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Genetically dissecting the role of CRHR2 and UCN2 in the control of emotional behavior

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3V	third ventricle
2Cb	second cereberallar lobule
5HIAA	5-hydroxyindoleacetic acid
5HT	5-hydroxytryptamine, serotonin
$5HT_{1A}$	serotonin receptor type 1A
AAV	adenoassociated virus
ACTH	adrenocorticotropic hormone
AG	adrenal gland
AHA	anterior hypothalamic area
Amb	ambiguous nucleus,
AOB	accessory olfactory bulb
AON	anterior olfactory nucleus
Arc	arcuate hypothalamic nucleus
ASR	acoustic startle response
ASV-30	antisauvagine-30
BAC	bacterial artificial chromosome
Bar	Barrington's nnucleus
Bidest	bidestilated
BLA	basolateral amygdaloid nucleus
BNST	bed nucleus of the stria terminalis
Bp	base pair
BSA	bovine serum albumin
Camk2a	calcium/calmodulin-dependent protein kinase type II alpha chain
cc	central canal
cDNA	coding deoxyribonucleic acid

CeA	central amygdaloid nucleus
CKO	conditional knockout
cmp	counts per minute
CNS	central nervous system
CoA	cortical amygdaloid nucleus
COE	conditionally overexpressing
Cort	corticosterone
cp	choroid plexus
CPu	caudate putamen
Cre	cre-recombinase
CRH	corticotropin-releasing hormone
CRH-BP	corticotropin-releasing hormone binding protein
CRHR1	corticotropin-releasing hormone receptor type 1
CRHR2	corticotropin-releasing hormone receptor type 2
CSDS	chronic social defeat stress
Ctrl	control
Cx	cortex
DaLi	dark light box test
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DBB	dorsal diagonal band nucleus
dCTP	deoxycytosine triphosphate
Den	dorsal endopiriform nucleus
DEPC	diethylpyrocarbonate
DH	dorsal horn
DG	dentate gyrus
dGTP	deoxyguanosine triphosphate

DMF   dimethylformamide

DMH   dorsomedial hypothalamic nucleus

DMSO   dimethyl sulfoxide

DNA   deoxyribonucleic acid

dNTP   deoxyribonucleotides triphospahte

DRC   dorsal raphe nucleus caudate part

DRD   dorsal raphe nucleus dorsal part

DRI   dorsal raphe nucleus intermediate part

DRN   dorsal raphe nucleus

DRVL   dorsal raphe nucleus venrtolateral part

DTT   dithiothreitol

dTTP   deoxythymidine triphosphate

E   epinephrine

EDTA   ethylendiaminetetraacetate

EGTA   ethylene glycol tetraacetic acid

EPM   elevated plus maze test

EtOH   ethanol

EW   Edinger-Westphal nucleus

FST   forced swim test

FLP   flippase

GABA   gamma-aminobutyric acid

Gad2   glutamate decarboxylase type 2

GAD65/67   glutamate decarboxylase 65/67

Glu   glutamate

GFP   green fluorescent protein

GPe   globus pallidus

GR   glucocorticoid receptor

GRE	glucocortiocoid response element
GrO	glomerural layer of the olfactory bulb
Hip	hippocampus
HPA	hypothalamic-pituitary-adrenal axis
HPLC	high pressure liquid chromatography
Hsp90	heat shock protein 90
i.c.v.	intra cerebro ventricular
IC	interior colliculus
IO	inferior olive nucleus
KO	knockout
K_i	inhibitor's dissociation constant
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
LH	lateral hypothalamic nucleus
LS	lateral septal nucleus
LSO	lateral superior olive
MeA	medial amygdaloid nucleus
MePO	medial preoptic area
MnR	median raphe nucleus
mUCN2	mouse urocortin 2
mUCN3	mouse urocortin 3
mPFC	medial prefrontal cortex
mRNA	messenger ribonucleic acid
MS	medial septal nucleus
MVH	mediovetral hypothalamic nucleus
NAc	nucleus accumbens
NE	norepinephrine

NREM	non-rapid eye movment
NTE	NaCl-Tris-EDTA buffer
NTS	nucleus of tractus solitarus
o.n.	over night
OB	olfactory bulb
OE	overexpression
OF	open field test
opt	optic tract
PAG	periaqueductual gray
PB	parabrachial nucleus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	perifornical nucleus, paraform alhedyde
PirCx	piriform cortex
Pit	pituitary
PMV	premammillary nucleus
PPI	prepulse inhibition test
PPTg	pedunculopontie tegmental nucleus
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of hypothalamus
R	red nucleus
r/h CRH	rat/human corticotropin releasing hormone
REM	rapid eye movment
RN	raphe nuclei
RNA	ribonucleic acid
rUCN	rat urocortin 1
rUTP	ribouridine-5'triphosphate

RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
RTN	reticular thalamic nucleus
S	subiculum
S.E.M.	standard errors of the mean
SC	superior colliculus
SCP	stresscopin
SFi	septofimbria
Slc6a2	noradrenaline transporter
SN	substantia nigra
SON	supraoptic nucleus
Sp5n	spinal trigeminal nucleus
SPO	superior paraolivary nucleus
SRP	stresscopin related peptide
SSC	standard saline citrate
SWA	slow wave activity
Syn-GFP	synaptophysin-green fluorescent protein
TAE	triethanolamine
TH	tyrosine hydroxylase
TPH2	tryptophan hydroxylase type 2
TST	tail suspension test
UCN1	urocortin 1
UCN2	urocortin 2
UCN3	urocortin 3
UTR	untranslated region
VH	ventral horn
vHip	ventral hippocampus

VMH	ventromedial hypothalamic nucleus
vSub	ventral subiculum
VTA	ventral tegmental area
VTg	ventral tegmental nucleus
WT	wild-type

Abstract

Stress is one of the major risk factors contributing to development of psychiatric disorders. Corticotropin-releasing hormone (CRH), its related neuropeptides, urocortins (UCN, UCN2, UCN3) and their receptors, CRHR1 and CRHR2, are well known components of the central stress response system. CRHR2 is present in numerous limbic structures like the lateral septum, the bed nucleus of stria terminalis, the medial amygdala as well as in the raphe nucleus. It has been shown that total loss of CRHR2 function increases anxiety-related behavior and passive stress coping in mice. In addition, activation of the receptor is thought to be necessary for the proper stress recovery. Nevertheless, local manipulation of CRHR2 activation suggests that its modulatory effect on anxiety is highly dependent on the brain area where the receptor is stimulated or inhibited.

Here we assessed the impact of selective CRHR2 selective deletion form either, central nervous system (CNS), GABAergic, Camk2a positive or serotonergic neurons on emotional behavior.

First, by combining immunostaining methods with a CRHR2 reporter mouse line, CRHR2^{Venus}, we characterized the neurotransmitter identity of CRHR2 positive neurons. We found that the majority of CRHR2 expressing neurons are of GABAergic, Camk2a or serotonergic identity. In the next step we generated a set of conditional CRHR2 knockout mouse lines in which CRHR2 is removed from CNS (CRHR2$^{CKO-CNS}$) or selectively either GABAergic (CRHR2$^{CKO-GABA}$), Camk2a positive neurons during adulthood (CRHR2$^{CKO-Camk2a}$) or serotonergic neurons (CRHR2$^{CKO-5HT}$). By subjecting mutant animals to several behavioral paradigms we found that conditional knockout mice show distinct signatures of anxiety-related or/and stress coping behavior.

In the second part of this study we investigated effects of CRHR2 activation in the dorsal raphe nucleus. We showed that direct stimulation of raphe CRHR2 increases passive stress coping behavior. To figure out what structure could provide the agonist for CRHR2 in the DRN we traced neurons directly projecting to the raphe nuclei. We confirmed that noradrenergic neurons of the locus coeruleus, the native site of UCN2 expression, establish synapses in the area of the DRN. In respect to previous results we generated a second transgenic mouse line, in which UCN2 is exclusively overexpressed in noradrenergic neurons. As a consequence, animals showed decreased active stress coping behavior, what is in contrast to the phenotype observed in CRHR2$^{CKO-5HT}$ mice.

Our results indicate that central functions of CRHR2 are highly segregated between several neurotransmitter systems. Most importantly, we explicitly showed that selective deletion of CRHR2 from the serotonergic system bears some properties of anxiolytic and antidepressant actions, while its activation in the DRN promotes behavioral dispair in both naïve and in genetically modified mice (UCN2-COE^{NE}).

Zusammenfassung

Stress ist einer der Hauptrisikofaktoren für die Entstehung psychischer Erkrankungen. Das Corticotropin-releasing Hormon (CRH), seine verwandten Neuropeptide die Urocortine 1-3 (UCN1, UCN2, UCN3) und ihre Rezeptoren – CRHR1 und CRHR2 – sind bekannte Komponenten der zentralen Stressantwort. Der CRHR2 findet sich in zahlreichen limbischen Strukturen wie zum Beispiel dem lateralen Septum, dem Bettkern der Stria Terminalis, der medialen Amygdala sowie im Rahphe Kern. Es konnte gezeigt werden, dass der komplette Verlust der CRHR2-Funktion Angstverhalten und passives Stressbewältigungsverhalten in der Maus erhöht. Außerdem wird angenommen, dass eine Aktivierung des Rezeptors für eine adäquate Stresserholung wichtig ist. Nichtsdestotrotz deutet die lokale Manipulation des CRHR2 darauf hin, dass der modulatorische Effekt auf Angstverhalten stark von der Hirnregion abhängt, in welcher der Rezeptor stimuliert oder inhibiert wird.

Hier wurde der Einfluss einer Deletion des CRHR2 im gesamten zentralen Nervensystem (ZNS) oder selektiv von GABAergen, Camk2a positiven oder serotonergen Neuronen auf emotionales Verhalten untersucht.

Zuerst wurde die Neurotransmitteridentität von CRHR2 positiven Neuronen durch Kombination von Immunfärbemethoden mit einer CRHR2-Reportermauslinie – CRHR2Venus – charkterisiert. Die Mehrheit CRHR2-exprimierender Neuronen sind GABAerg, Camk2a positiv oder serotonerg. Im nächsten Schritt wurden konditionale CRHR2-Knockout-Mauslinien generiert, in denen der CRHR2 entweder im gesamten ZNS (CRHR2$^{CKO-CNS}$) oder selektiv von GABAergen (CRHR2$^{CKO-GABA}$), Camk2a Neuronen im adulten Tier (CRHR2$^{CKO-Camk2a}$) oder von serotonergen Neuronen (CRHR2$^{CKO-5HT}$) deletiert wurde. Die Untersuchung der Tiere mit Hilfe verschiedener Verhaltenstest offenbarte, dass die konditionalen Knockout-Mäuse spezifische Signaturen Angst-ähnlichen und/oder Stressbewältigungs-Verhaltens zeigten.

Im zweiten Teil der Arbeit wurden Effekte der CRHR2-Aktivierung im dorsalen Raphekern (DRN) untersucht. Es konnte gezeigt werden, dass die direkte Stimulation des CRHR2 im DRN passives Stressbewältigungsverhalten verstärkt. Um herauszufinden, welche Struktur den Agonisten im DRN bereitstellt, wurden Neuronen zurückverfolgt, die direkt zum DRN projizieren. Es konnte bestätigt werden, dass noradrenerge Neuronen des Locus coeruleus – dem Kern endogener UCN2-Expression – Synapsen in der Region des DRN ausbilden. Im Kontext der vorherigen Ergebnisse wurde eine zweite transgene Mauslinie generiert, die UCN2 ausschließlich in

noradrenergen Neuronen Überexprimiert. Als Konsequenz zeigten die Tiere ein reduziertes aktives Stressbewältigungsverhalten, was gegensätzlich zu dem Phänotyp der CRHR2$^{CKO-5HT}$ Mäuse ist.

Diese Ergebnisse weisen darauf hin, dass die zentralen Funktionen des CRHR2 spezifisch für verschiedene Neurotransmittersysteme sind. Insbesondere konnte gezeigt werden, dass die selektive Deletion des CRHR2 von serotonergen Neuronen Anzeichen anxiolytischer und antidepressiver Effekte beinhaltet, während seine Aktivierung im DRN mittels pharmakologische oder genetischer Methoden Verzweiflungs-ähnliches Verhalten.

The Three Oddest Words

When I pronounce the word Future,
the first syllable already belongs to the past.

When I pronounce the word Silence,
I destroy it.

When I pronounce the word Nothing,
I make something no non-being can hold.

By *Wisława Szymborska*
(translated by S. Baranczak & C. Cavanagh)

1 Introduction

1.1 Depression and anxiety

Mood and different forms of anxiety disorders are the most commonly occurring mental diseases. According to the World Health Organization's report from 2012 around 350 million people worldwide are suffering from one form of depression. The estimated risk to encounter an affective disorder during the lifespan is up to 15% (www.who.int). Although the different forms of depression vary between each other the core symptoms always include depressed mood and loss of interest. In addition, weight change (loss or gain), sleep disturbances (excessive sleepiness or hyposomnia), psychomotor disturbances (agitation or retardation), fatigue, feeling of guilt and worthlessness, executive dysfunctions as well as suicidal idealizations may occur (DSM-IV). Commonly depression is comorbid with anxiety disorders, their presence often precedes and accelerates the occurrence of a full blown affective illness.

Despite of great progress in the treatment of systemic diseases, e.g. hypertension or diabetes, psychiatric disorders are still the most difficult to deal with. Many reasons underlie the current difficulties with respect to successful treatment of mood disorders ranging from multifactorial nature and their frequent relation to other conditions. Unfortunately, mechanisms standing behind psychiatric disorders are still poorly understood.

1.1.1 Neurobiology of depression and anxiety disorders

Although for many centuries mood disorders were considered as a 'soul's disease' and left untreated, nowadays it is well known that mood and anxiety disorders have their biological correlates and should be appropriately medicated as soon as diagnosed. The classic monoaminergic theory of depression points out the particular role of monoamines such as serotonin, dopamine and noradrenaline in modulation of mood and anxiety. Monoamine neurotransmitters originate from cell bodies localized in monoaminergic brainstem centers, the ventral tegmental area, the locus coeruleus and the raphe nucleus, which then form respective neurotransmitter systems innervating the forebrain. It has repeatedly been shown that during periods of depression brain levels of dopamine and noradrenaline are decreased, what accounts for symptoms such as apathy, fatigue, executive dysfunctions or sleep disturbances. These can be successfully reverted by treatment with antidepressant drugs. The role of the serotonergic

system in anxiety and affective disorders deserves special attention as the changes within it are often the most prominent.

1.1.2 Serotonergic dysfunction in anxiety and mood disorders

The serotonergic system of the brain plays a unique role in modulation of mood and anxiety. It is worth to mention that only 10% of the entire body serotonin is produced in the brain, while most of it is stored in the digestive tract and blood platelets. In the brain serotonin synthetizing neurons are localized in the brainstem and divided into 9 groups: B1-B9 (Kiyasova and Gaspar, 2011). The most important and attracting major attention are groups delineated as the dorsal (DRN) and median raphe (MnR) nuclei. These densely packed serotonergic nuclei innervate most of the forebrain structures (Lowry et al., 2008).

Based on anatomy and functional topology the dorsal raphe nucleus is divided into several subregions; the rostal, dorsal, ventral, ventrolateral, intrafascicular and caudal parts (Hale et al., 2012). All of these encompass serotonergic neurons positive for tryptophan hydroxylase type 2 (Tph2), a rate-limiting enzyme responsible for synthesis of serotonin. Moreover, within the raphe complex also other neurotransmitters like GABA (Gamma-aminobutyric acid), dopamine or to lesser extent neuropeptides: corticotropin-releasing hormone (CRH), substance P, galanin and cholecystokinin are produced (Michelsen et al., 2007). According to tracing studies, dorsal and median raphe project primarily to forebrain limbic structures. Thus disturbances of DRN and MnR physiology produce pathophysiological changes in mood, clearly recognizable as symptoms of depression and anxiety (Michelsen et al., 2007;Waselus et al., 2011). In this regard decreased release of serotonin to the ventromedial prefrontal cortex results in depressed mood, feelings of guilt and worthlessness. Sleep disturbances are caused by altered serotonergic neurotransmission within the prefrontal cortex, basal forebrain thalamus and hypothalamus. Conversely, the serotonergic control of activity of the hypothalamus accounts for the weight changes observed during periods of depression, projections to the ventromedial prefrontal cortex, orbital frontal cortex and amyglada may account for suicidal ideations.

Although the first models suggested that serotonin is anxiogenic, nowadays it is assumed that it also can be anxiolytic. Two paradigms describing the impact of serotonin on anxiety are currently accepted (Graeff and Zangrossi, Jr., 2010;Gordon and Hen, 2004). The first paradigm segregates anxiety responses depending on the nature of the threat i.e. distinct or proximal. The distinct threats activate amygdala and result in response suppression e.g. freezing. In contrast proximal threats require immediate responses, e.g. escape, and involve processing within the dorsal peri-

aqueductual gray. According to that model, increased release of serotonin into the amygdala (distinct threat) induces inhibitory anxiety responses i.e. suppression of exploration. Increased serotonin release into the dPAG (dorsal periqueduqtual gray) would decrease anxiety response related to escape. The second paradigm segregates between anxiety-related behaviors and fear-related behaviors. Anxiety-related behaviors depend on the septo-hippocampal system and rely on a potential threat. Here, anxiety is reflected as 1) inhibition of exploratory behaviors, 2) replacement with approach-avoidance and 3) threat-evaluation behaviors. Serotonin released into the hippocampus inhibits the septo-hippocampal system and reduces anxiety. In contrast to anxiety, fear-related behaviors involving the amygdala and PAG, rely on immediate presence of a threat and provoke a fight or flight reaction.

Strong impact of the serotonergic system on affective and anxiety disorders has been documented by changes observed in patients. It has been shown that in patients suffering from major depression and anxiety disorders the turnover of brain serotonin is significantly elevated. In addition, post-mortem studies indicate that expression of Tph2 in suicide victims is also increased. Changes in serotonin turnover and release are associated with altered expression of serotonergic receptors. Essentially, binding to 5-HT$_{1A}$ receptor has been shown to be reduced in the dorsal raphe, the anterior cingulate cortex, ventrolateral cortex, and orbitofrontal cortex in patients suffering from major depression (Hale et al., 2012).

Up to now it is not completely clear what makes some individuals more prone to develop depression or anxiety disorders. In case of depression it is thought that the genetic risk accounts to 40% (Levinson, 2006). Most likely the interplay between genes and environment contributes to development of psychiatric disorders and stress is a significant factor in this interaction (Hornung and Heim, 2014;Keers and Uher, 2012;Uher, 2014).

