-responsive, anxiety-producing neurocircuitry during chronic stress (Makino et al., 1994). Expression of the CRH system is regulated in a complex manner by glucocorticoids (Bruhn et al., 1986). There is evidence that during periods of stress, glucocorticoids may augment PVN CRH gene expression (Plotsky et al., 1986).

In a similar way to other hypothalamic peptides known to regulate their own secretion, CRH autoregulates CRH neurons in the PVN. We found that expression of CRH in the PVN was slightly decreased in COR-Cam mice, and was decreased by 12 % and 23 % in heterozygous and homozygous COR-Nes mice respectively (Figure 16A and B). In COR-Cam mice, CRH is not overexpressed in the PVN (Figure 10 B and C), leaving the HPA axis unaffected. Therefore, the decreased CRH expression in the PVN was thought as endogenous CRH. Although CRH is overexpressed in the entire CNS in COR-Nes mice (Figure 10E and F), CRH expression in the PVN was also found to be decreased. This might be due to counter regulatory effects on endogenous CRH. To understand the molecular mechanism behind this observation, further investigation is needed. These results were consistent with that of Metallothionein promoter CRH-Tg mice (Stenzel-Poore et al., 1992), Stenzel-Poore and colleagues found that in contrast to the robust expression of CRH mRNA

elsewhere in the central nervous system, the strength of the hybridization signal in the PVN was equivalent, or only marginally elevated. When they used a transgene-specific oligonucleotide probe, they found the transgene expressed in the PVN, indicating a reduction of the endogenous CRH. Decreased CRH expression in the PVN of our CRH overexpressing mice also exhibited marked glucocorticoid down-regulation.

It should be noted that the alteration of CRH expressing neurons in various regions of the brain depends on the type and intensity of the stressor. On the other hand, the effects of glucocorticoids on CRH gene expression are diverse depending on the tissue and the level of gene expression when the glucocorticoids affect the cell (Makino et al., 1994). The CRH neurons in the CeA project to the BNST, the lateral hypothalamus and midbrain reticular formation (Sakanaka et al., 1986), medial and lateral parts of the parabrachial nucleus (Moga and Gray, 1985), and the mesencephalic nucleus of the trigeminal nerve patterns. In the amygdala, the effects of glucocorticoids are not consistent *in vivo,* investigators have demonstrated that glucocorticoids can positively or negatively regulate amygdalar CRH levels (Makino et al., 1994). Kalin and colleagues (Kalin et al., 1994) reported that acute restraint increased CRH mRNA in the CeA using both RNase protection assay and in situ hybridization (Hsu et al., 1998). A study using *in vivo* microdialysis demonstrated increased CRH levels in the CeA following restraint stress or ethanol withdrawal (Merlo Pich et al., 1995) (Merali et al., 1998) (Richter et al., 2000). Some stressors, which contain more physical or metabolic components, such as salt-loading (Watts, 1992), cold or starvation (Makino et al., 2001) can lead to decreased CRH mRNA in the CeA. Chronic administration of the triazolobenzodiazepine agonist alprazolam decreased CRH mRNA in the CeA and CRH-R1 mRNA expression and receptor binding in the basolateral amygdala (Skelton et al., 2000), whereas adrenalectomy does not affect amygdalar CRH expression (Beyer et al., 1988). Expression of CRH in the forebrain (COR-Cam) increased CRH expression slightly in the CeA in heterozygous and homozygouse mice (Figure 16C), however, overexpression of CRH in the entire CNS (COR-Nes) decreased CRH expression in the CeA by 25 % and 30 % in heterozygous and homozygous mice respectively (Figure 16D).

The mechanisms underlying positive or negative glucocorticoid effects on CRH in the CeA are uncertain, but differential combinations of glucocorticoid-responsive neurotransmitters or transcription factors in the CeA or the PVN may be responsible

for it.

The careful measurement of protein levels is a critical step in understanding how environmental effects on gene expression are translated into actual changes in the organism's behavior. In the total brain, the CRH content was increased about 1.2-fold in heterozygous and 1.6-fold in homozygous COR-Cam mice (Figure 13A) compared to control mice. The CRH content was increased 1.4-fold in heterozygous and 2.1-fold in homozygous COR-Nes mice compared to control mice (Figure 13B). CRH overexpression at the protein level in various brain regions confirmed the pattern of oeverexpression observed by *in situ*. CRH is overexpressed strongly in the hippocampus, cortex and thalamus in COR-Cam mice (Figure 14A), and in the hippocampus, cerebellum, cortex and thalamus in COR-Nes mice (Figure 14B).

4.2.2. Expression of CRH-R1 and CRH-R2 mRNA

CRH acting upon its receptors at hypothalamic and extra-hypothalamic sites mediates behavioral and autonomic responses to stress (Holmes et al., 1987) (Vale et al., 1983a).

Information about the regulation of the expression of CRH itself, both in the hypothalamus and limbic regions, has been forthcoming (Hatalski et al., 1998) (Hatalski et al., 2000) (Makino et al., 1994) (Smith et al., 1997). Imaki and Mansi also found that central administration of CRH increases both CRH mRNA and CRH-R1 mRNA in the PVN (Imaki et al., 1996) (Mansi et al., 1996). CRH may be capable of up-regulating CRHR-1 and enhance its own biosynthesis in the PVN in a paracrine or autocrine manner. In this context, positive effects of CRH on its own receptor (CRHR-1), and vice versa, may represent one mechanism of persistent activation of CRH neurons in the PVN during stress. The regulation of CRH-R1 and CRH-R2 expression in many regions of brain has remained relatively unexplored. The majority of studies investigating regulation of CRH receptors have centered on the PVN. CRH-R1 mRNA expression in the PVN is extremely low, but the expression of both CRH and CRH-R1 mRNA in the parvocellular PVN is substantially increased by stress (Luo et al., 1994) (Makino et al., 1995), icv administered CRH (Imaki et al., 1996) (Mansi et al., 1996) and immune challenge (Rivest et al., 1995). Only limited information is available on the regulation of CRH receptors in limbic structures such as hippocampus, amygdala, and cortex. In essence, a single study has suggested that

chronic stress in adult rats resulted in a decrease in CRH-R1 mRNA in frontal cortex while increasing CRH-R1 expression has been found in the hippocampus (Iredale et al., 1996). Brunson and colleagues found that, compared to vehicle-treated controls after CRH administration in immature rats, CRH-R1 mRNA levels were significantly upregulated in cingulate cortex at the 4 h time point, in layers II/III of frontal cortex by 2 h (returned to control values by 4 h), in the CA3 pyramidal cell layer of hippocampus at the 4 h time point, no changes in layer V/VI of neocortex, CA1 and CA2 of hippocampus and in the amygdala.