1.2 Stress as an environmental factor contributes to development of psychiatric disorders.

1.2.1 Stress response

In general, stress is defined as the body's response to any kind of challenge that evokes a stress response. The stress response is a highly preserved reaction preparing an organism to deal with the stressor by either active or passive coping; fight or flight reaction. The stress response starts

in the hypothalamus where the decision of initiation of the stress reaction is made. On its way it involves multiple organs and tissues, like pituitary and adrenal glands. Their graduate stimulation forms a self-regulating loop called hypothalamus-pituitary-adrenal (HPA) axis (Figure 1).

Hypothalamus-pituitary-adrenal axis
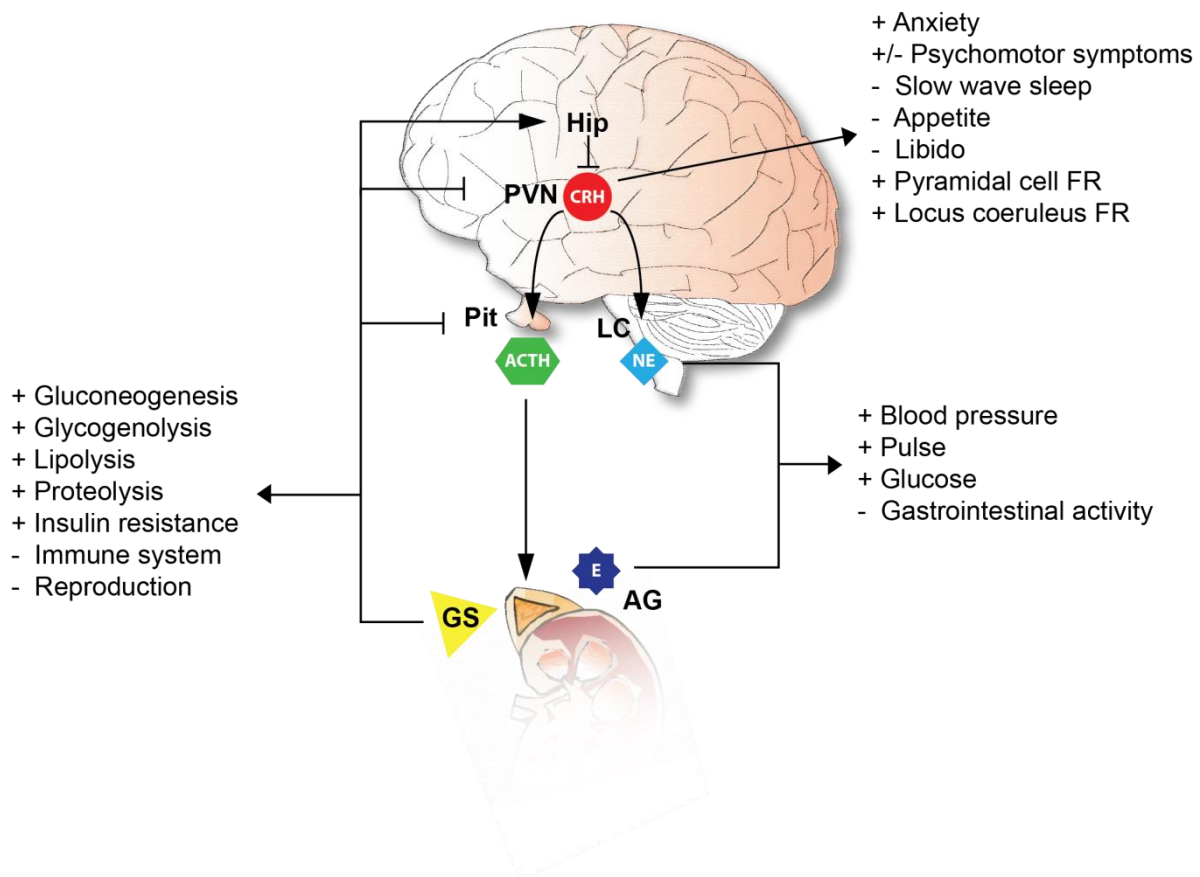
Figure 1. HPA axis regulation and its peripheral effects.
Activation of the HPA axis starts in the brain with release of CRH from the PVN. The main peripheral mediators of the HPA axis are glucocorticoids which also mediate a negative feedback. PVN, Paraventricular nucleus of hypothalamus; Pit, pituitary; CRH, corticotropin-releasing hormone, ACTH, adrenocorticotropic hormone; GS, glucocorticoids; AG adrenal glands; E, epinephrine; NE norepinephrine, LC locus coeruleus; Hip, hippocampus; FR, firing.

Upon the stress exposure the paraventricular nucleus of the hypothalamus (PVN) releases CRH into the anterior pituitary gland. In turn the anterior pituitary releases the adrenocorticotopin hormone (ACTH) into the blood stream. ACTH reaches the adrenal glands stimulates production and future release of glucorticoteroids, cortisol in primates or corticosterone in rodents. In parallel to the HPA axis activation, the parasympathoadrenomedulary system is stimulated.

Glucocorticoids serve as a negative feedback message to the pituitary and brain. They decrease the activation of the PVN and inhibit the HPA axis. Cortisol and corticosterone display a wide range of actions from metabolic effects aiming to mobilize energy sources through increasing glycogenolysis, gluconeogenesis, lipolysis, proteolysis and insulin resistance, decreasing immunological response to psychological effects such as changes in attention, memory, arousal or sleep pattern and increasing fear and anxiety. Effects of glucocorticoids are mediated by two types of nuclear receptors, namely the mineralocorticoid and glucocorticoid receptors. Under basal, non-stress, conditions the activity of the HPA axis depends mostly on mineralocorticoid receptors which show high affinity towards gluco- and mineralocorticoids. With increasing concentration of circulating glucocorticoids, glucocorticoid receptors are gradually activated producing both immediate and long-lasting cellular changes.

1.2.2 Regulation of the HPA axis

As the stress response involves numerous tissues, its effects are long-lasting and costly for the organism, it is crucial to maximally optimize mechanisms governing its initiation and termination. The regulation of the HPA axis depends on multiple levels of signal integration and transduction. It comprises stressor perception and recognition within the cortical and subcortical brain centers, passing the information into integration centers to finally evoke changes in neuronal excitability, neuropeptide release and gene expression.

1.2.2.1 Afferent projections to the PVN – initiation and integration of the stress signal

The PVN is the most important site of HPA axis activation; here the final decision about the humoral stress response takes place. The PVN consists of the medial parvocellular part which expresses and releases CRH and vasopressin (AVP), the posterior magnocellular division which produces oxytocin and vassopresin, the dorsal, medial and lateral regions of the parvocellular subregions, which project to the brainstem and spinal cord and integrate information passed on to the peripheral nervous system (Herman et al., 2002).

It has been shown that the regulation of the stress response and HPA axis activity depends on many, often distinct, brain regions (Figure 2); the later septum, hippocampus, ventral subiculum, central and medial nucleus of the amygdala, prefrontal cortex and most importantly the paraventricular nucleus of the hypothalamus itself (Choi et al., 2007;Choi et al., 2008b;Choi et al., 2008a;Herman et al., 2005;Herman and Mueller, 2006;Singewald et al., 2011).

Figure 2. Hierarchical arrangement of stress-related pathways.
Green arrows indicate stimulation of targets, red and brown dashed lines indicate inhibition of targets. AHN, anterior hypothalamic nucleus, BNST bed nucleus of stria terminalis, CeA central amygdaloid nucleus, DMH dorsomedial hypothalamus, Glu glutamate, LS lateral septum, MeA medial amygdaloid nucleus, mPFC medial prefrontal cortex, NE norepinephrine, NTS nucleus of tractus solitarus, PBN parabrachial nucleus, PMV premammillary nucleus, POA preoptic area, PVN paraventricular nucleus of hypothalamus.Adapted from (Herman et al., 2005).

Although numerous brain sites are stimulated during stress not all outsent information reaches the PVN. The majority of brain structures connect with the PVN indirectly through relay centers, i.e. the bed nucleus of the stria terminalis (BNST), the nearest vicinity of the PVN - peri-PVN, or the nucleus of the tractus solitarus. Despite that, PVN neurons express a wide range of receptors such as serotonergic, adrenergic alpha receptors, glutamatergic NMDA receptors and GABAergic receptors, most of the direct input to the PVN is inhibitory in its neurotransmitter nature. For instance the posterior part of BNST, the peri-PVN, the dorsomedial hypothalamus and the preoptic area mostly consist of GABAergic neurons projecting directly to the PVN. This kind of inhibitory input modulating the PVN's CRH positive neurons can serve a tonic, dynamic or mixed

capacity. Due to that the BNST mediated brake on PVN neurons can be modulated in both directions depending on the nature of signals reaching the BNST. The BNST receives stimulatory, glutamatergic input from the ventral subiculum. Upon activation of vSub, the GABAergic brake on the PVN is intensified, blunting the humoral stress response. On the contrary, GABAergic input to the BNST originating in the MeA causes local inhibition of BNST GABAergic neurons resulting in disinhibition of the PVN.

One third of the entire input to the PVN is GABAergic, nevertheless stimulatory projections to CRH positive neurons are also present (Cullinan et al., 2008). It has been shown that the NTS, anterior hypothalamic nucleus and ventral premammillary nucleus provide strong stimulatory, glutamatergic input to the PVN enhancing its activity (Herman et al., 2004). Also serotonergic and noradrenergic neurotransmission stimulate CRH release (Jorgensen et al., 1998).

1.2.2.2 Termination of the stress response

De novo synthetized glucocorticoids are systemic messenger of the humoral stress response. Along with the increasing concentration of circulating glucocorticoids inhibition of the HPA axis take place. Actions of glucocorticoids are primarily mediated by a glucocorticoid receptor complex comprising the steroid receptor and various chaperones including heat shock proteins. Upon binding of cortisol cytoplasmatic glucocorticoid receptors release chaperones, form homodimers and translocate to the cell nucleus. There they either activate gene expression through binding to specific glucocorticoid response elements (GRE) on DNA strands or repress it by direct interaction with DNA or interference with transcription factors. Besides transcriptional effects, activated GRs exert fast forward responses mediated by their interaction with ion channels and other membrane proteins. Although GRs are expressed in almost the entire brain, the highest concentration is found in hippocampus, cortex, hypothalamus and the DRN. Most important for the negative feedback regulation of the HPA axis are GRs localized in the hippocampus and cortex as their stimulation decline activity of CRH positive neurons in the PVN and enable termination of the stress response. Nevertheless glucocorticoids might inhibit the HPA axis acting on GRs localized directly in the PVN and pituitary (Laryea et al., 2013;Schmidt et al., 2009;Sterlemann et al., 2008).

1.3   Beyond the monoaminergic theory of depression

The corticosteroid receptor hypothesis of depression was first introduced by Holsboer and it underscores the special role of the stress system in anxiety and depression (Holsboer, 2000). According to this, chronic activation of glucocorticoid receptors during periods of stress accounts for morphological and functional changes in the CNS, like decreased volume of the hippocampus and shrinking of neuronal processes, which cause increased anxiety-like behavior and vulnerability to develop depression-like behaviors. Indeed clinical data from a subpopulation of depressive patients indicate dysregulation of the HPA, axis i.e. basal hypercortisolemia and resistance to the dexamethasone suppression test. In depressed patients CRH levels have been shown to be increased in the cerebrospinal fluid. In addition increased expression of CRH was found in the PVN of patients suffering from depression along with enhanced CRH binding sites in the prefrontal cortex of suicidal victims. Taking together changes in the central CRH/UCN1 system along with a dysregulation of the HPA axis were shown to coincidence with episodes of depression, indicating importance of that system in regulation of mood and anxiety (Holsboer, 2000).

1.3.1   The CRH/UCN system in the brain

The CRH/UCN1 system in the brain (Figure 3) consists of ligands, i.e. CRH, urocortin 1 (UCN1), urocortin 2, also called stresscopin-related peptide (UCN2, SRP) and urocortin 3 also called stresscopin (UCN3, SCP) as well as their cognate receptors, i.e. CRHR1 and CRHR2 (Risbrough and Stein, 2006;Reul and Holsboer, 2002). Beside CRH receptors, CRH and UCN1 bind to the corticotropin-releasing hormone binding protein (CRH-BP), which is the biggest reservoir for CRH within the brain (Keck et al., 2005).
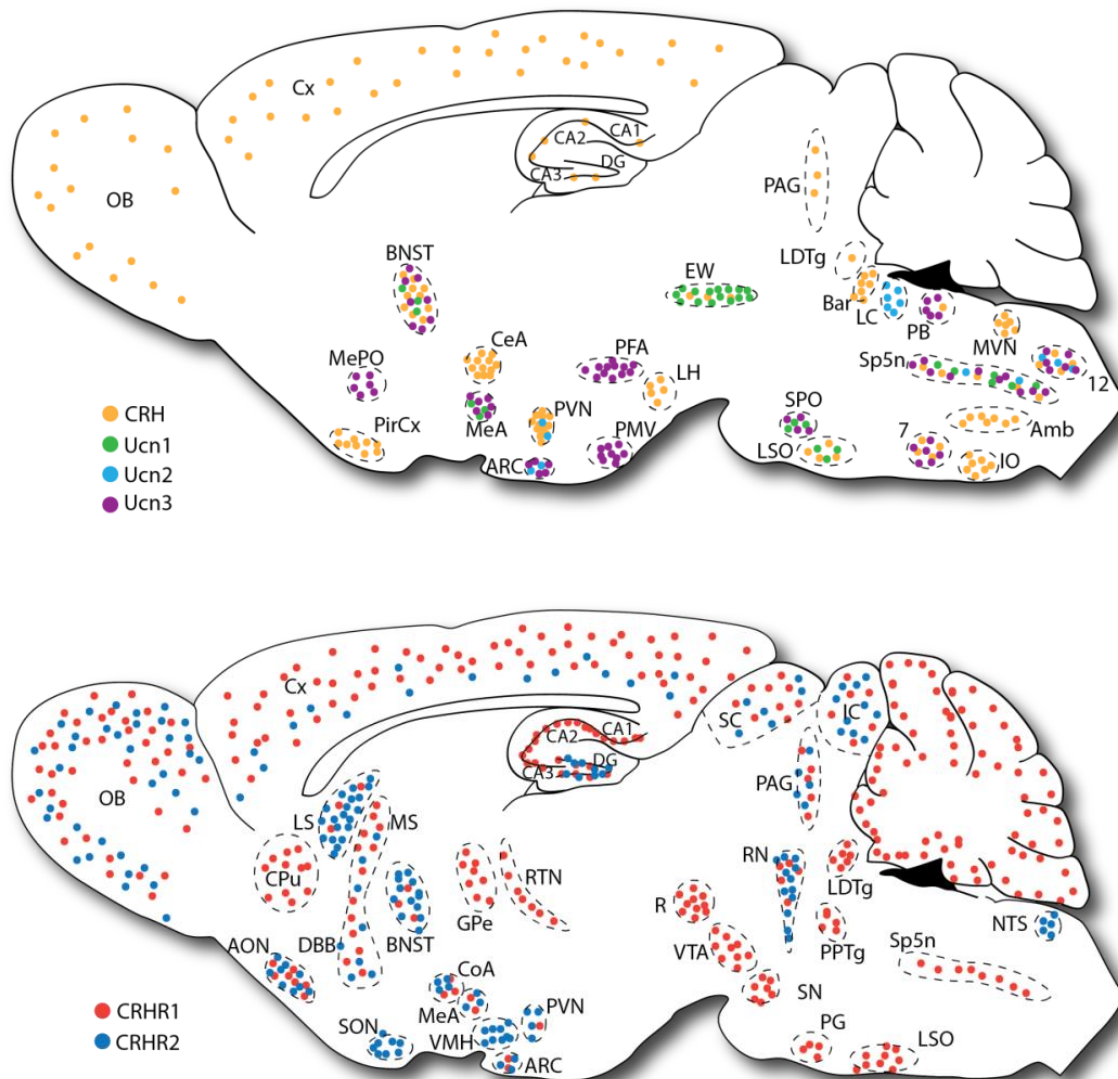
Figure 3. Expression pattern of CRH/UCN system components.

A, expression of CRH-related ligands B, expression of CRH receptors in murine brain. Amb ambiguous nucleus, AON anterior olfactory nucleus, Arc arcuate hypothalamic nucleus, Bar Barrington's nnucleus, BNST bed nucleus of the stria terminalis, CeA central amygdaloid nucleus, CoA cortical amygdaloid nucleus, CPu,caudate putamen, Cx cortex, DBB dorsal diagonal band nucleus, DG dentate gyrus, EW Edinger Westphal nucleus, GPe globus pallidus, IC interior colliculus, IO inferior olive nucleus, LC locus coeruleus, LDTg laterodorsal tegmental nucleus, LH lateral hypothalamic nucleus, LS lateral septal nucleus, LSO lateral superior olive, MeA medial amygdaloid nucleus, MePO medial preoptic area, MS medial septal nucleus, MVH mediovetral hypothalamic nucleus, NTS nucleus of tractus solitarus, OB olfactory bulb, PAG periaqueductual grey, PB parabrachial nucleus, PFA perifornical nucleus, PirCx piriform cortex, PMV premammillary nucleus, PPTg pedunculopontie tegmental nucleus, PVN paraventricular nucleus of hypothalamus, R red nucleus, RN raphe nuclei, RTN reticular thalamic nucleus, SC superior colliculus, SN substantia nigra, SON supraoptic nucleus, Sp5n spinal trigeminal nucleus, SPO superior paraolivary nucleus, VMH ventromedial hypothalamic nucleus, VTA ventral tegmental area. Adapted from (Reul and Holsboer, 2002).