Dysfunctioning of CRH and its receptors has been linked to the development of stress-related disorders, such as mood disorders. We analyzed CRH-R1 and CRH-R2 expression in CRH overexpressing mice, which are excellent models to analyze how chronic hyperactivity of CRH itself is able to regulate CRH receptors *in vivo*.

CRH-R1 mRNA was decreased in the neocortex, CA1, CA2 and CA3 of hippocampus and the reticular nucleus of the thalamus (RT) in both CRH overexpressing mouse lines (Figure 17 and 18). These findings may reflect one mechanism to adapt to the changed physiological demands caused by CRH hyperactivity. Such loss of CRH-R1 may be also due to increased exposure to CRH, involveing the consequences of receptor occupation and activation by CRH, as well as "downstream" cellular activation. Vale and colleagues reported that exposure of pituitary cells to CRH *in vitro* led to sustained decreases in CRH-R1 mRNA levels (Pozzoli et al., 1996). Aguilera and colleagues showed that infusion of CRH leading to increased levels of circulating peptide caused CRH-R1 down-regulation and desensitization (Aguilera, 1994). Down-regulated CRH-R1 expression in the cortex in CRH overexpressing mice is particularly interesting, because alteration of both CRH and its receptors in the cortex has been demonstrated in a number of human disorders (Imaki et al., 1993). For example, reduced CRH receptor binding capacity in frontal cortex has been reported in depressed suicide victims (Imaki et al., 1995), in whom cerebrospinal fluid CRH levels are increased (Roseboom et al., 2001). Other laboratories showed that CRH-R1 mRNA levels in the hippocampal CA3 pyramidal layer were highly enhanced by high dose icv CRH administration (Luo et al., 1994) (Makino et al., 1995). CRH-R1 mRNA was lower expressed in the CA1, CA2 and CA3 of hippocampus in our CRH overexpressing mice compared to control mice, this probably because of different patterns of stress, chronic stress in CRH overexpressing mice and acute stress (icv) by infusion of CRH.

Very limited reports exist about how CRH regulates its receptor in dentate gyrus, where new neurons are generated even in the adult. CRH-R1 mRNA was increased in the dentate gyrus in both CRH overexpressing mouse lines. This upregulation may have important physiological consequences. Brunson and colleagues found high dose of CRH infused icv to developing rats leaded to hippocampal pyramidal cell injury and contributed to stress related neurological disorder (Brunson et al., 2001).

CRH-R1 mRNA in the pontine gyrus was decreased in COR-Cam mice. When CRH overexpressed in the entire brain (COR-Nes), CRH-R1 mRNA in the pontine was decreased only in heterozygous mice, whereas, increased in homozygous mice (Figure 17B, C, E, F and Figure 18A and B). Finally, we did not find any regulation of CRH-R1 in PVN by CRH overexpression.

Bronson and colleagues found that CRH-R2 binding and mRNA levels were not altered by CRH administration (Brunson et al., 2001). Whereas, we found CRH overexpression also modulated CRH-R2 mRNA expression in both CRH overexpressing mouse lines. CRH overexpression in the forebrain (COR-Cam) increased expression of CRH-R2 in the thalamus. Increased CRH-R2 mRNA expression was also found in the septum, dentate gyrus, choroids plexus only in heterozygous COR-Cam mice. However, CRH-R2 expression in the choroid plexus was decreased in homozygous COR-Cam mice (Figure 19B and C and Figure 20A). CRH overexpression in the entire brain (COR-Nes) decreased CRH-R2 mRNA expression in the CP. CRH-R2 mRNA was increased in lateral septum and thalamus in homozygous COR-Nes mice compared to control (Figure 19E and F and Figure 20B). The decrease of CRH-R2 mRNA is most likely the consequences of the excess occupation by its ligand CRH an effect similar as observed for CRH-R1. Moreover, increased CRH-R2 mRNA expression in the cortex, septum, dentate gyrus and choroids plexus was found only in heterozygous COR-Cam mice, while CRH-R2 expression in the dentate gyrus and choroids plexus was decreased in homozygous COR-Cam mice (Figure 19A-C and Figure 20A). The two members of the CRH receptor family demonstrate differing regulatory mechanisms, consistent with their subserving distinct and separate functions.

Our results in terms of CRH receptors expression under conditions of CRH excess are different from previous reports. This is mostly likely due to the experimental setup. Other studies used (1) adult or immature rats (Luo et al., 1994) (Konishi et al., 2003). (2) stressed the mice by immobilization or forced swimming or

icv CRH administeration (Konishi et al., 2003) (Imaki et al., 1996) (Mansi et al., 1996); (3) or even studied this point *in vitro* by cell culture (Iredale et al., 1996). In our case, the results were obtained from transgenic mice. Firstly, mice are different from rats. Even for the mice, differences in CRH receptors gene expression have been observed between different strains, for example, C57BL/6 mice show a higher baseline and stress-induced increases in CRH-R1 mRNA levels than DBA mice (Giardino et al., 1996). Moreover, these various strains display a differential responsiveness to stress with regard to CRH system gene regulation. Secondly, CRH overexpression in our animal model was chronic, it was different from the acute effect of immobilization or forced swimming stress and icv exogenous CRH administeration. Finally, it is much easier to understand that the *in vivo* interactions of CRH with its receptors differed from the ones observed *in vitr*o.

CRH regulated CRH receptors in a region-specific manner. This is in agreement with the *in vitro* studies where it has been shown that CRH regulates its receptor in a cell type dependent way, for example, Duman and colleagues (Iredale et al., 1996) reported that CRH treatment decreased CRH-R1 mRNA in CATH.a cells, a neuron-derived cell line. However, Kalin and colleagues (Roseboom et al., 2001) found CRH treatment did not change CRH-R1 mRNA in the human neuroblastoma cell line IMR-32.

As CRH is considered to be the prime mediator of psychopathological processes and neuroendocine symptoms of affective disorders, strategies targeted against the biosynthesis of CRH and its receptors have a strong potential to provide insight into the underlying mechanisms.