1.3.1.1 CRH and its role in anxiety and mood disorders

CRH is a small 41-amino acid neuropeptide expressed in peripheral tissues i.e. lungs, adrenal glands, heart, skin and most importantly in the central nervous system. In the rodent brain the highest concentration of CRH positive neurons is found in the PVN, in the neocortex, LS, BNST, the parabrachial nucleus, the dorsal vagal complex, the central nucleus of amygdala, the Edinger-Westphal nucleus, the inferior and superior olivary nucleus, the Barrington nucleus but also in the raphe nuclei and the locus coeruleus (Wang et al., 2011). Most important for the initiation of the humoral stress response are CRH positive neurons of the PVN. However, it has repeatedly been shown that CRH present in other limbic structures accounts for anxiety- or depression-related behaviors, increased acoustic startle response and deficits in information gating (Lu et al., 2008;Regev et al., 2011;Regev et al., 2012;Sink et al., 2013;Liang et al., 1992a). Insight to the involvement of CRH in regulation of mood and anxiety was obtained from pre-clinical studies utilizing genetically modified animals and pharmacological experiments. Crucially it has been shown that i.c.v. application of CRH, induces anxiety-like behaviors and increases the startle amplitude in naïve animals confirming its primary impact on behavior (Jones et al., 1998;Liang et al., 1992b;Spina et al., 2002). The first animal models of CRH overexpression (CRH-OE) displayed signs of HPA axis hyperactivation such as increased basal levels of ACTH and corticosterone. Due to that CRH-OE mice develop a Cushing's syndrome-like phenotype, i.e. excess fat accumulation, muscle atrophy, thin skin and hair loss. Moreover, CRH-OE mice exhibit significantly increased anxiety-related behavior (Heinrichs et al., 1997;van Gaalen et al., 2002;Coste et al., 2001;Groenink et al., 2002). In line with this finding, conditional overexpression of CRH in all peripheral tissues and in the central nervous system fully recapitulates outcomes from CRH-OE mice in terms of anxiety-related behavior and dysregulation of the HPA axis. Additionally, when overexpression of CRH is further restricted to the pituitary, conditional CRH overexpressing mice still show some features of HPA axis hyperactivity, yet no change in anxiety-like behavior (Dedic et al., 2012). On the other hand, in mice conditionally overexpressing CRH either in the central nervous system, in GABAergic neurons of the forebrain or in principal forebrain neurons no change in basal anxiety-related behavior was found. Instead central CRH overexpression decreases passive stress coping behavior (Lu et al., 2008). The same phenotype is observed when exogenous CRH is applied i.c.v into naïve animals (Dunn and Swiergiel, 2008;Swiergiel et al., 2008). Besides transgenic mice overexpressing CRH continuously throughout their lifespan also site-specific overexpression models, generated by infusion of viral constructs in adult animals,

were described highlighting the special role of CRH in modulation of different aspects of anxiety and depression-like behavior in particular brain regions, especially in BNST and CeA (Keen-Rhinehart et al., 2009;Regev et al., 2011;Regev et al., 2012;Sink et al., 2013). In contrast to CRH overexpressing animals showing obvious behavioral phenotypes under basal settings, mice deficient in CRH, although not able to activate the HPA axis, do not display gross behavioral alterations neither under stress nor non-stress conditions (Dunn and Swiergiel, 1999;Muglia et al., 1996;Muglia et al., 2001;Venihaki and Majzoub, 2002;Sterlemann et al., 2008;Weninger et al., 1999b). Despite the obvious fact that CRH KO mice do not produce any CRH they still respond positively to blockade of CRH receptors. In a study by Weninger and colleagues CRH KO mice were presented a foot shock followed by infusion of CRH receptor antagonist (Weninger et al., 1999a). The freezing behavior was scored afterwards. Unexpectedly mice pretreated with non-selective as well as CRHR1 selective antagonist showed a decreased percentage of time spent freezing, raising the possibility of compensatory effects within the CRH system, i.e. excess production of other ligands taking over the role of CRH. Described experiment also emphasizes the special role of CRH receptors in anxiety and the stress response, which are still functional despite the lack of CRH.

1.3.2 CRH receptors

Actions of CRH and related peptides are mediated by two G protein-coupled, 7 transmembrane receptors, CRHR1 and CRHR2. Although they share 70% of amino acid homology and both are transmembrane metabotropic receptors they differ in distribution (Figure 3), affinity to CRH and urocortins, and thus most likely in function (Deussing and Wurst, 2005;Groenink et al., 2008;Radulovic et al., 1999;Reul and Holsboer, 2002;Risbrough et al., 2003).

1.3.2.1 Corticotropin-releasing hormone receptor type 1 (CRHR1)

Since CRH was discovered it was obvious that its effects must be mediated through some kind of receptor, but back that time probably nobody suspected what a big career its most prominent receptor, CRHR1, would make in the field of the stress neurobiology.

CRHR1 consists of seven transmembrane domains and is coupled to the G_s protein (Spiess et al., 1998). It is expressed in high concentration in the CNS and in the peripheral tissues such as skin

or pituitary (Kuhne et al., 2012). In the CNS CRHR1 was localized mostly in the cortex, olfactory bulb, hippocampus, different amygdala divisions, basal ganglia, reticular thalamic nucleus, arucate nucleus, periaqueductal gray as well as in the cerebellum (Kuhne et al., 2012;Potter et al., 1994;Van Pett K. et al., 2000). In addition, based on available co-localization studies and deletion patterns of CRHR1 in conditional CRHR1 mutant mice, it is known that the majority of CRHR1 is expressed in glutamatergic neurons (Refojo et al., 2011). It is thought that CRHR1 is the main mediator of CRH actions, as its affinity toward this type of receptor is many-fold higher than toward CRHR2. In addition, the majority of CRH effects can be reversed by treatment with selective CRHR1 antagonists (Spiess et al., 1998;Takahashi, 2001;Bittencourt and Sawchenko, 2000). There is no doubt that the strongest link between mood, anxiety disorders and stress comes from animal studies engaging CRHR1 blockade or deletion in conjunction with CRH application or overexpression. Among others it has been shown that the central application of CRH, increases the acoustic startle response as well as, similarly to CRH overexpression, decreases prepulse inhibition and both of these phenotypes can be successfully reversed by treatment with CRHR1 antagonist (Chalmers et al., 1995;Dirks et al., 2003;Groenink et al., 2008;Lee and Davis, 1997b;Liang et al., 1992a;Liang et al., 1992b;Risbrough et al., 2004). On the other hand mice lacking CRHR1 showed decreased anxiety-like behavior. Nevertheless, knowing the importance of CRHR1 in activation of the HPA axis, the impaired hormonal stress response leading to decreased release of glucocorticoids for long time confounded the interpretation of these results (Contarino et al., 1999;Smith et al., 1998;Timpl et al., 1998). The final answer was provided by Mueller et al. with generation of conditional CRHR1 knockout mice. In these mice CRHR1 is removed from forebrain principal neurons, resulting in decreased anxiety-like behaviors, but unaffected in the pituitary preserving intact functionality of the HPA axis and thus a physiological stress response (Muller et al., 2003). In addition, conditional CRHR1 knockout mice unveiled that in some instances activation of CRHR1 might also have anxiolytic consequences (Refojo et al., 2011). Using a transgenic knock-in mouse reporter line where GFP (green fluorescent protein) is expressed in CRHR1 neurons, Refojo at al. first identified the neurotransmitter identity of neurons expressing CRHR1 in order to create a series of conditional CRHR1 knockout mutants using Cre/LoxP system-mediated gene disruption. Along this line glutamatergic, GABAergic, dopaminergic, serotonergic and central nervous system specific conditional CRHR1 knockout mice were generated. As expected glutamatergic CRHR1 knockout showed decreased anxiety-related behavior, but surprisingly deletion of CRHR1 in dopaminergic neurons resulted in an opposite phenotype, i.e. increased anxiety-related behavior (Refojo et al.,

2011). This study highlighted for the first time that activation of CRHR1 depending on its localization within the brain and neurotransmitter system, might produce opposite behavioral outcomes. Deciphering such mechanisms by using conventional genetic tools, where a protein or gene of interest is removed not only from the entire brain, yet from the whole organism, is impossible. Taking into account the complex nature of the brain, its precise organization and wiring, conditional mutagenesis plays a crucial role in investigating functions of brain molecules and their contributions to modulation of behaviors and mental processes.

1.3.2.2 Corticotropin-releasing hormone receptor type 2 (CRHR2)

CRHR2 is the second type of transmembrane receptor through which CRH may exerts its actions. Up to now 4 splice variants of CRHR2 were identified, i.e. CRHR2$_\alpha$, CRHR2$_\beta$, CRHR2$_\gamma$, and short, soluble cytoplasmatic form (Catalano et al., 2003;Chen et al., 2005). Moreover, these splicing variants differ not only in the length of translated protein but most importantly in localization within the body. The CRHR2$_\alpha$ variant is predominantly expressed in the central nervous system while the CRHR2$_\beta$ variant is present in the peripheral tissues. The CRHR2$_\gamma$ splice variant is present only in humans and has not been found in other species so far (Kostich et al., 1998).

In striking contrast to the central expression of CRHR1, CRHR2 mRNA is present mostly in subcortical structures and to far lesser extent in cortical layers. Within the forebrain CRHR2 mRNA is present in the olfactory bulb, the deepest layers of cortex, claustrum, endopiriform nucleus, subiculum and the dentate gyrus of hippocampus, in the medial and cortical amygdala, the lateral septum, the posterior part of the bed nucleus of the stria terminalis, septofimbria, nucleus accumbens, preoptic and posterior paraventricular nucleus, medial preoptic area, ventromedial hypothalamus, premammillary nucleus, supramammillary nucleus and in the lateral hypothalamus. In the brainstem CRHR2 mRNA was described to be expressed in the inferior colliculus, mesencephalic nucleus, nucleus of the solitary tract and most importantly in the dorsal and medial raphe nuclei (Van Pett K. et al., 2000;Day et al., 2004;Chalmers et al., 1995;Bittencourt and Sawchenko, 2000).
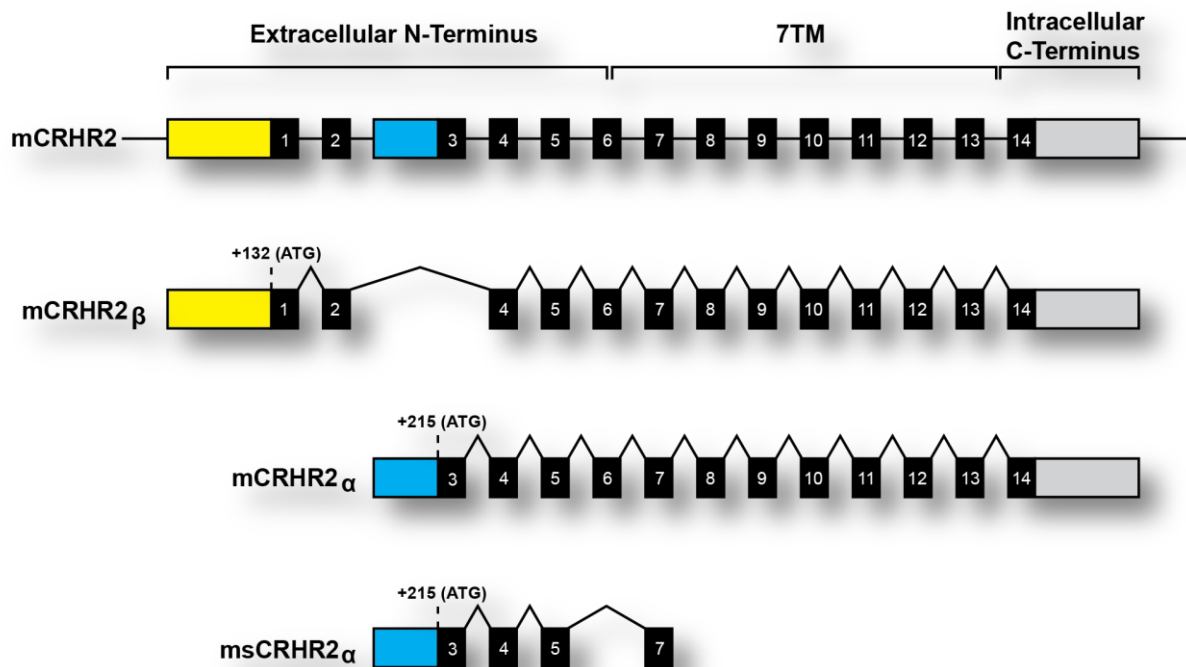
Figure 4. Splice variants of the mouse CRHR2 gene.
Black boxes exons, yellow, blue and gray boxes, 5' UTR and 3' UTR, 7TM 7 transmembrane region. Adapted from (Chen et al., 2005).

In accordance with the distribution pattern it has been confirmed that within the lateral septum CRHR2 mRNA is present in GABAergic neurons and in the dorsal raphe nucleus majority of neurons expressing CRHR2 mRNA are serotonergic (Anthony et al., 2014;Day et al., 2004).

In peripheral tissues CRHR2 was localized in the pituitary, heart and skeletal muscles, veins, skin, lungs, pancreas and digestive tract (Kageyama et al., 2003;Van Pett K. et al., 2000;Kuperman et al., 2011). Interestingly, CRHR2 is also found in the choroid plexus, a structure present in the brain ventricles and responsible for production and clearance of CSF.

1.3.2.2.1 CRHR2 and its role in stress response regulation

Since CRHR2 was first cloned in 1995 by Lovenberg et al. (Anthony et al., 2014;Lovenberg et al., 1995) and its expression pattern determined within the brain, it became obvious that it is involved in regulation of emotional, hormonal and behavioral stress responses. Indeed, localization within structures controlling the HPA axis regulation (LS, pBNST, peri-PVN or subiculum) processing the emotional information (mPFC) or social behavior (MeA) or even food intake (LH and MVH) resulted in many publications concerning its functions within these brain

regions and its impact on behavior. Importantly the role of CRHR1 was already established as anxiogenic and 'pro-depressive receptor'. Five years after publication of Lovenberg's study, three independent groups generated and described the first CRHR2 knockout mice (Bale et al., 2000;Coste et al., 2000;Kishimoto et al., 2000). Although in all three models of CRHR2 deficiency expression of CRHR2 is disrupted by truncating transmembrane domains either 4 to 7, 3 to 5 or 5 to 7, a clear sex-independent behavioral phenotype was found only by Bale et al. (Bale et al., 2000). They showed that the deletion of CRHR2 in both males and females increases anxiety-like behavior, measured in the elevated plus maze test as well as in the open field test, and dysregulates HPA axis making mice significantly more sensitive to restraint stress (Bale et al., 2000). In line with this publication, Kishimoto et al. also observed increased anxiety-related behavior, however, only in male CRHR2 KO mice. These results were further supported in pharmacological experiments included in this study in which naïve mice were pretreated i.c.v. with anti-sauvagine-30 a selective CRHR2 antagonist. Animals which received anti-sauvagine-30 showed increased anxiety-like behavior in the elevated plus maze test. No change in HPA axis sensitivity was detected between CRHR2 KO and WT mice (Kishimoto et al., 2000). In contrast to both previous studies Coste et al. found no behavioral difference between CRHR2 KO and WT mice, although they confirmed the dysregulation of the HPA axis in CRHR2 KO mice similar to Bale and colleagues (Bale et al., 2000;Coste et al., 2000).

Behavioral differences in these three models of CRHR2 knockout might be explained in many ways. First of all, the background strain and age of animals, their handling, housing and differences in protocols of performed tests might account for the observed discrepancies. The order of the tests and time intervals between them, the long term adaptation mechanisms and finally differences in breeding schemes resulting in differential maternal care may also confound the final results. Possible influence of mothers genotype on maternal care was further investigated by Bale et al., who showed that either full or even partial, heterozygous, deletion of the CRHR2 gene in a mouse dam results in increased anxiety-like behavior in the male offspring independently of genotype, when tested in adulthood (Bale et al., 2002a).

Independently of anxiety-related behavior it was numerously shown that the full deletion of CRHR2 impairs the stress-coping behavior inducing a state of behavioral despair (Bale et al., 2000;Coste et al., 2006;Liebsch et al., 1999;Todorovic et al., 2009). The latter is supported by a study where CRHR2 antisense oligonucleotides applied to naïve rats mimicked the phenotype of CRHR2 KO mice i.e. increasing the time spent floating in the forced swim test (Liebsch et al.,

1999). On the other hand i.c.v. infusion of urocortin 3 fragments, a selective CRHR2 agonist, has been shown to increase active stress coping behavior in the FST (Tanaka et al., 2011b).

Taken together, it seems that the role of CRHR2 in behavioral and emotional stress regulation is opposite to the role of CRHR1. Since activation of CRHR1 is necessary to begin the stress response, stimulation of CRHR2 is required for proper stress extinction and to initiate the recovery process. This view is also supported by series of following pharmacologic and genetic studies.

1.3.2.2.2 Stress recovery - CRHR2 in the bed nucleus of the stria terminalis

Despite the complex nature of pharmacological experiments involving application of CRHR2 agonists and antagonist it has been shown that central application of urocortin 3, and its biologically active fragments, decreases anxiety-related behavior and increases active stress-coping behavior (Tanaka et al., 2011a;Telegdy et al., 2011;Valdez et al., 2003;Tanaka and Telegdy, 2008b). In addition, human urocortin 2, another selective CRHR2 agonist, is able to induce delayed anxiolytic-like effects (Valdez et al., 2002) and activation of septal CRHR2 decreases electric shock-induced freezing without disturbing general locomotion or promotion of analgesia (Bakshi et al., 2002). In this regard it was shown that CRHR2 KO mice are not able to recover properly from the stress event proceeding behavioral testing (Henry et al., 2006;Issler et al., 2014). Inability to initiate a proper recovery processes after stress not only increases the risk for mood disorders but in some extreme situations, especially when a stress event is of great magnitude, might lead to development of post-traumatic stress disorder (PTSD). PTSD is one of anxiety-spectrum disorders characterized by frequent re-experience of trauma, hyperarousal, hypervigilance, insomnia or nightmares, increased startle response and hypocortisolemia. It was proposed that this sort of sustained fear is controlled by the BNST. The posterior part of the BNST, consisting mostly of GABAergic neurons, is especially rich in CRHR2 and plays an intriguing role in susceptibility to PTSD and regulation of its symptoms (Elharrar et al., 2013;Lebow et al., 2012;Van Pett K. et al., 2000). Along this line, it has been shown that animals which are more susceptible to develop PTSD-like symptoms show decreased expression of CRHR2 mRNA in the posterior part of the BNST following the fear-incubation process, i.e. re-exposure to a cue reminding the trauma. Conversely, lentivirus mediated overexpression of CRHR2 in the pBNST of susceptible rats significantly alleviates PTSD symptomology (Elharrar et al., 2013). In contrast to

that, it has also been reported that in mice which develop steady PTSD-like symptoms, CRHR2 mRNA in pBNST was 4-fold higher compared to resilient animals. Also in this model virus mediated the pBNST specific knock-down of CRHR2 protected mice from developing PTSD-like symptoms (Lebow et al., 2012). Summarizing both studies: well balanced levels of CRHR2 in the pBNST are crucial for a proper response to stressful events and stress recovery.

1.3.2.2.3 In search of the anxiogenic role of CRHR2 - the lateral septum

CRHR2 mRNA has been found to be expressed in many brain regions which might affect emotional behavior in opposite ways, i.e. increasing vs. decreasing anxiety-like responses. Despite the general view that activation of CRHR2 is thought to be anxiolytic, an entirely opposite picture has been drawn with respect to its role in the lateral septum. The lateral septum is a site of CRHR2 expression within GABAergic neurons (Anthony et al., 2014;Chalmers et al., 1995;Van Pett K. et al., 2000). The first pharmacologic approach aiming to decipher functions of CRHR2 in the LS was focused on selective and local application of CRH or CRH receptor antagonists. It has been shown that activation of CRHR2 in the lateral septum intermediate impairs learning in the fear conditioning paradigm and conversely blockade of septal CRHR2 enhances it (Radulovic et al., 1999). Moreover, intra septal infusion of urocortin 1 (UCN1), a non-selective CRH receptor agonist, increases not only anxiety-related behavior in the EPM, but also grooming and stereotypic movements, i.e. stress-associated behaviors (Bakshi et al., 2007a). Interestingly, Radulcovic et al. presented that both, prior infusion of CRH into the lateral septum and immobilization stress, result in increased anxiety-related behavior measured in the EPM test. These effects were then successfully reversed by intra-LS pretreatment with the CRHR2 selective antagonist antisauvagine-30 (Radulovic et al., 1999). Subsequent pharmacological studies confirmed that the increase in the anxiety-like behavioral responses to prior stress exposure might be alleviated by intra septal pretreatment of animals with CRHR2 antagonist (Todorovic et al., 2007), but also that the highly specific CRHR2 ligand urocortin 2 (UCN2) infused into the LS in high doses induces anxiety-like response. When animals are exposed to prior immobilization, even a low dose of UCN2, which alone does not evoke anxiety-like behavior is sufficient to significantly promote anxiety-related responses (Henry et al., 2006). A recent study from Anthony et al. (Anthony et al., 2014) combining a CRHR2-specific cre line with optogenetic and neuron tracing methods provides a clean summary of existing pharmacological studies collected so far. This study confirms that CRHR2 expressing septal

GABAergic neurons which project to the anterior hypothalamic area (AHA) promote anxiety-like responses when activated either in combination with or without immobilization stress. On the other hand their inhibition during the immobilization or during the following behavioral testing time, mitigates effects of stress. In addition, these septal CRHR2 positive GABAergic neurons have been shown to control the activity of the HPA axis as their activation promotes corticosterone release and inhibition exerts opposite effect (Anthony et al., 2014).

1.3.2.2.4 Linking stress and regulation of serotonergic system – CRHR2 in the dorsal raphe nucleus

The raphe complex is the primary localization of serotonergic neurons in the brain. A link between stress and malfunction of the brain serotonergic system has been made many decades ago and since then it has being continuously studied. In this regard presence of CRH-expressing neurons and CRH receptors within the raphe is no surprise. CRHR1 is expressed in a very sparse population of serotonergic neurons of the raphe nuclei. Nevertheless, it was found in many GABAergic cells in which its activity is regulated sex-specifically and thereby might differentially affect the susceptibility to develop mood disorders observed among men and women (Howerton et al., 2014;Refojo et al., 2011). In contrast, CRHR2 is expressed mostly in serotonergic neurons of the dorsal and medial raphe nuclei, although its presence was also reported in GABAergic neurones in the caudal part of the dorsal raphe nucleus (Day et al., 2004;Lukkes et al., 2008;Refojo et al., 2011;Lukkes et al., 2011). Topographically the highest expression of CRHR2 was found in the dorsal and ventral parts of the dorsal raphe nucleus, where up to 58% of serotonergic neurons express CRHR2 mRNA (Lukkes et al., 2011). Despite the fact that serotonergic neurons express mostly CRHR2, CRHR1 localized on GABAergic neurons also plays an important role in regulation of the dorsal raphe function, counterbalancing in part effects of CRHR2 activation. It has been shown that activation of CRHR2 with high doses of CRH increases the firing rate of serotonergic neurons. This effect is opposite to activation of CRHR1 receptors localized in GABAergic raphe neurons (Kirby et al., 2000;Kirby et al., 2008;Lowry et al., 2000;Price et al., 1998). Interestingly low dose of UCN2 infused into the raphe nucleus also inhibits the firing rate 5HT neurons, while high doses of UCN2 increases their activity (Pernar et al., 2004). Finally, selective activation of CRHR2 within the dorsal raphe nucleus seems to correlate with increased serotonin release in target structures. In this respect Amat et al. demonstrated that intra dorsal raphe infusion of UCN2 not only activates serotonergic neurons as quantified by c-

Fos expression, but corresponds with increased 5HT released in the BLA. Both effects can be blocked by pretreatment with the CRHR2 selective antagonist ASV-30 (Amat et al., 2004;Amat et al., 2004). Moreover, also high doses of CRH infused into the dorsal raphe nucleus have been shown to increase 5HT release in the CeA and mPFC and at the same time produce freezing behavior (Forster et al., 2006). It is remarkable that CRH overproduction or i.c.v. delivery induce opposite effects, i.e. increase in general activity, locomotion and alertness, actions mediated by activation of CRHR1 (Jones et al., 1998;Liang et al., 1992b;Lu et al., 2008). Along with previously described indirect inhibitory action of CRHR1 on firing of serotonergic neurons, intra-dorsal raphe injections of low doses of CRH were shown to significantly decrease 5HT release in striatum (Price et al., 1998). The same pattern of activation of serotonergic neurons is still observed even when agonists of CRHR2 are delivered i.c.v. instead of intra-raphe infusion (Bittencourt and Sawchenko, 2000;Hale et al., 2010;Staub et al., 2006). Corresponding increase in extracellular 5HT most likely contributes to mechanism by which those compounds, i.e. CRH, UCN1, UCN2 and UCN3, exert their behavioral effects (De Groote L. et al., 2005;Jones et al., 1998;Pelleymounter et al., 2002;Pelleymounter et al., 2004;Spina et al., 2002). It is surprising that despite the rich literature discussing molecular or net consequences of CRHR2 activation within serotonergic neurons not much is known about its behavioral implications. Indirect evidences come from studies concerning animal models where a change of raphe CRHR2 mRNA expression was found in correlation with observed behavior. In this regard CRH overexpression and morphine withdrawal both induce an increase in CRHR2 expression in the dorsal raphe nucleus (Lunden and Kirby, 2013;Korosi et al., 2006). Up to now only a few studies were able to directly pinpoint effects of raphe CRHR2 activation on animal's stress-related behavior. Among them convincible evidence was presented recently by Issler and colleagues proving that CRHR2 KO mice show indeed changes in tissue concentrations of serotonin, and its metabolite 5-hydroxyindoloacetic acid, in various brain regions along with alterated expression of genes governing serotonergic system's functions i.e. Tph2 and Slc6a4. Those changes possibly contribute to impaired stress recovery observed in mutant CRHR2 KO mice (Issler et al., 2014). In contrast Hammack et al. showed that activation of CRHR2 in the dorsal raphe mediates behavioral effects of inescapable shock in a model of uncontrollable stress, i.e. resulting in induction of behavioral despair (Hammack et al., 2003b;Hammack et al., 2003a). In another study the blockade of CRHR2 in the median raphe nucleus has been shown to decrease freezing behavior in animals exposed to the same context where they were previously stressed. This phenotype was then correlated with blunted increase in ventral hippocampal 5HT levels

(Ohmura et al., 2010). Since stress is a major factor precipitating psychiatric diseases it is of special interest to gain more insight into the role of raphe CRHR2 in regulation of the serotonergic system with respect to anxiety- or stress-related emotional and behavioral responses.

1.3.3 CRH-related peptides with affinity toward CRHR2 – Urocortins

CRH is not the only endogenous neuropeptide able to interact with CRH receptors. Urocortins, i.e. CRH-related peptides were first identified in fish and then found also in mammals (Skelton et al., 2000). Amino acids sequence of urocortins differs from that of CRH, thus they also differ in binding affinities towards CRH receptors and CRH-BP (Fekete and Zorrilla, 2007a).

Peptide	CRHR1; K_i(nM)	CRHR2; K_i(nM)			rCRH-BP; K_i(nM)
		CRHR2α	sCRHR2α	CRHR2β	
r/hCRH	0.95	15 (9-19)	23	13.5 (10-17)	0.54
rUCN1	0.32 (0.16-0.32)	1.5 (0.8-2.2)	6.6	0.62 (0.41-0.62)	0.98
mUCN2	>100	0.58 (0.16-2.1)	113	0.66 (0.25-0.66)	4.4
mUCN3	>>100	9.6 (5.0-14.2)	>200	1.8	>2000

Table 1. Affinity of CRH and urocortins towards CRH receptors and CRH-BP.
Adapted from (Fekete and Zorrilla, 2007b)

1.3.3.1 Urocortin 1 (UCN1)

UCN1 is a 40 amino acid neuropeptide that was first identified in fish (Skelton et al., 2000;Vaughan et al., 1995). Subsequently cloned from rodents it shares 45% sequence identity to rat CRH (Pan and Kastin, 2008). UCN1 binds with a much higher affinity to both CRH receptors than other ligands, yet also to CRHR-BP. In the brain it is present in the non-preganglionic Edinger-Westphal nucleus, lateral superior olive, supraoptic nucleus, magnocellular part of the PVN, lateral mammillary nucleus, lateral and ventromedial hypothalamus, substantia nigra, dorsal raphe nucleus and various motor nuclei (Bittencourt et al., 1999;Kozicz et al., 1998;Morin et al., 1999;Yamamoto et al., 1998). In peripheral tissues UCN1 was found in heart, spleen, thymus and gastrointestinal tract (Baigent and Lowry, 2000;Kimura et al., 2002;Martinez et al., 2004). Notably, it has been shown that regulation of expression UCN1 in the Edinger-Westphal

nucleus is modulated by benzodiazepines, stress, CRH and CRHR2 deficiency (Bale et al., 2000;Kozicz et al., 2004;Weninger et al., 2000;Dirks et al., 2002;Kozicz et al., 2008a;Kozicz et al., 2008a). Additionaly, a 9-fold increase in expression of UCN1 the EW was found in male suicide victims (Kozicz et al., 2008b). In line with that UCN1 in the centrally projecting EW nucleus has been shown to be involved in addictive behaviors i.e. alcohol consumption, mood regulation and stress adaptation (Giardino et al., 2011;Kozicz, 2007;Ryabinin et al., 2012;Kozicz et al., 2008a). When UCN1 is infused i.c.v. its effects resemble those of both CRH receptors activation with underscored effects of CRHR1 stimulation. This includes increase in anxiety-related behavior and acoustic startle response, decrease in food and water intake and increase in the HPA axis activation (Jones et al., 1998;Spina et al., 2002;Spina et al., 1996;Bagosi et al., 2014). Interestingly UCN1 deficient mice show increased anxiety-like behavior, similar to CRHR2 KO mice, impaired acoustic startle response and hearing deficits (Bale et al., 2000;Vetter et al., 2002;Wang et al., 2002). Expression and physiology of UCN1 is in an close interrelationship to CRH and its receptors. In some instances UCN1 might substitute functions of CRH, or by UCN1 up-regulated expression compensate CRH deficiency, in others counterbalance them enabling proper stress response and stress adaptation.

1.3.3.2 Urocortin 2 (UCN2)

UCN2, also called stresscopin-related peptide is a highly selective CRHR2 agonist, although its basal expression level is very low. It was found in the paraventricular nucleus of the hypothalamus, supraoptic, arcuate and motor nuclei. Its expression also has been confirmed in the locus coeruleus (Chen et al., 2006;Reyes et al., 2001). It is of particular interest as it has been described that noradrenergic neurons project to the dorsal raphe nucleus, what creates the possibility that UCN2 is endogenous agonist for CRHR2 present therein (Kim et al., 2004;Kozicz, 2010;Samuels and Szabadi, 2008). Besides the CNS UCN2 was found in peripheral tissues i.e. skeletal muscles, skin, adrenals, heart, kidneys, spleen, lungs, stomach, testis, thyroid and bone marrow (Chen et al., 2004;Hsu and Hsueh, 2001). There is a strong link between regulation of UCN2 expression and glucocorticoid levels, indicating its possible role in stress adaptation and recovery processes (Chen et al., 2003;Chen et al., 2004;Tillinger et al., 2013). When UCN2 is infused i.c.v. it increases the acoustic startle response and percentage of prepulse inhibition, decreases food intake and promotes anxiogenic-like responses (Pelleymounter et al., 2002;Pelleymounter et al., 2004;Reyes et al., 2001;Risbrough et al., 2003;Risbrough et al.,

2004;Skorzewska et al., 2011;Ohata and Shibasaki, 2004). The first UCN2 knockout mice were generated by Chen et al. (Chen et al., 2006). It has been shown that upon total UCN2 deletion the anxiety-like response under basal condition is not affected in neither sex, yet female UCN2 KO mice show decreased passive stress coping behavior along with increased basal daily rhythms of ACTH and corticosterone. Most importantly, lifelong deficiency of UCN2 results in compensatory changes in expression of CRHR2 in the LS, VMH and the dorsal raphe nucleus pointing out brain centers through which it might exerts its actions (Chen et al., 2006). In addition, Breu et al. showed that male UCN2 KO mice display reduced aggressiveness, underscoring UCN2 gender-specific phenotype differences (Breu et al., 2012). Up to 2014 no UCN2 overexpressing mouse line has been published.

To address possible stress adaptation functions of urocortins, especially UCN1 and UCN2, a double UCN1/UCN2 knockout mouse model was published by Neufeld-Cohen and colleagues (Neufeld-Cohen et al., 2010a). Double UCN1/UCN2 KO mice were generated by breeding single UCN1 and UCN2 KO mice. Despite increased expression of CRH in the PVN and increased corticosterone response to immobilization stress, double KO mice showed decreased anxiety-like behavior already under basal conditions. Decreased anxiety-related behavior was also reported after stress exposure indicating improved behavioral stress recovery. Interestingly, those mice showed genotype and sex-specific changes in concentration of 5HT, and its metabolite 5HIAA, in stress related brain regions i.e. basolateral amygdala (BLA), ventral hippocampus (vHip), lateral endopiriform nucleus (Lent), subiculum (S) and the dorsal raphe caudate (DRC) indicating aberrant serotonergic neurotransmission.

1.3.3.3 Urocortin 3 (UCN3)

It is thought that urocortin 3, or stresscopin, is the most selective CRHR2 agonist. As in case of all CRH-related peptides it is present in the brain, i.e. in the median preoptic nucleus, the rostal perifornical area, BNST, MeA in paraolivaty nucleus as well as in peripheral tissues, skin, small intestine and pancreas (Deussing et al., 2010;Lewis et al., 2001;Li et al., 2003). In addition, UCN3 positive fibers were found in amygdala, lateral septum, pBNST, arcuate and medial preoptic nuclei and in the ventromedial hypothalamus, prominent sites of CRHR2 expression (Anthony et al., 2014;Li et al., 2002). When UCN3 is infused i.c.v. it decreases food intake and locomotor activity, increases prepulse inhibition, and increases blood glucose and insulin levels, activates HPA axis, but does not affect anxiety-related behavior (Ohata and Shibasaki, 2004;Pelleymounter

et al., 2002;Pelleymounter et al., 2004;Risbrough et al., 2004;Ushikai et al., 2011;Jamieson et al., 2006). Moreover it has been shown that UCN3 might either activate or inhibit the HPA axis in a dose-dependent manner (Bagosi et al., 2013;Jamieson et al., 2006). Nevertheless, in another set of studies it was also shown that centrally applied UCN3 decreases anxiety-related behavior under basal condition as well as after ethanol administration, and increases active stress-coping behavior (Tanaka and Telegdy, 2008a;Tanaka and Telegdy, 2008b;Tanaka et al., 2011c;Telegdy et al., 2011;Valdez et al., 2003;Venihaki et al., 2004). Interestingly, mice overexpressing UCN3 show increased anxiety-related behavior under non-stress conditions and attenuated anxiety-like response upon acute immobilization, changes related to disrupted serotonergic system function (Neufeld-Cohen et al., 2012). Increased basal anxiety-related behavior was also reported in mice where overexpression of UCN3 is restricted to the perifornical area (Kuperman et al., 2010). Up to now two UCN3 KO mouse lines were created (Deussing et al., 2010;Li et al., 2007). UCN3 KO mice do not show changes in anxiety-related behavior or HPA axis regulation under non-stress conditions, although they exhibit alterations in social discrimination (Deussing et al., 2010). Additionally it has been shown that UCN3 KO mice as well as perifornically overexpressing UCN3 mutants exhibit changes in energy homeostatis and some metabolic effects (Chao et al., 2012;Chen et al., 2012).

The very intriguing triple knockout model of urocotins, including UCN1, UCN2 and UCN3, was presented by Neufeld-Cohen and colleagues (Neufeld-Cohen et al., 2010b). They showed that triple urocortin mutants (tUCN) under non-stress conditions did not differ from wild-type controls in terms of anxiety-related behavior, but displayed impaired recovery 24 hours after stress exposure reflected by changed serotonergic neurotransmission and gene expression within the amygdalar complex (Neufeld-Cohen et al., 2010b).

1.4   Transgenic mouse models

The central CRH/UCN system presents an important neurocircuitry though which stress, both physiological and psychological, influences emotions, mood and behavior. Since CRH was characterized by W. Vale and colleagues in 1981, other components of this system, CRH receptors CRHR1 and CRHR2, CRH-BP and urocortins, UCN1, UCN2 and UCN3 were gradually discovered. Transgenic mouse models are important tools providing insights into their functions.