It can be gathered from these studies that the state of knowledge about CRH receptor gene regulation in response to stress is less consistent and less well characterized than that about the CRH gene. It seems that the findings with repeated stress and CRH receptor mRNA levels are not easily reproduced across laboratories. In general, much less is known about the different experimental conditions that influence the expression of the CRH receptors genes. Some of the aforementioned discrepancies in the literature may derive from subtle methodological differences regarding the strength and, perhaps, anatomic pattern of the signal being measured. The type and duration of the stressor, the post-stress delay before animal sacrifice, the strain and age of the subjects, and the time of day for testing and sacrifice are just

among a few of the many factors that can profoundly affect the nature of stress-induced gene expression changes.

4.2.3. Expression of AVP mRNA

Evidence has accumulated during the last two decades indicating that AVP is co-expressed in some of CRH neurons. AVP plays an important role in augmenting the actions of CRH in activating the HPA axis in the chronic stress condition (de Goeij et al., 1992). *In vivo* and *in vitro* studies have shown that the rate of release of immunoreactive AVP from median eminence terminals increases in response to repeated or chronic stress (de Goeij et al., 1991) (Aguilera et al., 1993). Basal levels of CRH and AVP expression are under feedback inhibition by glucocorticoids because bilateral adrenalectomy markedly increases the number of CRH neurons that coexpress vasopressin (Ma et al., 1997). Furthermore, overexpression of AVP after adrenalectomy was suppressed by supplementation with glucocorticoids in parallel with CRH, indicating a potent inhibition effect of glucocorticoid on vasopressin expression (Itoi et al., 1987).

No change of AVP expression was observed in both CRH overexpressing mouse lines compared to respective control mice although corticorsterone levels are slightly increased. We can not explain why and how AVP escapes from glucocorticoid-mediated negative feedback. One explanation might be that chronic effect leads to adaption/normalization of AVP.

4.2.4. Expression of GR mRNA

Glucocorticoids, final products of the HPA axis, are secreted from the adrenal cortex and exert a negative feedback effect on the biosynthesis and release of CRH in the PVN and ACTH in the anterior pituitary, resulting in the termination of stress-induced HPA axis activation (Akana et al., 1992a) (Akana et al., 1992b).

Chronic stress negatively influences cognitive performance (McEwen, 1999) (McEwen and Sapolsky, 1995), and glucocorticoids participate in this effect. Long-term treatment of patients with glucocorticoids and chronic increased glucocorticoid levels in patients with Cushing's syndrome are associated with cognitive dysfunction, including memory deficits (Avishai-Eliner et al., 2002). As previously mentioned,

chronically increased glucocorticoid levels in aged rats and humans correlate with a decreased hippocampal volume and memory deficits (Lupien et al., 1998).

In addition, glucocorticoids are importantly involved in modulating fear and anxiety-related behavior (Korte et al., 1996) (Korte, 2001). Disruption of glucocorticoid receptor signaling in the central nervous system reduces anxiety-related behavior in mice (Tronche et al., 1999).

Several *in vitro* studies have shown a glucocorticoid-induced decrease in GR transcription (Okret et al., 1986) (Dong et al., 1988) (Vedeckis et al., 1989). We also found GR was down-regulated in both CRH overexpressing mouse lines. This kind of glucocorticoid-dependent reduction of GR mRNA in multiple regions in the brain may be associated with suppression of stress-induced hyperactivity of the HPA axis at the level of the PVN, anterior pituitary, but also at the hippocampal level (De Goeij et al., 1992). Thus, it has been suggested that the adaptive function of the HPA axis is critically dependent on glucocorticoid feedback mechanisms to dampen the stressor-induced activation of the HPA axis and to shut off further glucocorticoid secretion (Jacobson and Sapolsky, 1991).

Our investigation to the altered expression of CRH and molecules related to the CRH-system in CRH overexpressing mice provided new insights into their involvement in the hyperactivity of the CRH system and the HPA axis in depression. Furthermore, elucidating molecular mechanisms of persistent activation of the central CRH and system could lead to the development of more effective drugs for stress-accociated disorder by shutting down the vicious circle via multiple feedback loops upon these system, by the functional modulation of either corticosteroid receptors or CRH receptors.

4. 3. Hyperactivity of HPA axis in CRH overexpressing mice

CRH plays a major role in the regulation of basal and stress-induced activation of the HPA axis (Muglia et al., 2001). CRH stimulates the release of ACTH from corticotrope cells of the anterior pituitary gland, which in turn stimulates secretion of glucocorticoids from the cortex of the adrenal gland.

Targeted manipulations of specific CRH system elements (peptides, receptors, or binding protein) have proven as very useful to understand HPA axis biological actions during both physiological and pathological conditions. Dysregulation of the HPA axis

is a well-known phenomenon observed in depressed patients (Blackburn-Munro and Blackburn-Munro, 2001). To investigate the effect of long-term central CRH overexpression on HPA axis regulation, basal plasma corticosterone concentrations were measured. Corticosterone in the morning was slightly increased in the heterozygous and homozygous COR-Cam mice compared to control mice (Figure 24A). The increased corticosterone may be caused by long-term hypersecretion of CRH in the extra hypothalamic sites. For example, CRH cells located in the CeA project to the BNST (Sakanaka et al., 1986), the latter of which sends CRH projections to PVN, in addition, the PVN also receives CRH input from various other hypothalamic areas, as well as from the dorsal raphe (Champagne et al., 1998), subsequently leading to activation of the HPA axis. HPA axis activation resulted in increased corticosterone release. In COR-Nes mice, corticosterone in the morning was increased 1.8- and 2.4-fold in heterozygous and homozygous mice respectively compared to control mice (Figure 24B). Corticostrone levels were increased in COR-Nes mice compared to COR-Cam mice, most likely because CRH is overexpressed in the PVN in COR-Nes mice. Furthermore, CRH was much strongly and more widely overexpressed in COR-Nes mice than in COR-Cam mice. In both CRH overexpressing mouse lines, the increased corticosterone levels mediate negative feedback at multiple levels of the HPA axis to return the system to homeostasis. Especially at the level of parvocellular neurons of the PVN, increased corticosterone levels might lead to a down-regulation of CRH expression in the PVN (Figure 16A and B). These increased corticosterone secretion indicates a slightly hyperactive HPA axis. The hyperactive HPA axis was associated with CRH-R downregulation and desensitization, indicating that CRH receptor number is not a major determinant of corticotroph responsiveness *in vivo*. In presence of CRH, a small number of CRH-R1 are required for the pituitary gland to respond to most stressors (Perrin and Vale, 1999) (Turnbull and Rivier, 1997) (Venihaki and Majzoub, 1999), and other factors must be responsible for the facilitation of ACTH response during chronic stress (Aguilera, 1994). Moreover, it seems that the enhanced corticosterone levels is CRH-dose dependent. Corticosterone in the morning was slightly increased in heterozygous COR-Cam mice, and increased 1.6-fold in homozygous COR-Cam mice. Corticosterone was increased by 1.8- and 2.4-fold in heterozygous and homozygous COR-Nes mice respectively (Figure 24A and B). In contrast, the two previously published CRH overexpressing mouse lines displayed significantly elevated

corticosterone levels: 4.4-fold in Thy-1 promoter driven CRH-Tg mice (Groenink et al., 2002) and 10-fold in Metallothionin-promoter driven CRH-Tg mice respectively (Stenzel-Poore et al., 1992).