1.4.1 Conventional vs. conditional mouse models

Conventional transgenic mouse models are characterized by life-long disruption of gene function. Both deletion and overexpression of genes are possible. Nevertheless, changes in the expression of one gene often cause compensatory effects in other genes and systems or in the most severe cases lethality. Moreover, in conventional mouse models of gene overexpression ectopic, unwanted expression of a protein of interest may occur. These additional changes create obstacles in interpretation of obtained data and often require more control experiments (Jaisser, 2000). Alternative to conventional mutagenesis avoiding some of its caveats and enabling very precise site or time specific gene manipulation is conditional mutagenesis. In conditional mouse models recombination of a gene of interest takes place only in specific tissues organs or cells (Lewandoski, 2001a). In these models essential parts of the targeted gene are flanked by loxP sites (floxed). In the presence of active cre-recombinase the DNA fragment between loxP sites is recombined (Wilson and Kola, 2001). Deletion, inversion or translocation of DNA fragments is possible. Expression of cre-recombinase is driven by highly specific promoters which restrict the activity to certain tissue or cell types (Kuhn and Torres, 2002). With this method it is possible to generate animals in which the gene of interest is manipulated only in a particular tissue, for instance neuronal tissue (Tronche et al., 1999), or even more specifically, i.e. in neurons of a certain neurotransmitter type like serotonergic (Scott et al., 2005), GABAergic (Morin et al., 1999), dopaminergic (Zhuang et al., 2005), noradrenergic (Stubbusch et al., 2011), neurons and etc. An additional feature of conditional mutagenesis is the possibility to generate specific knockout or overexpressing animal during adulthood (Erdmann et al., 2007;Lewandoski, 2001b). Due to genetic manipulation starting in the adulthood it is possible to pass by problems of developmental compensatory changes or eliminate lethality when proper gene function is necessary for the early life survival. Such site or temporal restricted gene manipulations allow investigating functions of broadly expressed genes, separately in certain body compartments. It is useful when either global deletion of a gene is lethal or the same gene exerts opposite functions in different tissues - cases impossible to study with conventional mutagenesis. Spatiotemporal gene manipulation might be accessed either by introduction of a modified cre recombinase, fused to the estrogen receptor ligand binding site which can be activated through binding to the exogenously delivered drug, tamoxifen or by generation of animals where expression of the gene of interest is occuring or prohibited by time the period when the antibiotic, tetracycline, is administrered (Erdmann et al., 2007;Lewandoski, 2001b). The

specificity of protein action and expression is of particular interest in highly organized organs like the brain. For this reason conditional animal models play a crucial role in studying mechanism underlying CNS functions health and disease (Gaveriaux-Ruff and Kieffer, 2007).

2   Aims of the thesis

Previous data strongly suggest an involvement of CRHR2 in development of anxiety and mood disorders. Although conventional CRHR2 KO mice are available and their phenotype suggests an anxiolytic role of CRHR2 activation, several other studies point towards on anxiogenic and pro-depressive role of the receptor. Such discrepancies might be due to opposite functions CRHR2 exerts within the different separate brain regions, neurotransmitter systems, central and peripheral tissues or possible compensatory effects. Therefore, the following aims were addressed in this study:

1) What is the neurochemical identity of CRHR2 expressing neurons?

2) What are the effects of central CRHR2 deletion?

3) What is the effect of neurotransmitter type specific or temporally controlled selective CRHR2 deletion on anxiety-related behaviour and neurophysiology?

4) What is the possible effect of selective CRHR2 overactivation?

To answer these questions we first determined the neurochemical identity of CRHR2 expressing neurons by double in situ hybridization and double immunohistochemistry. Consequently we generated a set of conditional CRHR2 knockout mice, devoid of CRHR2 either in the entire central nervous system, forebrain GABAergic neurons, serotonergic neurons of the raphe complex or lacking of CRHR2 in Camk2a positive neurons but beginning in the adult life. Mice were comprehensively phenotyped in terms of anxiety-related and stress coping behavior, HPA axis regulation and possible molecular changes. Furthermore, mice selectively overexpressing UCN2, a CRHR2 selective agonist, were created and phenotyped.

3   Materials and Methods

3.1   Materials

3.1.1   Buffers and solutions

All chemicals and solutions were purchased from Sigma-Aldrich, Munich, Germany or Carl Roth, Karlsruhe, Germany, if not mentioned otherwise.

3.1.1.1 Buffers for agarose gel electrophoresis

1 x Tris acetate EDTA (TAE) buffer

4.84 g	Tris(hydroxylmethyl)-aminomethane (TRIS)
1.142 ml	Acetic acid
20 ml	0.05 M Ethylendiaminetetraacetate (EDTA), pH 8.0
800 ml	H_2O_{bidest}

adjust pH to 8.3 with acetic acid

ad 1 l	H_2O_{bidest}

6 x DNA loading buffer (orange)

1 g	Orange G
10 ml	2 M TRIS/HCL, pH 7.5
150 ml	Glycerol
ad 1 l	H_2O_{bidest}

3.1.1.2 Solutions for *in situ* hybridization (ISH)

H_2O-DEPC

1 ml	Diethylpyrocarbonate (DEPC) (Sigma-Aldrich)
ad 1 l	H_2O_{bidest}

2 x autoclave

10 x Phosphate buffered saline (PBS)

1.37 M   NaCl

27 mM   KCl

200 mM Na_2HPO_4 x 12 H_2O (Merck, Darmstadt, Germany)

20 mM KH_2PO_4 (Merck)

pH 7.4

add 1 ml DEPC/l, ad H_2O_{bidest}

incubate over night (o.n.), 2 x autoclave

20 % Paraformaldehyde (PFA)

20% w/v Paraformaldehyde

in 1 x PBS-DEPC

pH 7.4

10 x Triethanolamine (TEA)

1.0 M   TEA

pH 8.0

add 1 ml DEPC/l, ad H_2O_{bidest}

incubate o.n., 2 x autoclave

20 x Standard saline citrate (SSC)

3 M   NaCl

300 mM   Sodium citrate

pH 7.4

add 1 ml DEPC/l, ad H_2O_{bidest}

incubate o.n., 2 x autoclave

Hybridization mix (hybmix)

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	Dextran sulphate
0.02 g	Ficoll 400
0.02 g	Polyvinylpyrrolidone 40 (PVP40)
0.02 g	Bovine serum albumin (BSA)
5 ml	tRNA (10 mg/ml, Roche Diagnostics GmbH, Mannheim, Germany)
1 ml	carrier DNA (salmon sperm, 10 mg/ml, Sigma-Aldrich)
4 ml	5 M dithiothreitol (DTT, Roche)

store as 1 to 5 ml aliquots at -80°C

Hybridization chamber fluid

250 ml	Formamide
50 ml	20 x SSC
200 ml	H_2O_{bidest}
5 M	DTT/DEPC
7.715 g	DTT
4 ml	H_2O-DEPC

shake falcon tube until the powder is nearly solved

ad 10 l	H_2O-DEPC

5 x NTE

146.1 g	NaCl
50 ml	1 M TRIS/HCl, pH 8.0
50 ml	0.5 M EDTA, pH 8.0

add 1 ml DEPC/l, ad 1 l H_2O_{bidest}

incubate o.n., autoclave

3 M NH$_4$OAc

3.0 M Ammonium acetate (NH$_4$OAc)

ad H$_2$O$_{bidest}$

Alcohol (dehydration)-solutions

Alcohol (dehydration)-solutions alcohol conc.	vol. of EtOH 100 % (ml)	vol. of 3 M NH$_4$OAc (ml)	vol. of H$_2$Obidest (ml)
30 % EtOH/ NH4OAc	150	50	300
50 % EtOH/ NH4OAc	250	50	200
70 % EtOH/ NH4OAc	350	50	100
96 % EtOH	480 + 20 ml H$_2$Odest		
100 % EtOH	500		

Cresyl violet staining solution

2.5 g   Cresyl violet (Merck)

0.102 g   Na-acetate

1.55 ml   Acetic acid

ad 500 ml H$_2$O$_{bidest}$

adjust to pH 3.5

filtrate

3.1.1.3 Immunohistochemistry solutions

Blocking solution

5 %   BSA

0.1 %   Triton

Solution for antibodies

5 %   BSA

0.01%   Triton

Cryoprotection solution

125 ml   ethyleneglycol

125 ml   glycerol

250 ml   PBS (1x)

3.1.1.4 LacZ staining solutions

LacZ-fix

4%   paraformaldehyde (PFA)/PBS, pH 7.4

0.005 M   ethylene glycol tetraacetic acid (EGTA)

0.001 M $MgCl_2$

diluted in 0.1 M PBS, pH 7.4

LacZ wash buffer

0.002 M $MgCl_2$

0.01%   deoxycholate

0.02%   Nonidet P40 (NP40)

diluted in 0.1 M PBS, pH 7.4

LacZ staining solution

0.1%   X-Gal (stock solution in DMF)

0.005 M   potassium-ferrocyanide

0.005 M   potassium-ferricyanide

Diluted in LacZ wash buffer

3.1.1.5 Solutions for western blotting

<u>10 x TBS</u>

200 mM   TRIS/HCl

1.35 M   NaCl

ad H_2O_{bidest}

pH 7.6

<u>TBS/T</u>

100 ml   10 x TBS

1 ml T   ween20 (BioRad)

ad 1 l H_2O_{bidest}

<u>Blocking solution and solution for antibodies</u>

0.2 %   I-Block (Applied Biosystems)

0.1 %   Tween-20

ad 1 x PBS

pH 7.4

<u>Lämmli 4x</u>

40 %   Glycerol (v/v)

0.25 M   TRIS/HCl

8 %   SDS

0.008 %   Bromphenoleblue

ad H_2O_{bidest}

add 7.5 %   ß-mercapto-EtOH prior to use.

Running buffer

25 mM   TRIS/HCl

190 mM   Glycine

0.1 %   Sodium dodecyl sulfate (SDS)

ad H_2O_{bidest}

3.1.1.6 HPLC Solutions

All solutions must be filtered through 0.2 µm filter and degassed before use.

Acetate buffer

992.7 ml   HPLC grade water

3.0 g   sodium acetate

4.3 ml   glacial acetic acid

pH adjusted to 5.0

0.1 M Perchloric acid

991.36 ml   HPLC grade water

8.64 ml   70% perchloric acid (69-72%)

NB. Pipette PCA in fume hood

Mobile phase

9.53 g   potassium dihydrogen orthophosphate dihydrate

200 mg   octanesulphonic acid (OSA)

35 mg   EDTA

870 ml   HPLC grade water

Allow to dissolve then add 130 ml HPLC grade methanol (13%)

pH to 3.4 with orthophosphoric acid

<u>**Automatic seal wash/needle wash for autosampler**</u>

160 ml   HPLC grade water

40 ml   HPLC grade methanol (20%)

<u>**Acetate buffer containing DHBA at 500 pg/20µl**</u>

Add 25µl of 1mg/ml stock solution to 1 L of acetate buffer.

3.1.2   Oligonucleotides for genotyping

Name	Sequence 5'-3'	Amplicon [bp]	Detection
<u>CRHR2</u>			
CKO2-GT1	AACCCTGCATCCACA AACAT	359 *(CKO-GT1/ GT3)*	Wildtype
CKO2-GT2	TGGTGTGGGAAA GGGTTC	478 *(CKO-GT1/ GT3)*	Floxed allele after Flp
CKO2-GT3	GAGTCCTGG GTTATG GCTGA	300 *(CKO-GT1/ GT2a)*	LacZ reporter
CKO2-GT4	AATGAAGCCCAGATT GTTGG	563 *(CKO-GT1/ GT4)*	Mutant (CKO)
<u>Cre</u>			
CRE-F	GATCGCTGCCAGGATATACG	574	Cre transgene
CRE-R	AATCGCCATCTTCCAGCA		
<u>Thy1</u>			
Thy1-F1	TCTGAGTGGCAAAGGACCTTAGG	372	Thy1 gene, control
Thy1-R1	CCACTGGTGAGGTTGAGG		band
<u>CRHR2</u>			
R2-GT1	ACGAAACCCAGTCTGACTAGC	500 *(R2-GT1/ GT2)*	Wildtype
R2-GT2	GCCACGGAACCTTGAACTTTG	233 *(R2-GT1/ NEO)*	R2 KO
R2-NEO	ACGAGTTCTTCTGAGGGGATC		
<u>Camk2aCreERT2</u>			
i-Cre 1	GGTTCTCCGTTTGCACTCAGGA	290 *(i-Cre 1/ 2)*	Wildtype
i-Cre 2	CTGCATGCACGGGACAGCTCT	375 *(i-Cre 1/3)*	Cre-transgene
i-Cre 3	GCTTGCAGGTACAGGAGGTAGT		
<u>Nestin Cre</u>			
Nes-Cre r	CGGGAAACCATTTCCGGTTA	595	Cre-transgene
191f	ACTTTGGTTCTTCTCTTCTGCACCCGGATG		
<u>Rosa 26 (UCN2)</u>			
ROSA-1	AAAGTCGCTCTGAGTTGTTAT	398 *(ROSA-1 / 6)*	Wildtype
ROSA-2	GCGAAGAGTTTGTCCTCAACC	320 *(ROSA-1 / 2)*	Mutant (UCN2-COE)
ROSA-6	GCTGCATAAAACCCCAGATG		
<u>Venus</u>			
Venus fwd.	TACGGCCTGCAGTGCTTC	364	Venus present
Venus rev.	GGGTGTTCTGCTGGTAGTGG		

3.1.3 Oligonucleotides for qPCR

Name	Sequence 5'-3'
CRH	
CRH frw	GGAGGCATCCTGAGAGAAGTC
CRH rev	CATGTTAGGGGCGCTCTC
CRHR1	
CRHR1 frw	GGGCCATTGGGAAACTTTA
CRHR1 rev	ATCAGCAGGACCAGGATCA
C-fos	
C-fos frw	CAGCCTTTCCACTACCATTCC
C-fos rev	ACAGATCTGCGCAAAAGTCC
RLP19	
Rlp frw	GCATCCTCATGGAGCACAT
Rlp rev	CTGGTCAGCCAGGAGCTT

3.1.4 mRNA probes for ISH

Name	antisense	sense	vector	GeneBank acc. no.	Complem. Region [bp]
CRHR2	Sp6	T7	pCRII-Topo	NM_001288618	400-595
c-fos	Sp6	Sp6	pCRII-Topo	NM_010234	608-978
UCN2	T7	Sp6	pCRII-Topo	NM_145077	1-1002

3.1.5 Antibodies

specificity	Produced in	Company	Dilution
Thyptophan hydroxylase	Mouse	Sigma (TO678)	1:500
Calbindin k-28D	Rabbit	Swant (CD38)	1:1000
Tyrosine hydroxylase	Sheep	Abcam (AB113)	1:500
Camk2a	Rabbit	Abcam (AB52476)	1:500
GFP	Chicken	Abcam (AB13970)	1:1000
GFP	Rabbit	Genetex (GTX20209)	1:1000

Anti-mouse (594)	goat	Invitrogen (A31553)	1:1000
Anti-rabbit (488)	goat	Invitrogen (A21441)	1:1000
Anti-sheep (594)	goat	Invitrogen (A21099)	1:1000
Anti-chicken (488)	goat	Invitrogen (A11042)	1:1000

3.1.6   Animals

CRHR2^{CKO} mice

Conditional CRHR2 knockout mice were obtained from the International Knockout Mouse Consortium   (http://www.mousephenotype.org/martsearch_ikmc_project/about/eucomm).   In these mice exon 4 of CRHR2 the gene is flanked by two loxP sites and can be removed by cre-recombinase, causing disruption of the receptor expression in cre-positive cells. In order to obtain conditional CRHR2 mice with specific deletion of CRHR2 in the central nervous system CRHR2^{CKO} mice were inbred to mice expressing cre recombines under the Nestin promoter (Tronche et al., 1999). For the deletion of CRHR2 in GABAergic, Camk2a or serotonergic neurons, CRHR2^{CKO} mice were inbred to Dlx5/6-Cre (Monory et al., 2006;Ruest et al., 2003), Camk2aERT2-Cre (Erdmann et al., 2007) or ePet-Cre (Scott et al., 2005) mice respectively.

CRHR2^{Venus} mice

CRHR2^{Venus} mice were obtained from the Gensat project (www.gensat.org). In CRHR2^{Venus} mice a bacterial artificial chromosome (BAC) containing the DNA of the enhanced yellow fluorescent protein Venus (Nagai et al., 2002) was introduced into the CRHR2 gene in exon 3. Due to the presence and preservation of all regulatory elements directing expression of CRHR2 in BAC, the Venus protein is expressed in cells endogenously producing CRHR2. Additionally, a membrane localization signal added to in the Venus sequence, the Venus is also localized to neuronal processes i.e. dendrites and axons.

Nat-Cre mice

Nat-Cre mice used for the virus injection and to generate UCN2-COE^{NE} animals were obtained from the Gensat project (www.gensat.org) and described before (Kuhne et al., 2012). In this mouse line Cre-recombinase is driven by the noradrenaline transporter promoter and thus expressed in noradrenergic neurons of the locus coeruleus.

UCN2-COE[NE] mice

Conditional UCN2 overexpressing mice were generated by Ailing Liu (unpublished data). The targeting procedure is based on the strategy used to generate conditional CRH overexpressing mice (Lu et al., 2008). The Rosa26 (*R26*) locus was engineered to harbor a transcriptional terminator sequence flanked by loxP sites, followed by a UCN2-IRES-Tau-LacZ expression unit (R26[flopUCN2], flop: floxed stop). Cre-mediated excision of the transcriptional terminator leads to the expression of UCN2 and β-galactosidase driven by the endogenous *R26* promoter. To obtain UCN2 overexpression selectively in noradrenergic neurons, R26[flopUCN2/flopUCN2] mice were bred to Nat-Cre mice. In the F2 generation homozygous UCN2 overexpressing mice (UCN2-COE[NE]) and control littermates (UCN2-COE[Ctrl]) were obtained.

CRHR2 KO mice

Constitutive CRHR2 KO mice used to investigate the endocrine stress response were described before (Coste et al., 2000). Briefly, CRHR2 KO mice in place of endogenous CRHR2 gene contain a mutated CRHR2 allele encompassing thymidine kinase and neomycin resistance cassettes what causes disruption of functional CRHR2 protein expression.

3.2 Methods

3.2.1 Preparation and analysis of nucleic acids

DNA preparation from mouse tail tissue

For genotyping PCRs of transgenic mice, tail biopsy was digested in 100 µl 50 mM NaOH for 30 min at 80°C followed by a neutralization step using 30 µl 1M Tris-HCl (pH 7.0) and stored at 4°C. 2 µl of the lysates was used as PCR genotyping reaction template.

Agarose gel electrophoresis

For DNA gel electrophoresis 2% agarose (Invitrogen) in 1 x TAE buffer was boiled until dissolved. Clear agarose solution was cooled down to RT, 0.1 µg/ml ethidiumbromide (Carl Roth) was added and agarose was poured into a gel electrophoresis chamber (PeqLab). DNA Samples mixed with 1/6 of 6 x DNA loading buffer were loaded into prepared gel wells. As a size marker the DNA smart ladder (Eurogentec, Brussels, Belgium) was used. Electrophoresis was carried out with 130 V, 300 mA for 2 h. The DNA fragments were detected with UV light and documented using a gel documentation system (Biometra, Göttingen, Germany).