Evidences from both clinical and preclinical studies strongly supports that CRH from both hypothalamic and extrahypothalamic neurons is hypersecreted in depression. 40 years ago, Board and colleagues (Board et al., 1956) reported that plasma cortisol concentrations were elevated in a majority of patients with major depressive disorder, a finding that has been repeatedly replicated. Therefore, our CRH overexpressing mice may serve as an animal model for the HPA axis abnormalities occurring in major depressive disorder.

4.4. CRH overexpression decreased body weigh

Numerous studies demonstrate a role for CRH in appetite regulation, energy balance, and the etiology of eating disorders. Enhanced CRH within the CNS, is known to suppress appetitive behavior (Gosnell et al., 1983) (Spina et al., 1996), to decrease food consumption (Morley and Levine, 1982), to blunt energy storage by reducing energy intake and to augment energy expenditure resulting in decreased weight gain, and reduced body fat and protein content (Richard et al., 2000). CRH overexpression in the forebrain (COR-Cam) decreased body weight slightly compared to control mice (Figure 25A); CRH overexpression in the entire brain (COR-Nes) decreased body weight significantly compared to control mice (Figure 25B). Both CRH overexpressing mouse lines did not show any signs of Cushing-like syndrome even when they were 9 months old. Our mice are different compared to Stenzel-Poore group's CRH-Tg mice (Stenzel-Poore et al., 1992), which showed Cushingoid features: truncal obesity with large adipose deposits, muscle wasting, bilateral symmetric hair loss, and abnormally transparent skin. Our CRH overexpressing mice showed decreased body weight compared to control mice comparable to the second published CRH-Tg mouse line (Dirks et al., 2002b). However, the latter also showed Cushing-like syndrome after 5-6 months of age with fat deposition and hair loss. Studies from our CRH overexpressing mouse lines and previous published other two CRH-Tg mouse lines showed the correlation between body weight, Cushing syndrome and CRH content. The Metallothionein (MT) promtor driving CRH transgene overexpressed CRH in all areas of the brain and in the peripheral tissues

(Stenzel-Poore et al., 1992). Thy-1 promotor driving CRH transgene overexpressed CRH in the entire CNS. For both CRH-Tg mice, it is not known how many copies and where the transgene was inserted into the genome. In our CRH overexpressing mice, only a single copy of murine CRH cDNA was inserted into ROSA26 locus, CRH was overexpressed 1.2- and 1.6-fold in heterozygous and homozygous COR-CAM mice respectively, and 1.4- and 2.1-fold in heterozygous and homozygous COR-Nes mice. However, CRH was overexpressed 3.2 fold even in hemizygous MT CRH-Tg mice (not data shown). Therefore, our results strongly suggested that CRH overexpression decreased body weight in dose dependent manner. The Cushing-like syndrome showed by other two previously CRH-Tg mice might be caused by the significant increased corticosterone.

4.5. Acoustic startle response

The neuropeptide CRH functions as a key mediator of the responses to stress (Vale et al., 1981), it has been increasingly recognized, however, in addition to its neuroendocrine effects, CRH acts as a neuromodulator in stress-associated limbic regions to propagate and integrate stress-induced behaviors including arousal, motor function, food intake, reproduction and anxiety-related behavior (Heinrichs and De Souza, 1999) (Dunn and Berridge, 1990b). Because CRH is released from limbic neuronal terminals during stress, this regulation might play a crucial role in the mechanisms by which stress contributes to human neuropsychiatric conditions such as depression or posttraumatic stress disorder.

Chronically elevated levels of CRH in the brain appear to be associated with alterations commonly associated with major depressive disorder, as well as with sensorimotor gating deficits commonly associated with schizophrenia (Conti et al., 2002). Centrally administered CRH in rats increased acoustic startle response (Jones et al., 1998) (Liang et al., 1992) (Swerdlow et al., 1986). There are only a few reports showing that acoustic startle reactivity is increased after chronic stress (Gewirtz et al., 1998) (Servatius et al., 1994), whereas others report no alterations in startle reactivity after sustained stress (Sipos et al., 2000).

In the present study, we used startling acoustic stimuli to investigate the consequences of chronic CRH hyperactivity on sensory information processing mechanisms in mice.

4.5.1. Increased startle magnitude in COR mice was dose-dependent

The startle reflex is a fast, short-latency response, involuntary contraction of facial and body muscles accompanied by eye-lid closure as well as an arrest of ongoing behaviors and an acceleration of the heart rate, evoked by sudden and intense acoustic stimuli (Koch, 1999). The ASR has been used as a behavioral tool to assess the neuronal basis of behavioral plasticity and to model neuropathological dysfunctions of sensormotor information processing. The ASR is also enhanced in human patients suffering from anxiety disorders (Grillon et al., 1994) (Grillon et al., 1996), in humans anticipating shock (Grillon et al., 1991), in the presence of an unpleasant odor (Ehrlichman et al., 1995), or while viewing aversive pictures (Lang, 1995) (Patrick et al., 1996). As a matter of fact, an enhanced startle response is one of the diagnostic criteria for post-traumatic stress disorder (Diagnostic and Statistical Manual IV of Mental Disorders, American Psychiatric Association).