3.2.1.1 Polymerase chain reaction (PCR)

Standard PCR

To amplify DNA for genotyping of transgenic mice, polymerase chain reaction PCR was performed using the Thermoprime Plus DNA polymerase (ABgene, Hamburg, Germany) as follows:

Standard PCR reaction:

2 µl   genomic DNA template

2.5 µl   10 x reaction buffer IV (ABgene)

1.5 µl   25 mM $MgCl_2$ (ABgene)

0.5 µl   dNTPs (dATP, dTTP, dCTP and dGTP, 10 mM each, Roche Applied Science)

1.5 µl   10 µM primer fwd

1.5 µl   10 µM primer rev

0.25 µl   Thermoprime Plus DNA polymerase (5 U/µl, ABgene)

ad 25µl H_2O_{bidest}

PCR was carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems) with the following temperature settings:

Programme	Cycles	Temperature	Hold
Denaturation	1x	94°C	2 min
Ampflication:	35x		
Denaturation		94°C	30 sec
Annealing		x°C	30 sec
Elongation		72°C	1 min
Final elongation	1x	72°C	5 min
Cooling		4°C	∞

Annealing temperature (x) was chosen in dependence of the melting temperature of the primers.

3.2.1.2 RNA isolation

RNA was isolated from murine tissues using the TRIzol protocol (Invitrogen). Tissue was homogenized in ice cold 1 ml/mg TRIzol reagent using a TURRAX device (IKA Labortechnik, Staufen, Germany). Homogenate was incubated at room temperature for 5 min. After addition of 200 µl chloroform per 1 ml TRIzol, tube was shaken vigorously for 3 min and afterwards left at room temperature for additional 5 min. By centrifugation for 15 min at 13.000 rpm at 4°C (5403 centrifuge, Eppendorf) the phenol and water phases were separated. The aqueous phase containing the RNA was transferred into a fresh 1.5 ml Eppendorf tube and RNA was precipitated with 500 µl isopropanol/ml TRIzol for 2 h at -20°C. Precipitated RNA was centrifuged for 10 min at 13000 rpm at 4°C. Supernatant was discarded, the pellet was washed with 1 ml of cold 70% ethanol followed by centrifugation at 10000 rpm at 4°C for 10 min. The RNA pellet was dried at 37°C and dissolved in 20-50 µl H_2O_{bidest} at 65°C for 2 min. Final RNA concentration and quality was measured using Nanodrop (NanoPhotometer[TM], Implen GmbH, Germany).

3.2.1.3 Reverse transcription (RT) PCR

First strand cDNA synthesis from total RNA was performed using the SuperScript™ II reverse transcriptase kit from Invitrogen according to the provided protocol. To test for genomic DNA contaminations, one control reaction lacking the reverse transcriptase was carried out for each RNA sample.

Reverse transcription:

x µl   RNA (1 µg)

1 µl   oligo d(T) primer

y µl H_2O

11 µl   in total

The reaction mix was heated at 65°C for 2 min, then chilled on ice.

RT-mastermix:

4 µl   5x buffer (Invitrogen)

2 µl   0.1 M DTT (Invitrogen)

1 µl   10 mM dNTPs

1 µl   RNase inhibitor (40 U/µl; Roche)

8 µl of mastermix were added to each sample and the tubes were incubated at 40°C for 2 min. Subsequently, samples were incubated with 1 µl Superscript II for 60 min at 42°C followed by 15 min at 70°C and stored at -20°C.

3.2.1.4 Quantitative real-time PCR (qPCR)

Real-time PCR was carried out with the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer`s protocol in the Lightcycler 2.0 System (Roche).

qPCR Mastermix:

5.0 µl   QuantiFast SYBR Green PCR Mix (Qiagen, Hilden, Germany)

1.0 µl   primer forward (10 µM)

1.0 µl   primer reverse (10 µM)

1.0 µl H_2O_{bidest}

8 µl mastermix were pipetted in each capillary which was fixed in the LightCycler carousel, 2 µl DNA (1/10 diluted) were added and capillaries were closed immediately. Following programme was applied:

Programme	Cycles	Target [°C]	Hold [sec]	Slope [°C/s]	Sec target	Step size	Step delay	Acquisition mode
Preincubation	1	95	300	20	0	0	0	None
Amplification	40			20				
Denaturation		95	10	20	0	0	0	None
Annealing		60	30	20				None
Elongation		72	Y	20	0	0	0	Single
Melting Curve	1	95	0	20	0	0	0	None
		50	10	20	0	0	0	None
		95	0	0.1	0	0	0	Continuous
Cooling	1	42	30	20	0	0	0	none

Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using the real PCR efficiency calculated from an external standard curve, normalized to the housekeeping genes RLP19 and related to the data of control experiments.

3.2.2   Plasma corticosterone analysis

Two weeks before the experiment animals were left undisturbed with a 12 : 12 h light : dark cycle (lights on at 7:00 a.m.). Retrobulbar blood sampling was performed in the morning (7:30-

9:30 a.m.) or the evening (17.30-18.30 p.m.) under light isoflurane anesthesia. For evaluation of the endocrine response to stress, blood samples were collected immediately after 2 or 10-min restraint stress, for which the animals were placed in a 50 ml conical tube with bottom removed. For the recovery feedback measurement blood was sampled 90 min after end of 10 min of restraint stress. 100-300 µl blood was collected in ice-cold EDTA-coated tubes and centrifuged for 15 min at 8000 g, 4°C. After centrifugation plasma was transferred in 96 well plates and stored at -80°C until measurment of corticosterone concentrations in duplicate by ^{125}I-Radioimmunoassay according to manufacturer's protocol (RIA-Kit, ICN Biomedicals, Frankfurt/Main, Germany).

3.2.3   Tissue preparation

Mice were sacrificed by an overdose of isoflurane (Forene, Abbott, Wiesbaden, Germany), followed by decapitation. Brains were immediately removed. For *in situ* hybridization brains were immediately shock-frozen on dry ice and stored at -80°C until cutting on cryostat.

3.2.4   *In situ* hybridization (ISH)

For *in situ* hybridization 25 µm brain sections were cut on a cryostat and mounted on SuperFrost Plus slides (Menzel GmbH, Braunschweig, Germany). *In situ* hybridization using ^{35}S-labeld cRNA probes was performed according to a modified version of a previously described procedure (Dagerlind et al., 1992). Briefly, specific riboprobes were generated by standard PCR using T7 and SP6 primers with plasmids containing respective cDNAs as templates. Radiolabeled antisense cRNA probes were generated from obtained PCR products by *in vitro* transcription with ^{35}S-UTP using T7 and SP6 RNA polymerase. Hybridization was performed o.n. with a probe concentration of 3.5 to 7 million counts per slide at 55-60°C. After washing radioactive signal was visualised on light-sensitive radiographic film. Quantification of mRNA signals was conducted by ImageJ (http://rsb.info.nih.gov/ij/).

3.2.4.1 Radioactive labeling of probes

Probes were amplified from respective plasmids using the following PCR reaction:

2 µl   plasmid DNA template (1.5 µg)

3 µl   25 mM MgCl2 solution (ABgene)

5 µl   10 x reaction buffer IV (ABgene)

1 µl   10 mM dNTPs (Roche)

0.5 µl   Thermoprime plus DNA polymerase (5 U/µl, ABgene)

3 µl   10 µM Primer forward

3 µl   10 µM Primer reverse

add   50 µl H2O$_{bidest}$

PCR programme:

Programme	Cycles	Temperature	Hold
Denaturation	1x	94°C	2 min
Ampflication: Denaturation Annealing Elongation	35x	 94°C 67°C 72°C	 30 sec 30 sec 40 sec
Elongation	1x	72°C	5 min
Cooling down		4°C	∞

To prevent RNA degradation all precautions were taken to avoid RNase activity.

The *in vitro* transcription was pipetted as follows (mastermix for approximately 10 slides):

In vitro transcription mastermix:

1.5 µl   PCR product

13.5 µl   H$_2$O-DEPC

3 µl   10 x transcription buffer (Roche)

3 µl   NTP-mix (rATP/rCTP/rGTP 10mM, Roche)

1 µl   0.5 M DTT

1 µl	RNasin (RNAse-inhibitor, 40 U/µl, Roche)
6 µl	^{35}S-thio-rUTP (12.5 mCi/mM, 1250 Ci/mmol, Amersham)
1 µl	T7 or sp6 RNA polymerase (20 U/µl, Roche)

The reaction samples were gently mixed. Afterwards, samples were incubated at 37°C for 3 hours in total; after 1 h another 0.5 µl of RNA polymerase was added.

To destroy the DNA template, 2 µl of RNase-free DNase I (10 U/µl, Roche) was added and samples were incubated for 15 min at 37°C.

3.2.4.2 Purification of the riboprobes

For purification of riboprobes the RNeasy Mini Kit (Qiagen) was used according to the manufacturer´s protocol. RNA was diluted in 100 µl RNase-free water and 1 µl of the riboprobe was measured in 2 ml scintillation fluid (Zinsser Analytic, Frankfurt, Germany) in a beta-counter (LS 6000 IC, Beckmann Coulter). For *in situ* hybridization 35000 to 70000 cmp/µl and 100 µl/slide (7 million/slide) were required.

3.2.4.3 Pre-treatment of cryo-slides

Slides were taken out of the -20°C freezer and warmed up and dried for at least 1 h at room temperature (RT). For pre-treatment the following protocol was applied:

1. Fix	10 min	4% PFA/PBS	Ice cold
2. Rinse	3x 5 min	1x PBS/DEPC	
3.	10 min	0.1M triethanolamine.HCl 0.2 (pH 8.0) (TAE) 200ml	Add 600 µl acetic anhydrite to TAE while stirring bar is rapidly rotating
4. Rinse	2x 5 min	2x SSC/DEPC	
5. Dehydrate	1 min	60% EtOH/DEPC	
6.	1 min	75% EtOH/DEPC	
7.	1 min	95% EtOH/DEPC	

8.	1 min	100% EtOH/DEPC
9.	1 min	CHCl$_3$
10.	1 min	100% EtOH/DEPC
11.	1 h	Air dry (dust free)

3.2.4.4 Hybridization

An appropriate amount of hybridization mix (hybmix) containing the riboprobe was prepared. The hybridization mix containing the riboprobe was heated to 90°C for 2 min and snap cooled on ice. 100 µl of hybmix containing 3.5 to 7 milion cpm was pipetted onto the slides and coverslips were carefully mounted, avoiding air bubbles in between. The slides were placed into a hybridization chamber containing hybridization chamber fluid to prevent drying out of the hybmix. The chamber was sealed with adhesive tape. The slides were incubated in an oven (Memmert, Schwabach, Germany) at 55-60°C o.n. (up to 20 h).

3.2.4.5 Washing

After hybridization the coverslips were carefully removed and the following protocol was applied:

1.	4x 5 min	RT	4x SSC	
2.	20 min	37°C	1.0x NTE (20 µg/ml RNaseA)	Add 50 µl RNaseA (10 mg/ml) to 200 ml of NTE
3.	2x 5 min	RT	2x SSC/1mM DTT	50 µl of 5 M DTT/200 ml
4.	10 min	RT	1x SSC/1mM DTT	50 µl of 5 M DTT/200 ml
5.	10 min	RT	0.5 x SSC/1mM DTT	50 µl of 5 M DTT/200 ml
6.	2x 30 min	64°C	0.1x SSC/1mM DTT	50 µl of 5 M DTT/200 ml
7.	2x 10 min	RT	0.1x SSC	
8.	1 min	RT	30% EtOH in 300 mM NH$_4$OAc	
9.	1 min	RT	50% EtOH in 300 mM NH$_4$OAc	
10.	1 min	RT	70% EtOH in 300 mM NH$_4$OAc	
11.	1 min	RT	95% EtOH in 300 mM NH$_4$OAc	
12.	2x 1 min	RT	100% EtOH in 300 mM NH$_4$OAc	

13.	1 h	RT	Air dry (dust free)	

3.2.4.6 Autoradiography

Dried *in situ* sections were exposed to a special high performance X-ray (BioMax MR from Kodak) for three to seven days. Afterwards they were developed using automatic developing machine (SRX-101A, Konica Minolta).

3.2.4.7 Quantification of relative expression

Expression of mRNAs in different brain areas was quantified by measuring the OD on scanned ISH films using ImageJ.

3.2.5   Immunohistochemistry

Immunohistochemistry (IHC) was applied to enhance the native fluorescent signal of Venus and to perform double immunohistochemistry. 50 μm vibratome free-floating cuts were washed in 1x PBS 3 times for 5 min followed by blocking of non-specific binding with 5% BSA for 1 h at RT. Primary antibodies were incubated o.n. at 4°C. After removing an unbound primary antibodies by washing with PBS, slices were incubated for 2 h with respective fluorescently labelled secondary antibodies. After final PBS washing slices were mounted with DAPI (Vector Labs, Burlington, Canada).

3.2.6   Nissl staining

To confirm injection site brain sections were counterstained with the synthetic dye cresyl violet. This basic aniline dye is able to stain the RNA of the rough endoplasmatic reticulum, called Nissl substance, in the cytoplasm of neurons.

1.	15 min	0.5% Cresyl violet acetate
2.	1 min	H_2O
3.	2x 1 min	70% EtOH

4.	1 min	96% EtOH + 1 ml of Acetic acid
5.	1 min.	96% EtOH
6.	1 min	100% EtOH
7.	2x 5 min	Xylol

After the last step slides were mounted with DPX (317616-100ML, Sigma, Germany).

3.2.7   LacZ staining

Mice were killed by an overdose of isoflurane and then intracardially perfused using LacZ-Fix solution for 5-7 min, followed by a 1 min washing step with PBS. After preparation, the brain was additionally fixed for 1 h in LacZ-fix and then incubated overnight at 4°C in 20% sucrose/PBS. On the next day, the brain was frozen on dry-ice and cut at a cryostat (HM 560 M, Microm) in 50 μm thick sections and collected in cryoprotection solution. For staining, the sections were immersed for 5 min in LacZ-wash buffer and then incubated in LacZ staining solution at 37°C for up to 12 h in the dark. After washing with PBS, brain sections were incubated again in LacZ-fix to increase signal strength overnight at 4°C. Finally, sections were mounted on super frost plus slides (Menzel), immersed in xylol and embedded with DPX mounting solution (317616-100ML, Sigma, Germany).

3.2.8   HPLC monoamine measurement

Determination of monoamine content was performed in collaboration with the laboratory of Prof. Lowry (Boudler University, Colorado, USA) as described elsewhere (Evans et al., 2008). Specific brain regions were microdissected, kept in acetate buffer at 4 °C and centrifuged (~ 12,000 g) for 3 min. The pellet was separated and redissolved in 150 or 200 μl of 0.2 M NaOH for later protein assay (Pierce Protein Microassay Protocol, Perbio Science UK Ltd., Cramlington, UK). Samples in volume of 35 μl were placed in ESA 542 autosampler (ESA Analytical, Ltd., Huntington, UK) at 4°C with the column oven temperature of 29°C. 10 μl of each was injected to chromatographic system. Separation was done with an integrated precolumn/column system. Electrochemical detection was done with ESA Coulochem II multi-electrode detector with an ESA conditioning cell 5021 and an ESA analytical cell 5011 with electrodes set at − 0.10 and + 0.55 V.

Determination of 5HT and 5HIAA concentrations were obtained by comparison heights of appropriate standard peaks with heights of 5HT and 5HIAA peaks of examined samples using a computerized analysis system (EZChrom Elite for Windows, ver 2.8; Scientific Software, Inc., Pleasanton, California, US).

3.2.9 Animal protocols

3.2.9.1 Animal housing and breeding

Mice were housed 2-4 per cage and acclimated to standard laboratory conditions (light-dark cycle: 12:12 h, lights on at 7 a.m.; temperature: 21 ± 1°C; relative humidity: 50 ± 10 %) with food and water available *ad libitum*. All animal breeding and experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria.

All animals were genotyped according to the indicated protocol. The tail biopsies were taken at weaning, when animals were 3 to 4 week old.

3.2.10 Behavioral testing

In all experiments male mice were used, singly housed one week prior to the experiment and acclimated to standard laboratory conditions (light-dark cycle: 12:12 h, lights on at 7 a.m.; temperature: 22 ± 1°C; relative humidity: 55 ± 5 %) with food and water *ad libitum*. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Government of Bavaria, Germany.

Following the habituation period and seven days before starting behavioral experiments, animals underwent a general health check, including fur and general physical conditions as well as bodyweight analysis to ensure that behavioral findings are not confounded by the health condition of mice. Test performances in the open field, elevated plus-maze, dark-light box and tail suspension tests were recorded and analyzed using the ANY-maze software (Stoelting Co., Wood Dale, IL). Test apparatuses were cleaned with water before each trial.

3.2.10.1 Open field test (OF)

The open field test was used to characterize locomotor activity and anxiety-related behavior in a novel environment. The open field was made of grey PVC in dimensions of 48 x 48 x 40 cm. Light intensity was 15-20 lux. At the beginning of a test, each mouse was placed in the corner of the apparatus, facing the wall. Animal behavior was recorded by means of a video camera mounted above the apparatus. The distance covered by the animal was analyzed automatically using the ANY-maze software. In this process apparatus arena was virtually subdivided into two compartments i.e. an inner (20 x 20 cm) in the center and an outer zone. Measured parameters were total distance covered; number, time, distance and latency of inner zone exploration. The compartments were wiped out with water before each trial.

3.2.10.2 Elevated plus maze test (EPM)

The elevated plus maze test (EPM) was used to assess anxiety-related behavior. The apparatus was made of grey PVC and consisted of a plus-shaped platform with four intersecting arms, elevated 37 cm above the floor. Two opposing open (30 x 5 cm, 15-20 lux) and closed arms (30 x 5 x 15 cm, 8-10 lux) were connected by a central zone (5 x 5 cm). Animals were placed in the closed arm of the apparatus facing the closed the wall and were allowed to freely explore the maze for 10 min. Parameters of interest included open and closed arm time, latency to the first open arm entry and open arms entries.

3.2.10.3 Dark light box test (DaLi)

The dark/light box test (DaLi) was used to assess anxiety-related behaviour. The test box consists of black and white PVC and was divided into two compartments, connected by a tunnel (4 x 7 x 10 cm). The white compartment (30 x 20 x 25 cm) was brightly illuminated by cold light with an intensity of 680-700 lux; light intensity in the dark compartment (15 x 20 x 25 cm) was less than 5 lux. Each animal was placed in the dark compartment of the apparatus, facing the bright lit compartment. During the 10 min test, the time spent in dark and lit compartments, the latency until the first entry and the number of entries and distance in the lit compartment were assessed.

3.2.10.4 Forced swim test (FST)

The forced swim test (FST) was used to assess stress-coping behavior. Each animal was gently placed into a glass beaker (diameter 12 cm, height 24 cm) filled with water (temperature 23 ± 1°C) to a height of 12 cm and behaviour during a 6 min test period was recorded. The parameters floating (immobility except small movements to keep balance), swimming and struggling (vigorous attempts to escape) were recorded using the ANYmaze software and scored throughout the 6 min test period by a trained observer, blind to the animals genotype.