The ASR magnitude and latency are influenced by the stimulus intensity (Pilz et al., 1987) (Pilz et al., 1988), the interstimulus interval (Davis, 1970), ongoing motor behavior (Ison et al., 1986), and is variable among individuals (Plappert et al., 1993). It is also influenced by genetic differences (Glowa and Hansen, 1994), by the rhythm, by stress, fear, or other negative affective states (Davis, 1988). The ASR magnitude can be enhanced by conditioned and unconditioned aversive events (Davis et al., 1997b) (Davis et al., 1997a). It can be attenuated by the repeated presentation of startling stimuli (Davis et al., 1993). Icv infusion of CRH elicits a constellation of behavioral, physiological, and endocrinological changes normally observed after stress (Dunn and Berridge, 1990b). Interestingly, some of these changes are similar to symptoms seen in certain psychiatric disorders, such as post-traumatic stress disorder (PTSD). For example, infusion of CRH causes a profound, dose-related increase in the acoustic startle response (CRH-enhanced startle) (Swerdlow et al., 1986) (Liang et al., 1992). Similarly, patients with PTSD have elevated CSF levels of CRH and show increased startle responses under appropriate test conditions (Davis et al., 1993).

Our results showed that CRH increased startle magnitude in a dose-dependent manner. Increased startle magnitudes to increasing stimulus intensities were observed in both CRH overexpressing mouse lines (Figure 26A and B). Only mice over-expressing CRH just from a single R26 allele in the anterior forebrain (COR-Cam) showed significantly increased startle magnitude at high stimulus intensities (100-120

dB) (Figure 26A). This was consistent with previous reports that icv infusion of CRH produces a pronounced, dose-dependent increase of the acoustic startle reflex (Jones et al., 1998) (Liang et al., 1992) (Swerdlow et al., 1986). We also can say that, in some range, low dosage CRH increased startle magnitude and high dosage CRH inhibited it *in vivo*. This could explain why Thy-1 promoter CRH-Tg mice showed reduced startle reactivity (Dirks et al., 2002b). In addition, probably, it was also related to increased CRH expression in CeA in COR-Cam mice and decreased CRH expression in CeA in COR-Nes mice, because the CeA has been implicated in CRH-enhanced startle. CRH infused into CeA enhanced startle, however, electrolytic lesions of the CeA blocked CRH-enhanced startle (Liang et al., 1992). Furthermore, the difference in ASR between our two CRH overexpressing mouse lines and Thy-1 promoter CRH-Tg mice may be because of the different CRH overexpressing pattern. In Thy-1 promoter CRH-Tg mice, CRH was overexpressed strongly in cortex, CA3 of hippocampus, VMH and habenula. In our COR-Cam mice, CRH was overexpressed strongly in cortex, the whole hippocampus, dentate gyrus, amygdala, and striatum. In COR-Nes mice, CRH was overexpressed strongly in the entire brain.

The ASR is mediated by a relatively simple neuronal circuit located in the lower brainstem. Neurons of the caudal pontine reticular nucleus (PnC) are key elements of this primary ASR pathway what was confirmed by a series of studies in cats (Wu et al., 1988), rats (Koch et al., 1992) (Yeomans et al., 1993), and mice (Carlson and Willott, 1998). Detailed electrophysiological and neuroanatomical studies revealed that the subpopulation of giant (soma diameter >40 mm) reticulospinal neurons of the PnC receive direct acoustic input from different nuclei of the central auditory pathway, including the dorsal and ventral cochlear nucleus, the lateral superior olive and from neurons of the cochlear root nucleus, a ganglion located within the auditory nerve, reticular relay nuclei (RT), or the ventral tegmental area (VTA) (Kandler and Herbert, 1991) (Lingenhohl and Friauf, 1992) (Lingenhohl and Friauf, 1994) (Lee et al., 1996) (Davis et al., 1982). A long-lasting enhancement of the ASR has been shown by infusion of CRH into the PnC (Birnbaum and Davis, 1998), injection of α-helical CRH, the specific antagonist of CRH (Fendt et al., 1997) into the PnC prevented fear-potentiation of the ASR. Since the BNST projects to the PnC, it is conceivable that at least the BNST also involve the ASR. Moreover, the hippocampus is also involved in the enhancement of the ASR by CRH. Recent studies indicate that synaptic relays are interposed between the amygdala and the PnC, which are also important for fear-

potentiation of the ASR and for the enhancement of the ASR by footshocks. Therefore, it will be very important to investigate the expression of CRH in the PnC, BNST, amygdala and other brainstem regions (such as VTA, LC) in both CRH over-expressing mouse lines.

4.5.2. The CRH-R1 antagonist (NBI-30775) reversed CRH-induced startle response in COR-Nes but not in COR-Cam mice

The most influential theory of habituation is the *dual-process theory* (Groves and Thompson, 1970), which postulates the existence of two independent and opposing mechanisms in the central nervous system (habituation and sensitization), the net result of which is measured as the decline of the response magnitude across the different trials. Habituation is regarded as a form of non-associative learning, in which an animal learns to differentiate behaviorally meaningful from irrelevant stimuli. This habituation occurs during a single session of multiple stimuli (short-term or within-session habituation) and across several sessions (long-term or between-session habituation). Sensitization is the enhancement of a response following a strong stimulus that is probably mediated by heterosynaptic facilitation (Kandel et al., 1976). In another word, the term sensitization should be used for the enhancement of the ASR.

COR-Cam and COR-Nes mice did not show any normal habituation, only homozygous COR-Nes mice showed a deficit habituation in the trials 3-5 (Figure 27). Post hoc tests showed that, startle magnitude was significantly increased in block 2-3 in heterozygous COR-Cam mice (Figure 27A), and in block 5 in homozygous COR-Nes mice (Figure 27B) compared to control mice. A slightly increased startle magnitude was observed in homozygous COR-Cam and heterozygous COR-Nes mice. The increased startle reactivity suggests mechanisms of sensitization caused by CRH overexpression. This result is different with Dirks and collegues, who did not find any habituation in CRH-Tg mice. Their CRH overexpressing mice showed lower startle magnitudes than control mice through all trials (Dirks et al., 2002b). This perhaps was caused by different CRH dosage in CRH overexpressing mice as discussed above.

Recent efforts have been directed toward the development of CRH-R1 antagonists for the treatment of stress-related psychiatric disorders such as depression. The first clinical trial of such a compound was recently completed, and revealed that

administration of a CRH-R1 antagonist significantly reduced depression and anxiety scores in depressed patients (Zobel et al., 2000).