3.2.10.5 Acoustic startle response (ASR) and prepulse inhibition tests (PPI)

Acoustic startle response and prepulse inhibition testing was carried out using the set-up as described in Golub et al. (Golub et al., 2009). The mice were placed in a tubular enclosure and tested inside a SR-LAB™ (San Diego Instruments) apparatus with background noise set to 55 dB. For ASR, after an acclimatization period of five minutes, the mice were subjected to noise impulses of varying intensities (75 dB, 90 dB, 105 dB and 115 dB) in random order. The data depicted on the graphs represent the mean peak startle amplitude in mV + SEM in response to 30 pulses of each intensity, as well as 12 background noise (BG) measurements.

Prepulse inhibition was measured with the same set up as startle but according to previously described protocol (Douma et al., 2011). Briefly, after 5 min of acclimatization to the background noise (70 dB) animals were presented with startle stimuli (110 dB, 50 ms) preceded by a burst of withe noise (20 ms) of 72, 74, 78 or 86 dB with 100 ms difference between onset of the prepulse and startle. The test session consisted of 3 blocks of test trials (blocks 1 and 3, startle stimulus alone trials, block 2 startle stimulus alone, startle + prepulse and no-stimulus trials). Intertrial intervals varied from 25 to 35 sec, while the total test duration was 45 min. The mean startle-amplitude per prepulse intensity was calculated by subtracting from the startle amplitude at the 110 dB pulse the startle amplitude after prepulse presentation and dividing this by the startle amplitude at 110 dB x 100.

3.2.10.6 Sociability Test

The sociability test as originally described (Moy et al., 2004;O'Tuathaigh et al., 2007;Sankoorikal et al., 2006), was slightly modified to mainly asses the social preference between an unfamiliar sex and age matched mouse and an object of choice (in this case a dummy mouse). The apparatus contained a three chamber box (50 x 25 cm) with one center and two outer chambers (left and right chamber 19 x 25 x 40 cm; center chamber 12 x 25 x 40 cm). Two small openings with trap doors served as access points from one chamber to the other. The apparatus was filled with bedding and evenly illuminated with 3-5 lux. The paradigm consists of two separate stages.

Stage 1: Habituation

The animals were allowed to freely explore the apparatus on two consecutive days for 10 min. During this time, only the empty wire cages were present in the chambers.

Stage 2: Sociability trial

On the third day of testing, an unfamiliar male mouse was introduced into the left chamber, enclosed in the wire cage while a dummy mouse was placed in the opposite chamber (alternation occurred after 3 consecutive trials). The test mouse was placed in the middle chamber for 5 min with both trap doors being shut. These were then opened and the test animal was allowed to explore the rest of the apparatus for an additional 10 min. The time spent interacting with mouse and object was scored by a trained observer and the discrimination index was obtained as follows: interaction time with mouse (s) / (interaction time with mouse (s) + interaction time with object (s))

3.2.10.7 Chronic social defeat stress paradigm

The chronic social defeat was performed as previously described (Berton et al., 2006;Wagner et al., 2011). In brief; experimental mice (9-12 mice per group between 11-13 weeks of age) were submitted to chronic social defeat stress for 21 consecutive days. They were introduced into the home cage of a dominant CD1 resident for no longer than 2 min and until defeated. Following defeat, animals spent 24 hours in the same cage, which was separated via a holed steel partition, enabling sensory but not physical contact. Every day experimental mice were exposed to a new unfamiliar resident. Control animals were housed in their home cages throughout the course of

the experiment. All animals were handled daily; weight and fur status were assessed every 3-4 days (fur was evaluated as described (Mineur et al., 2003). Behavioral testing took place during the last week of the paradigm and included the open field, elevated plus maze, sociability and forced swim test.

3.2.10.7.1   Acute stress response and sampling during the CSDS

On day 19 of the chronic stress procedure animals were subjected to an acute stress challenge in the form of FST for 5 min. Blood samples were collected by tail cut 30 min or 60 min following the start of the FST, and corticosterone plasma concentrations were measured with a radioimmune assay according to the manufacturers instructions (MP Biomedicals Inc; sensitivity 6.25 ng/ml). Animal sacrifice was performed by decapitation on day 21 of the experiment. In order to obtain basal corticosterone levels, trunk blood was collected and processed. Thymus and adrenal glands were removed and stored in Ringer's solution. In order to determine the organ weight, additional surrounding tissue was removed and the thymus and adrenal glands were weighted.

3.2.10.8   Sleep phenotyping and analysis of sleep data

To monitor spontaneous sleep-wake behavior, mice were chronically implanted with electroencephalographic (EEG) and electromyographic (EMG) electrodes as previously described (Kimura et al., 2010;Romanowski et al., 2010). The baseline recording was conducted 2 weeks after recovery from surgery. The polysomnographic setup was as same as previously reported (Kimura et al., 2010;Romanowski et al., 2010). Polygraphic data were assessed by a LabView-based acquisition program (EGEraVigilanz, SEA, Cologne, Germany), in which a FFT algorithm, adapted from a report by (Louis et al., 2004) serves to carry out semiautomatic classification of vigilance states combined with power spectrum analysis. Vigilance states are defined as wake, non-rapid eye movement (NREM) sleep (NREMS), or rapid eye movement (REM) sleep (REMS), respectively, in 4 s epochs and manually corrected if necessary. Time spent in each vigilance state was expressed in percentage/2 h. Slow-wave activity (SWA) during NREMS was calculated between 0.5-4 Hz in the delta range at a 0.5 Hz-bin and normalized for each animal by the total power averaged from all epochs scored as NREMS throughout the 24 h period across all

frequency bins from 0.5 to 29 Hz, as previously described elsewhere (Baracchi and Opp, 2008;Franken et al., 1998). Sleep architecture and spectral distributions of EEG activity were also analyzed as previously described (Jakubcakova et al., 2011).

3.2.10.9 Physical restraint stress

Physical restraint stress was performed by immobilization of animals in a conical 50 ml tube with bottom removed. After 2 or 10 min of immobilization animals were subjected to blood sampling or returned to their home cage.

3.2.10.10 Fear conditioning (FC)

Fear conditioning was performed in conditioning chambers (ENV-307A, MED Associates) as previously described (Kamprath and Wotjak, 2004). Conditioning and context-dependent freezing were assessed in a chamber with a metal grid floor, which was thoroughly cleaned and sprayed with 70% Ethanol before the animals were introduced. A neutral context consisted of a plexiglas cylinder with bedding and was used to investigate cued, tone-dependent, freezing. The cylinder was cleaned and sprayed with 1% acetic acid. For foot shock application (day 0) mice were placed into the conditioning chamber for 3 min. After 180 s, a sine wave tone (80 dB, 9 kHz) was presented for 20 s, which co-terminated with a 2 s scrambled electric foot shock of 1.5 mA. The mice remained in the conditioning chamber for another 60 s. In order to measure freezing responses to the tone, mice were placed into the neutral environment (cylinder) on the following day (day 1). Three minutes later, a 3 min tone was presented (80 dB, 9 kHz). The animals were returned to their home cages 60 s after the end of tone presentation. Contextual (associative) fear was tested by re-exposing the animals to the conditioning grid chamber for 3 min on day 2. Re-exposure protocol was repeated 28 days later. As a measure of fear, freezing behavior was recorded and analyzed by an observer blind to genotype.

3.2.10.11 Tail suspension test (TST)

For the tail suspension test (TST) mice were suspended on a metal bar 60 cm above the floor by using adhesive tape attached 2 cm from the tip of the tail. During the 10 min of test duration

immobility and latency to the first immobile episode was scored. Animal was considered immobile when it hanged completely motionless.

3.2.10.12 Home Cage Activity

Activity in a familiar environment of the home cage was monitored by an automated infrared tracking system (Mouse-E-Motion 2.3.6, Infra-E-Motion, Hagendeel, Germany). Each mouse was tracked for at least 24 h to obtain an accurate score during the light and dark cycle.

3.2.10.13 Sucrose preference test

Mice had free access to two identical bottles, one containing 1% sucrose solution and another one with tap water for one week. Three days prior to the testing, animals were habituated to new drinking bottles. To prevent possible effects of side preference in drinking behavior, the bottle positions were swapped every two days. The consumptions of water and sucrose solution were estimated by weighing the bottles before and after swap. To compare sucrose preference between the different mouse lines, sucrose consumption is expressed as a percentage of the total fluid intake.

3.2.10.14 Dexamethasone suppression test

Animals were injected intraperitoneally with dexamethasone with a dose of 0.3 mg/kg B.W. (Fortecortin Inject 100 mg, Merk Pharma GmbH, Germany) at 11 am. 6 hours later blood samples were collected via retrobulbal bleeding under light isoflurane anesthesia. Samples were centrigued (10 min; 8000 rpm) at 4°C. Plasma was separated and used for corticosterone RIA measurement as described before.

3.2.11 Surgical protocols

3.2.11.1 FPA perfusion protocol

Animals were sacrificed by overdosing isofluran anaesthesia. Mouse was stably fixed to a perfusion table, chest cavity was open allowing access to heart. Perfusion needle was inserted into the main aorta though the left ventricle. Animal was perfused as follows:

1.	1 min	1x PBS ice-cold
2.	5 min	4% PFA ice-cold
3.	1 min	1x PBS ice-cold

After perfusion brain was gently extracted and allow to post-fixation in 4% PFA o.n. Following 12 h post-fixation the brain was transferred into 20% sucrose solution until sectioning on vibratom.

3.2.11.2 Cannulation in the dorsal raphe

Seven days before the experiment male adult mice (age 10-12 weeks) underwent cannulation into the dorsal raphe nucleus. Animals were anesthetized with isoflurane and fixed to a stereotaxic apparatus (TSE systems Inc., Germany). Entire surgery was done under mouse inhalation anaesthesia. The skull was exposed and bregma point localized. DRV coordinates aquatinted from the bregma point were following: anterio-posterior: +4.6, medio-lateral: +1.6, dorso-ventral +2.6, α=30°. After drilling a hole in the skull a stainless metal guide cannula (8 mm length, 0.6 mm diameter) was inserted into the brain and fixed to skull with a metal screw and dental cement (Dual Cement, Ivoclar Vivadent, Germany). Dental stiches (Ethican perma hand seide, J 5-0, C-1, Johnson and Johnson, Germany) were made to improve healing. Animals were allowed to Methacam® dissolved in drinking watter 0.25 mg/100 ml for three consecutive days after surgery.

3.2.11.3 mUCN2 application into the dorsal raphe

Mouse UCN2 (Phoenix Pharmaceuticals) was dissolved in bidestilated water. 25 min before the FST animals were briefly anesthetized with isoflurane and an injection needle (9 mm length, 0,3

mm diameter), connected to a 10 µl Hamilton syringe via PET tubing, was inserted into the guide cannula and volume 0.5 µl of vehicle or mUCN2 was injected over the time of 1 min. After injection, the needle was left inside for additional 30 s allowing full diffusion of the infusate. After removing of the injection needle animals were returned to their home cages and left undisturbed until the FST took place.

3.2.11.4 Histology

After completion of the microdialysis and the DRV injection experiments, animals were decapitated under isoflurane anaesthesia, brains were removed and sectioned with a cryostat. Sections were stained with cresyl violet for histological verification. In case of non-correctly implanted microdialysis probes or injections outside DRV, mice were excluded from the analysis.

3.2.11.5 Virus injection

Synpatophysin-eGFP construct encapsulated in adeno-associated virus (AAV) serotype 1/2 (*AAV-DIO-Syn-eGFP*), was provided by Dr. Valery Grinevich (Knobloch et al., 2012). A Nat-Cre mouse expressing cre-recombinase in noradrenergic neurons of the locus coeruleus was anaesthetized with isoflurane and placed in the stereotactic frame, scull was exposed and two intracranial holes were drilled (AP: +5.4 mm; ML: ±0.8 mm; DV:+3.75 mm). The mouse was bilaterally microinjected (World Precision Instruments) with 0.5 µl of virus (rate 50 nl/min). After injection each injection needle was left in place for additional 10 min to assure proper spreading of virus. After procedure, skin on the skull was closed with dental string (Ethican perma hand seide, J 5-0, C-1, Johnson and Johnson, Germany). For the next three days drinking water was exchanged for Methacam® dissolved in drinking watter 0.25 mg/100 ml.

3.3 Image acquisition

Images were taken using either a Zeiss Axioplan2 microscope (Göttingen, Germany) and digitalized using AxioVision Rel. 4.5, Adobe Photoshop CS2, and Adobe Illustrator CS2 image editing software (San Jose, CA) or an Olympus IX81 inverted laser scanning confocal microscope and the Fluoview 1000 software.

3.4 Statistical analysis

Data output and statistical analyse were performed with the computer programme GraphPad Prism 5.0. All results are shown as means ± standard error of the mean (SEM). Differences were considered significant at $p<0.05$. To examine ASR, PPI Two-way repeated measures ANOVA was employed. For analysis of 4 groups in behavioural and endocrine measurements during CSDS paradigm, Two-way ANOVA was conducted. Sleep architecture was analysed with One-way ANOVA. The appropriate post-hoc test was performed after acceptance of significant p-values. Analysis of behaviour results such as OF, EPM, DaLi, FST and ISH signal intensities was conducted with Student´s t-test.

4 Results

4.1 Venus expression in the CRHR2^{Venus} reporter mouse line reflects endogenous CRHR2

In order to characterize the expression of CRHR2 in the mouse brain and resolve the neurochemical identity of CRHR2 positive neurons we characterized a CRHR2 reporter mouse line (CRHR2^{Venus}). CRHR2^{Venus} mice were obtained from the Gensat consortium (www.gensat.org). In bacterial artificial chromosome (BAC) transgenic CRHR2^{Venus} mice expression of membrane anchored Venus (a variant of green fluorescent protein, GFP) is driven by CRHR2 regulatory elements. Therefore, CRHR2 expressing neurons are Venus positive in CRHR2^{Venus} mice. In the reporter mice neurons expressing CRHR2 simultaniously express Venus. In order to control that Venus is truly expressed in CRHR2 positive neurons, and ectopic Venus expression does not obscure subsequent exprements, we performed a double in situ hybridization with probes targeting CRHR2 and Venus mRNA. Based on the obtained results, i.e. high cellular alignment of both probes, we were able to confirm that CRHR2^{Venus} mice represent reliable model to study CRHR2 expression in vivo (results from this experiments are included in the bachelor thesis of Corinna Scheffel and the master thesis of Eva Planitscher).

Following fluorescent immunolabelling with an anti GFP antibody we found that in forebrain structures (Figure 6) strong Venus expression was present in the olfactory bulb (OB), prefrontal cortex (PFC), endopiriform nucleus (Den), nucleus accumbens (NAc), lateral septum (LS), septofimbria (SFi) and posterior part of the bed nucleus of the stria terminalis (BNST). The most intense GFP labeling was observed in LS, AOB, BNST and DEn, suggesting strong CRHR2 expression in these regions.
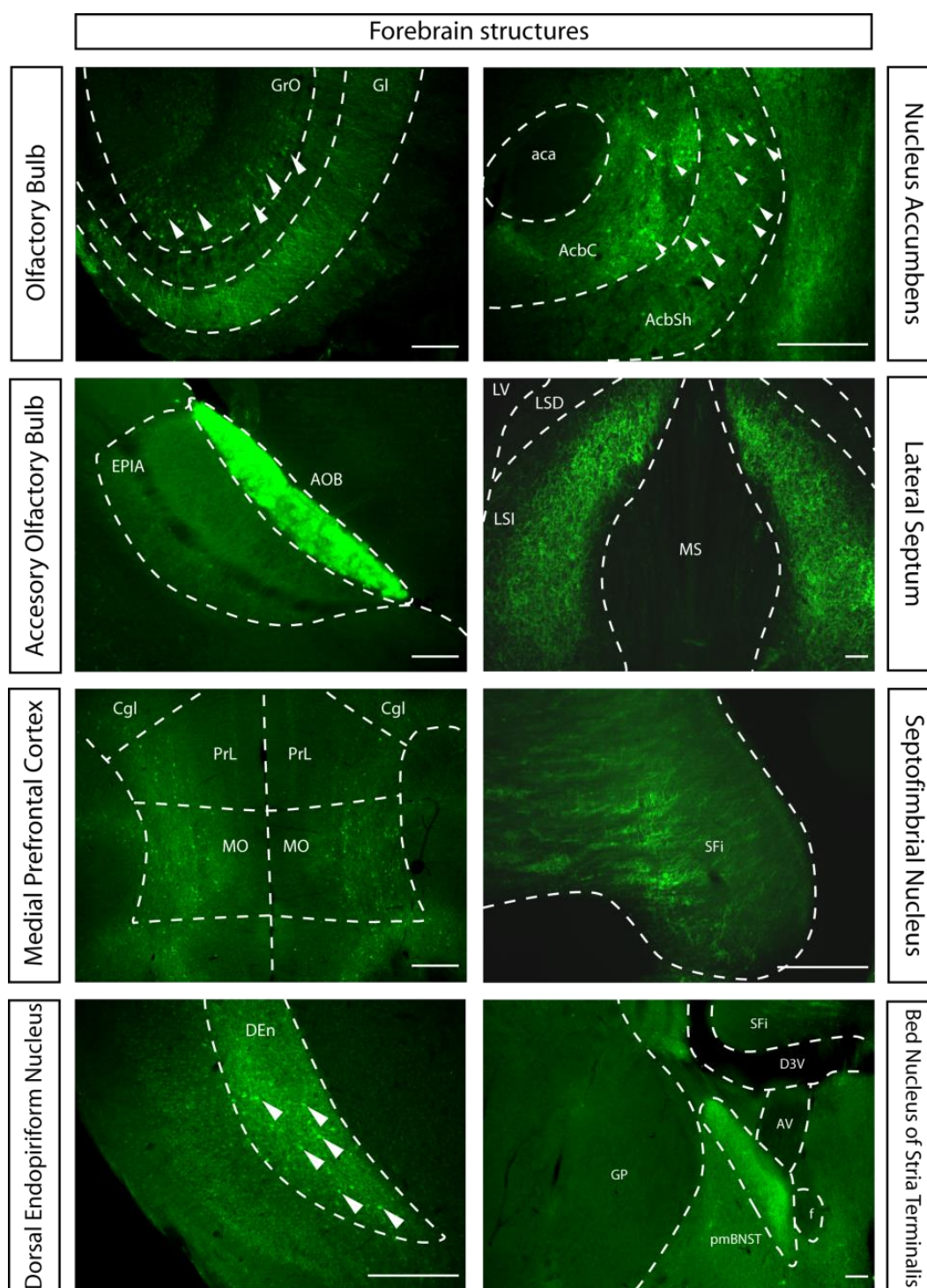
Figure 6. Expression of Venus reporter in forebrain structures of the brain of CRHR2^{Venus} mice.