To test whether such increased startle reactivity was due to an effect of the CRH overexpression in these transgenics, the CRH-R1 antagonist (NBI-30775) was infused into the lateral ventricles prior to testing startle magnitude and habituation. Administration of a dose of 20 mg/kg of the CRH-R1 antagonist (NBI-30775) decreased CRH-induced startle response in COR-Nes mice (Figure 28). The result was consistent with Geyer and colleagues result (Risbrough et al., 2004). NBI-30775 did not decrease startle magnitude in COR-Cam mice may be because NBI-30775 attenuated the increase in startle from the high but not low dose of CRH.

4.5.3. Impaired prepulse inhibition (PPI) in COR-Nes, but not in COR-Cam mice

Prepulse inhibition (PPI) is the reduction of the startle response that occurs if the startle stimulus is preceded 30–500 msec by a distinctive, non-startling stimulus (prepulse) (Graham, 1975). It is used as an operational measure for early sensorimotor gating mechanisms (Braff and Geyer, 1990), which is a neural mechanism theorized to maintain mental and behavioral integration by inhibiting irrelevant sensory and cognitive information and motor programs (Dulawa and Geyer, 2000). Levels of PPI are considered as an index for the integrity of sensorimotor gating mechanisms by measuring the extent to which information processing routines – which is elicited by the prepulse are interrupted by the subsequent startling stimulus (Dulawa and Geyer, 2000). PPI of the ASR is reduced in a variety of neuropsychiatric disorder that are characterized by a general reduction of the ability to gate intrusive sensory, motor or cognitive information, for example in schizophrenia, Tourette syndrome, obsessive-compulsive disorder, and Huntington disease and attention deficit disorder (Braff et al., 2001). Since PPI can be studied in rodents with procedures that are nearly identical to those employed in studies with human subjects, the paradigm is widely used to examine the potential roles of specific neurotransmitters in schizophrenia, as well as in pharmacological and pharmacogenetic studies.

No difference in PPI between heterozygous and homozygous COR-Cam mice was observed compared to control with respect to the prepulse intensity and the interval between prepulse and stimulus (Figure 29A and C). Homozygous COR-Nes

mice displayed impaired levels of PPI as a function of the prepulse intensity, and there was a difference between control and heterozygous and homozygous COR-Nes mice with respect to their percent PPI as a function of the interval between prepulse and stimulus (Figure 29B and D). Administration of a 20 mg/kg dose of the CRH-R1 antagonist (NBI-30775) reversed the impaired PPI (Figure 30B). Our results were consistent with Dirks and colleagues Dirks (Dirks et al., 2002), they also found impaired PPI in CRH-Tg mice with respect to the prepulse intensity and prepulse-startle stimulus interval. Our results also fitted to previous results that acutely administered CRH reduces PPI in rats and mice (Conti et al., 2002). Independent of its effects on startle reactivity, low dose of CRH decreased PPI at low prepulse stimuli intensity, high dose of CRH decreased PPI at all prepulse stimulus intensity. These findings also indicate that both acute and chronic elevated levels of CRH have similar disruptive effects on PPI.

The brain mechanisms underlying the mediation of PPI are still not fully understood. PPI of the acoustic startle response is perhaps altered by manipulations that affect brain monoamine neurotransmission. In both CRH overexpressing mouse lines, CRH was overexpressed in cortex, striatum, hippocampus and the central nucleus of the amygdala (Chalmers et al., 1995) (De Souza, 1987) (Radulovic et al., 1998), areas shown to be important for control of PPI (Swerdlow et al., 1994) (Bakshi and Geyer, 1999). Several investigators found that the nucleus accumbens septum (NAC) is one of the centers of convergence of several transmitter systems that regulate PPI. In rats, CRH (icv) increased both dopamine (DA) utilization and DA concentration in frontal cortex, NAC, hippocampus and amydala (Kalivas et al., 1987) (Lavicky and Dunn, 1993). The NAC receives a dense dopaminergic innervation from the VTA. Excessive DA receptor stimulation or a lesion-induced DA receptor supersensitivity in the NAC reduces PPI (Swerdlow et al., 1992). The NAC also receives a direct projection from the ventral hippocampus (Groenewegen et al., 1987). PPI-disruptive effect is also seen after transsynaptic stimulation of ACh release in the ventral hippocampus via the medial septum (Koch, 1996). Moreover, Leitner and colleagues found that lesions of the inferior/or superior colliculus disrupted PPI by auditory prepulses (Leitner et al., 1980) (Leitner et al., 1981) (Leitner and Cohen, 1985) (Li et al., 1998) suggesting that the ascending auditory pathway activates a PPI circuit at the level of the midbrain. Therefore, our CRH overexpressing models might help us to understand the molecular mechanisms underlying PPI.

The neurodevelopmental hypothesis of schizophrenia suggests that an abnormal development of the brain connectivity resulting from a genetic predisposition in combination with pre- or early postnatal environmental factors (Ellenbroek et al., 1998) (Lipska and Weinberger, 2000). In our CRH overexpressing mice, the onset of transgene expression occured about 20 days after birth in COR-Cam mice, and around embryonic 7.75-day stage in COR-Nes mice. The overexpression was retained throughout adult life. Hence, the early pre-/or post-natal onset of CRH overexpression and associated consequences for neuroendocrine, neurochemical, autonomic, and developmental processes, could be regarded comparable to other developmental manipulations such as maternal deprivation, neonatal hippocampus lesion, and isolation rearing. All these manipulations result in sensorimotor gating deficits in adult life (Geyer et al., 1993) (Ellenbroek et al., 1998) (Lipska and Weinberger, 2000).

Deficits in habituation of the startle response and impaired PPI have been demonstrated in patients with several neuropsychiatric disorders, most notably schizophrenia (Braff and Geyer, 1990) (Geyer et al., 1990). The results of the present study suggest that our CRH overexpressing mouse model might be an additional animal model to study the long-term effects of early postnatal homeostatic disturbances on sensorimotor gating. The effects of centrally overexpressed CRH on impaired PPI can be reversed by CRH receptor antagonists, supporting the usefulness of CRH receptor antagonists as a potential novel class of antidepressants and/or anxiolytics.

4.6. Less freezing exhibited by COR-Nes mice in the fear conditioning test

Multiple lines of evidence indicate a role for the CRH system in the neural pathways underlying learning and memory processes. In Alzheimer's disease (AD) patients, CSF levels of CRH may correlate with the degree of cognitive impairment in that greater cognitive deficits are associated with lower CSF CRH concentrations (Pomara et al., 1989). Reports showed that CRH can modulate learning and memory, either enhancing or impairing memory in a dose dependent and site specific manner (Koob and Bloom, 1985) (Radulovic et al., 1999).

In order to characterize learning and memory capacity under conditions of chronic constitutive CRH overexpression, COR-Nes mice were analysed in the fear-conditioning, forced swimming and tail suspension tests.

In the classical fear-conditioning task, animals form an association between a neutral conditioned stimulus (CS; e.g. tone, light or odour) and an aversive un-conditioned stimulus (US; e.g. electric foot shock or air puff). As a result of this association, the CS will evoke a variety of behavioral, endocrine and autonomic responses that are typically elicited in dangerous situations (LeDoux, 2000). In the last decades, fear conditioning has emerged as a leading paradigm for studying cellular correlates of learning and memory. Icv infusion of CRH elicits a pattern of behavioral changes typically observed during states of fear or anxiety (Dunn and Berridge, 1990a). Fear is a natural, adaptive change elicited by a potentially threatening stimulus, which prepares an animal to cope with provocation. Fear generally is elicited by an identifiable stimulus and subsides shortly after its offset, and is also used as an index of freezing.

The freezing time was reduced in heterozygous and homozygous COR-Nes mice compared to control mice although the difference in both tone-freezing and context-freezing tests was not significant (Figure 31). This result was consistent with the data from the Steckler's laboratory (van Gaalen et al., 2002), they found the MT CRH-Tg mice exhibited reduced freezing time in comparision with control mice. This reduced freezing time in COR-Nes mice perhaps was related to the decreased CRH expression in the CeA. A great deal of data indicate that the amygdala is critically involved in explicit cue conditioning (Henke, 1980) (Liang et al., 1986), the CeA projects directly to hypothalamic and brainstem target areas critically involved in specific signs and symptoms of fear. In addition, the natural pattern of behaviors produced by conditioned fear can be blocked by lesions of the amygdala and produced by electrical stimulation of the amygdala (Davis et al., 1997a).

The results from fear-conditioning consisted that CRH facilitated learning and memory, enhancing retention at low doses and impairing performance at higher doses (Koob and Bloom, 1985). Long-term or intense activation of the HPA axis impairs learning and memory (Cahill et al., 1994) (Luine et al., 1993) (McGaugh, 1983). Moreover, these behavior test results were perhaps also related to decreased GR and increased glucocorticoid in CRH overexpressing mice, because blockade of brain GR impairs the storage of new information (Oitzl and de Kloet, 1992), (Sandi et al., 1997).

Mutant mice with a point mutation in GR, which obliterates binding to DNA, are unable to store learned information (Oitzl et al., 2001). Moreover, mice exposed to chronic stress and high corticosterone deteriorate in spatial learning because high corticosterone induced structural changes in the hippocampus, which may impair memory encoding and or consolidation as sustained hypercortisolemia in patients with Cushing's syndrome, depression, or aging associated with a reduced hippocampal volume and impaired cognitive performance (Starkman et al., 1992) (Sheline et al., 1996) (Lupien et al., 1998).

4.7. More activity exhibited by COR-Nes mice in forced swimming test (FST)

The forced swimming test (also known as Porsolt's test; behavioral despair test) is probably the most widely and most frequently used to assess depression and anti-depressant-related phenotypes in genetically altered mice (Cryan et al., 2002) (Porsolt, 2000) (Seong et al., 2002), largely due to its relative reliability across laboratories and it's ability to detect activity in a broad spectrum of clinically effective antidepressants (Cryan et al., 2002). This test is based on the observation that rodents, following initial escape-oriented movements, develop an immobile posture, which can be regarded as an energy-conserving or stress-coping behavioral strategy, because the animal has learned that escape is impossible (Korte et al., 1996) in an escapable cylinder filled with water. Hughes and colleagues (Hughes et al., 1978) and Prince and Anisman (Prince and Anisman, 1984) reported that vigorous swimming is a maladaptive response style to an inescapable water situation, since this behavior may facilitate energy expenditure, and hence decrease the possibility of survival due to fatigue.

Since chronic hyperactivity of the CRH system is implicated in human affective disorders, including major depression (Mitchell, 1998), we predicted increased depression-like behavior in our CRH overexpressing mice – one animal models in which antidepressant-like behavior can be detected. However, we found heterozygous and homozygous COR-Nes mice spent less time in floating and more time in struggling in 24-26 °C warm water compared to control mice (Figure 32A and B). This result is in accordance to the data from the other two CRH-Tg mouse lines (van Gaalen et al., 2002) (Dirks et al., 2001) and this finding was also in agreement with

studies showing that icv CRH administration reduces immobility time in forced swimming in rats and it has been proposed that this may also be the result of increased anxiety (Butler et al., 1990).

Furthermore, heterozygous and homozygous COR-Nes mice showed decreased floating time compared to control mice in 32 °C warm water (Figure 32C), similarly in 24-26 °C warm water, however, they spent almost the same time in struggling as the control mice (Figure 32D). In addition, all the genotypes of COR-Nes mice showed less time in floating and struggling in 32 °C than in 25 °C warm water. Therefore, we can conclude water temperature influenced the degree of immobility time in the forced swimming test. Otomo and colleagues also found that mice showed less immobility in 35 °C water than in 25 °C water. They explained this effect by the low water temperature which decrease the body temperature, resulting in decreased behavior (Arai et al., 2000).

4.8. Less immobility exhibited by COR-Nes mice in tail suspension test (TST)

The tail suspension test is theoretically similar to the FST and acute antidepressant treatment will decrease immobility in this paradigm. Advantages of this test include its ability to detect a broad spectrum of antidepressants, it is inexpensive, methodologically unsophisticated and easily open to automation. This automation enables the assessment of additional parameters such as power and energy of movement (Porsolt et al., 1987) (Steru et al., 1987). Similar to the FST, it validity is questioned by one fact that acute antidepressant treatments reverse the behavioral 'depression'. Although both of FST and TST are similar in the constructs that they purport to assess, they are probably different in terms of the biological substrates that underline the observed behavior and often offer converging data on a potential antidepressant (Porsolt, 2000) (Bai et al., 2001) (Renard et al., 2003). Further, the TST avoids any possible confounds induced by hypothermic exposure that may be problematic in the FST, especially if a targeted gene is involved in thermoregulatory processes. Heterozygous and homozygous COR-Nes mice and control littermates were analyzed, a significant decrease of immobility was observed from 2-5 min during 6 min test in heterozygous and homozygous COR-Nes mice compared to control mice (Figure 33). Dirks and colleagues found that in tail suspension test,

CRH-Tg mice exhibited similar amount of immobility with control mice (Dirks et al., 2001). The difference between two CRH overexpressing mouse lines in tail suspension test may be caused by variety of procedural parameters such as experimental system. Another explanation was the different CRH-overexpression pattern as discussed above.

As CRH overexpressing mice overproduced CRH throughout postnatal development, numerous neurochemical and developmental changes may have occurred to compensate for the increased levels of this neuropeptide. Thus, it cannot be ruled out that the observed phenotype is not the direct consequence of chronically CRH excess, but rather the result of compensatory adaptations in other neurotransmitter systems involved in startle reactivity, startle and learning plasticity.

In summary, (1) Our model of CRH overexpressing mice is different with others because only one copy of the murine CRH cDNA was inserted into the genome; (2) The CRH overexpressing mice we have developed provide an opportunity to investigate the physiological consequences of CRH overproduction. (3) Chronic CRH overproduction in the transgenic mice appeared to be associated with chronic stress-like alterations, including altered HPA axis activity, reflected in increased basal plasma corticosterone concentrations, it may lead to the development of new and selective drugs targeting a range of psychiatric disorders associated with hyperactivity of the HPA axis. (4) In addition, CRH overexpressing mice provide a means to assess the relative importance of central CRH excess and glucocorticoid elevation in modulation of behavior and endocrine function. Such studies offer strong prospects for the development and testing of novel treatment agents and specific pharmacological interventions designed to block chronic CRH and CRH-R1 to predict anti-depressive drugs effects, which may be useful in treatment of anxiety and depression patients. (5) Furthermore, the mouse model of CRH overexpression may represent an important tool for the further investigation of psychotic depression, and could be a valuable tool for research addressing the impact of chronically elevated levels of CRH on information processing. (6) Finally, CRH overexpressing mice also allow the investigation of the specific role for each element of the CRH system in modulating ingestive behaviors.

Multiple studies indicate a crucial role for brain CRH pathways in mediating anxiety-like behaviors. Icv administration of CRH increases the expression of anxiety-like behaviors in rats and mice, and CRH receptor antagonist can block

anxiogenice effects of stressful events (Britton et al., 1986) (Berridge and Dunn, 1989) (Heinrichs et al., 1992). In humans, elevated CRH levels in the CSF have been demonstrated in pathological conditions characterized by heightened anxiety, such as post-traumatic stress disorders and alcohol withdrawal (Adinoff et al., 1996), (Bremner et al., 1997) (Baker et al., 1999), so it will be very interesting to investigate anxiety-related behaviors in our CRH overexpressing mice in the future.

Moreover, it is important to note that inactivation or overexpression of a gene affects the whole organism and may result in a compensatory response. In our CRH overexpressing mice, compensatory responses can develop from the early embryonic or postnatal stage. As a result, the phenotypical characterization of CRH over-expressing mice can not necessarily reveal the function of the gene in the wild-type situation, but it shows the reaction of the organism to the modification. Occasionally, this leaded to the paradoxical situation of conflicting phenotypes or the absence of expected phenotypes. Inducible and temporally controlled expression of transgenes in adult mice will partly circumvent this problem of compensation because the period of genetic interference is shorter, and many physiological systems are setup during embryonic development. The flexibility of the system allows us to generate tamoxifen-inducible CRH overexpressing *Cre*-mouse lines with the CamKIIα promoter, which is worth achieving in the next step.

5. Summary

CRH is a key regulator of the HPA system and has a prominent role in mediating neuroendocrine, behavioral, autonomic and immune responses to stress. Chronic CRH hyperactivity is implicated in human stress-related and affective disorders, including major depression. Central administration of CRH increases anxiety-like behavior and arousal in rodents. Existing transgenic mouse models of chronic CRH overexpression displayed elevated levels of ACTH and glucocorticoids accompanied by symptoms of Cushing-like syndrome (Stenzel-Poore et al., 1992) (Groenink et al., 2002) due to their widespread expression pattern – whole body or entire CNS respectively. Therefore, it can not be ruled out that part of the observed CRH effects e.g. on anxiety-related behavior may be mediated via its chronic activation of the HPA-system and resulting elevated plasma glucocorticoid levels.

The aim of our work was to study the function of CRH at specific sites in the CNS by its site-specific overexpression utilizing the R26 locus (Zambrowicz et al., 1997), which has proven as a useful tool to achieve ubiquitous expression during development or in the adult. These mice were crossed wit CaMKII α-Cre or Nestin-Cre transgenic mice resulting in a constitutive expression of CRH in all *Cre*-expressing cells of the anterior forebrain or within the entire CNS respectively. The specific pattern of CRH overexpression was initially demonstrated by monitoring β-galactosidase reporter gene activation. The detailed pattern of CRH overexpression was analyzed on the mRNA level by *in situ* hybridization and on the protein level by region specific RIA. We found that CRH overexpression led to disturbances of the central CRH/CRH receptor system reflected by alterations in expression levels of CRH-R1, CRH-R2 and endogenous CRH in specific brain regions. Additionally, GR was also found to be downregulated in the entire brain. The basal plasma corticosterone concentration in the morning was increased slightly in heterozygous and homozygous COR-Cam mice, as well as in heterozygous (1.8-fold) and homozygous (2.4-fold) COR-Nes mice, indicating a hyperactivity of the HPA axis despite high levels of CRH overexpression. Analysis of the acoustic startle response/reflex revealed increased startle activity in both CRH overexpressing mouse lines. Additionally, COR-Nes mice showed partially impaired PPI with respect to the prepulse intensity. These effects were reversable by the CRH-R1 specific antagonist (NBI-30775). Therefore our CRH overexpressing mice could be a valuable tool for research to address the impact of chronically elevated levels of CRH on information processing, and it is also a useful model to test the clinical potential of novel therapies that target CRH and its receptors. Moreover, heterozygous and homozygous COR-Nes mice displayed less freezing in the fear conditioning paradigm, less floating but more struggling in the forced swimming test and less immobility in the tail suspension test compared to control mice. In these three tests, the effect was correlated to CRH expression levels. These results suggested that CRH overexpression mice showed more activity compared to control mice, it can be proposed to be the result of increased anxiety.

In conclusion, our CRH overexpressing mice are a suitable model of CRH chronic hyperactivity and might give us the opportunity to investigate the consequences of CRH excess in situations of chronic stress.

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