Coronal brain sections. The scale bar corresponds to size of 250 μm. aca anterior commissure, AcbC nucleus accumbens core, AcbSh nucleus accumbens shell, AOB accessory olfactory bulb, AV anteroventral thalamic nucleus,Cgl cingulate cortex, CP caudate putamen, D3V dorsal 3rd ventricle, DEn dorsal endopiriform nucleus, EPIA external plexiform layer of the olfactory bulb, f fornix, Gl granular insular cortexof the olfactory bulb, GrO granular cell layer of the olfactory bulb, LSD lateral septal nucleus dorsal part, LSI lateral septal nucleus intermediate part, LV lateral ventricle, MS medial septal nucleus, MO medial orbital cortex, PrL prelimbic cortex, pmBNST posteriomedial part of the bed nucleus of the stria terminalis, SFI septofimbrail nucleus.
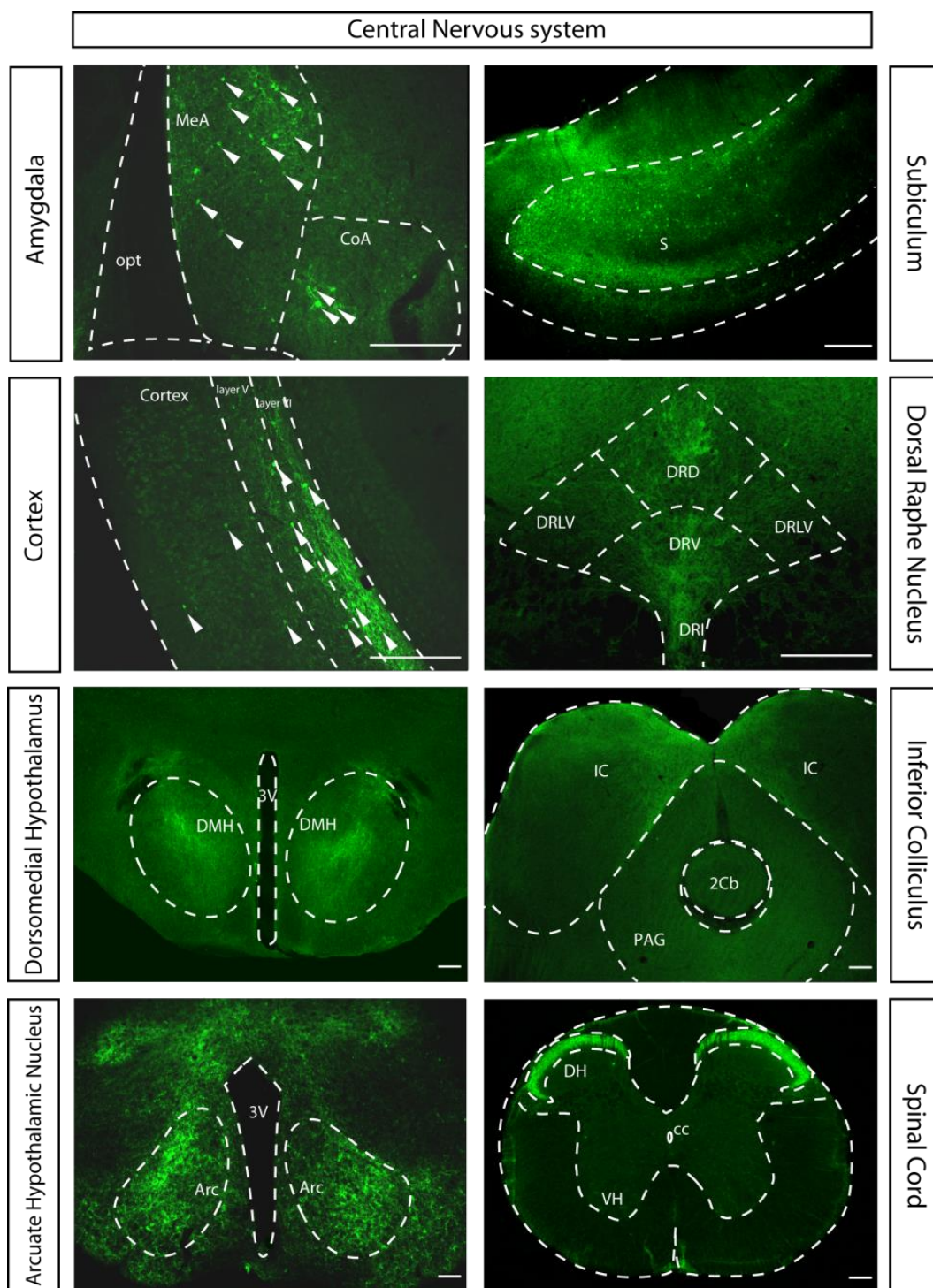
Figure 7. Expression of Venus reporter in the central nervous system of CRHR2^{Venus} mice.
Coronal brain secrions. The scale bare corresponds to size of 250 µm. 2Cb 2nd cerebellar lobule, Arc arcuate hypothalamic nucleus, cc central canal, CoA cortical amygdaloid nucleus, DH dorsal horn, DMH dorsomedial hypothalamic nucleus, DRD dorsal raphe dorsal nucleus, DRI dorsal raphe intermediate part, DRLV dorsal raphe ventrolateral part, DRV dorsal raphe ventral part, IC interior colliculus, MeA medial amygdaloid nucleus, Opt optic tract, PAG periaqueductal gray, S subiculum, VH ventral horn.

Venus signal was also found in cortical and medial amygdalar nuclei, the dorsomedial hypothalamic nucleus and the arcuate hypothalamic nucleus (Figure 7). In the caudal parts of the brain Venus was detected in the subiculum, dorsal and median raphe nuclei, inferior colliculus and also in the dorsal horns of the spinal cord.

This expression pattern of Venus fully recapitulates the expression of CRHR2 mRNA pattern in the wild-type mouse brain (Figure 8).
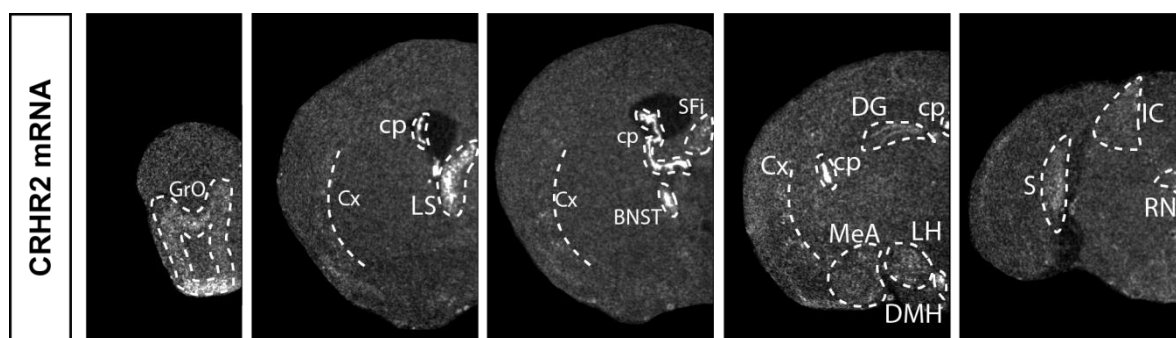
Figure 8. Expression of CRHR2 mRNA in the mouse brain.

Expression analysis of CRHR2 mRNA was performed by in situ hybridization with a radioactive antisense CRHR2 mRNA riboprobe. Depicted are coronal brain sections from an inverted autoradiography film. BNST bed nucleus of stria terminalis, cp choroid plexus, Cx cortex, DG dentate gyrus, DMH dorsomedial hypothalamus, ECIC external cortex interior colliculus, GrO Glomerular layer of the olfactory bulb, LH lateral hypothalamic area, LS lateral septum, MeA medial amygdaloid nucleus, RN raphe nuclei, S subiculum, SFi Septofimbria.

Taking into account that CRHR2 is expressed in many distinct brain sites, most likely CRHR2 positive neurons differ in their neurochemical identity. In order to resolve the neurochemical identity of CRHR2 expressing neurons we performed several double immunohistostainings using markers for GABAergic, serotonergic and Camk2a positive neurons. These results allow us to selectively direct CRHR2 inactivation using conditional mouse genetic tools.

4.1.1 CRHR2 is expressed in GABAergic neurons

To evaluate CRHR2 expression in the GABAergic system double immunolabelling for Calbindin K-28D, a GABAergic marker, and Venus was performed in the CRHR2[Venus] reporter mouse line (Figure 9). The majority of CRHR2 reporter co-localization with calbindin k-28D was observed in the pmBNST, DEn and cortical layers. In the LS and MeA the number of double positive cells was comparable with the portion of single Venus positive neurons. A small number of double immunolabelled neurons were also found in DRN and MnR (data not shown).
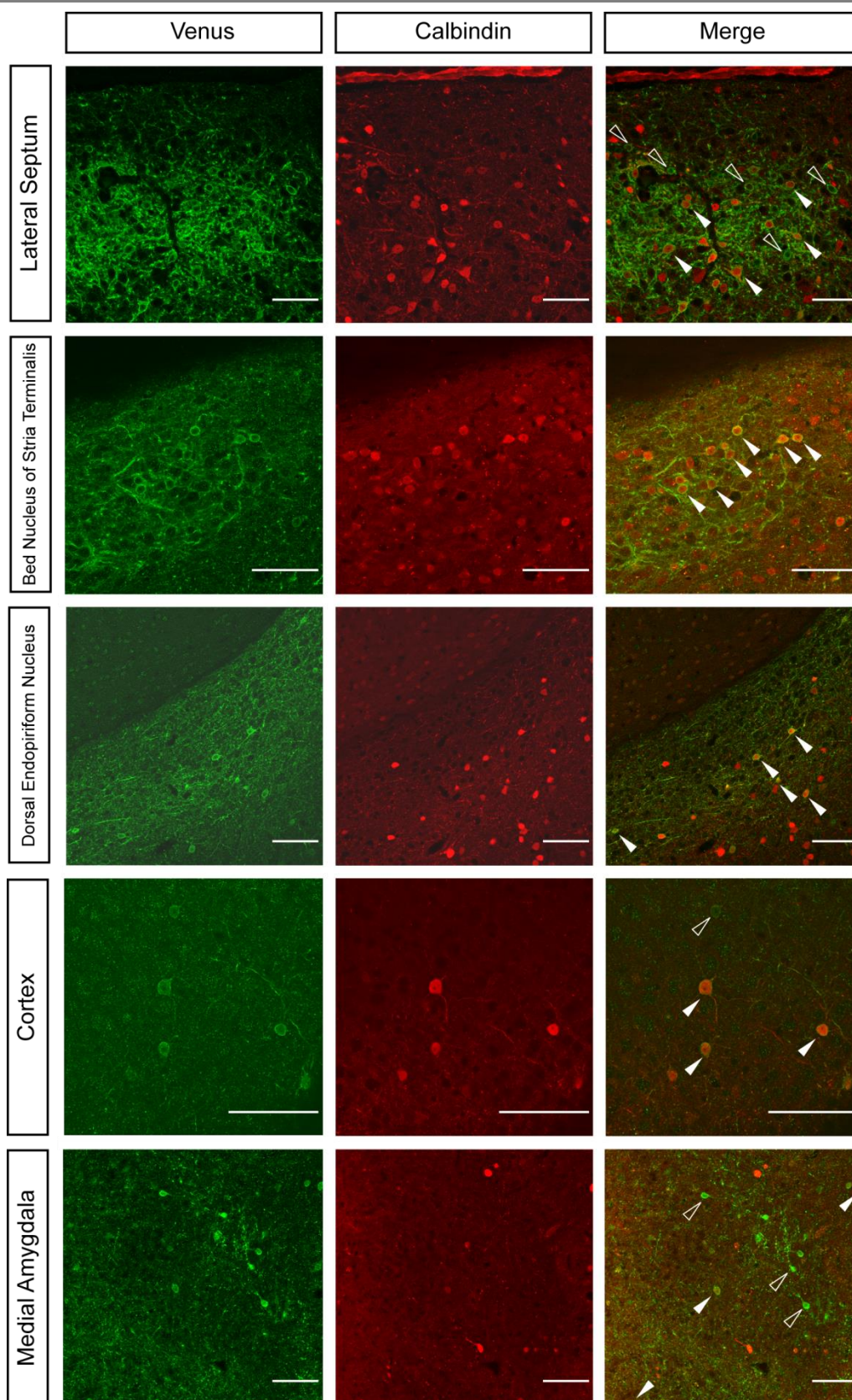
Figure 9. Expression of CRHR2 in Calbindin K-28D positive neurons.

Colocalization of Venus reporter with calbindin k-28D was found in the lateral septum, bed nucleus of stria terminalis, dorsal endopiriform nucleus, cortex and medial amygdala. Coronal brain sections. The scale bar corresponds to 100 µm. Filled arrowheads: double positive neurons, empty arrowheads, single positive neurons.

4.1.2 Expression of CRHR2 reporter in Camk2a positive neurons in the adult mouse brain

The establishment of inducible variants of cre-recombinase, by fusion to the ligand binding domain of the estrogen receptor, allows the temporally controlled inactivation of a gene of interest. One of the best inducible cre-drives is the Camk2a-CreERT2 line which enables inducible deletion of gene interest in principal forebrain neurons.

In order to identify neurons where an induction of conditional deletion of CRHR2 in adulhood is possible, double immunolabeling for Venus and Camk2a was performed in CRHR2^{Venus} mice. Double positive neurons were found in the lateral septum, subiculum and to smaller extent in the medial amygdala (Figure 10).
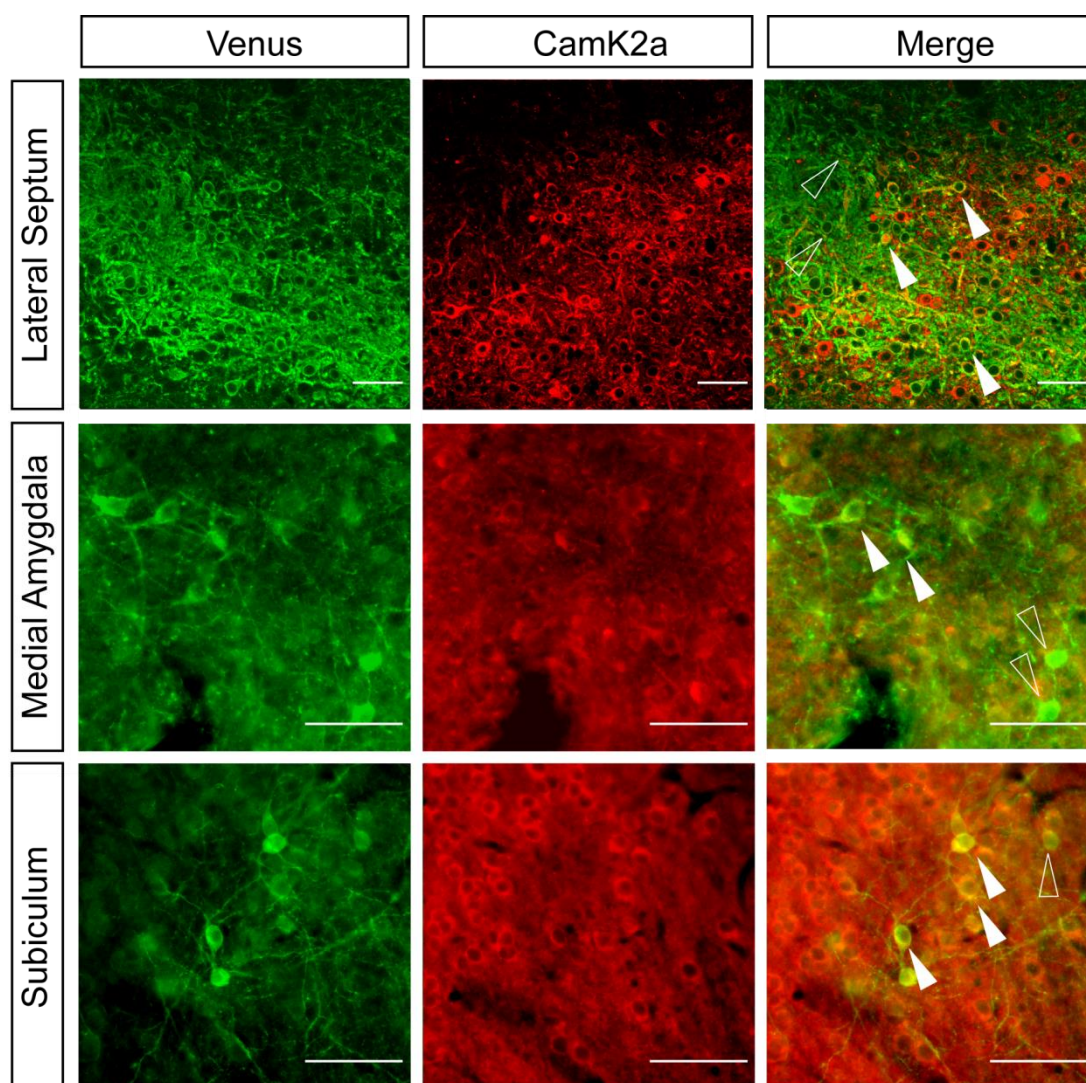
Figure 10. Localization of CRHR2 reporter Venus in Camk2a positive neurons (on previous page).
Filled arrows shows examples of double positive cells, empty arrows, signle Venus positive cells. Coronal brain sections. The scale bar corresponds to 100 µm.

4.1.3   CRHR2 is present in serotonergic neurons of the raphe complex

In order to study effects of CRHR2 activation within the dorsal raphe nucleus, presence of the receptor was confirmed in serotonergic neurons. For this reason raphe sections of CRHR2^{Venus} reporter mice were co-immunostained for Venus and tryptophan hydroxylase type 2 (TPH2), a marker for serotonergic neurons (Figure 11).

As expected only a subpopulation of neurons was double positive for Venus and TPH2, meaning that not all serotonergic neurons express CRHR2. These results are in accordance with in situ hybridization studies where, depending of the investigated raphe region, co-expression of CRHR2 and TPH2 mRNA reached up to 58% (Lukkes et al., 2011). In CRHR2^{Venus} reporter mice the highest amount of double labeled neurons was present in the dorsal and ventral portions of DRN, whereas almost no colocalization was observed in the ventrolateral part.
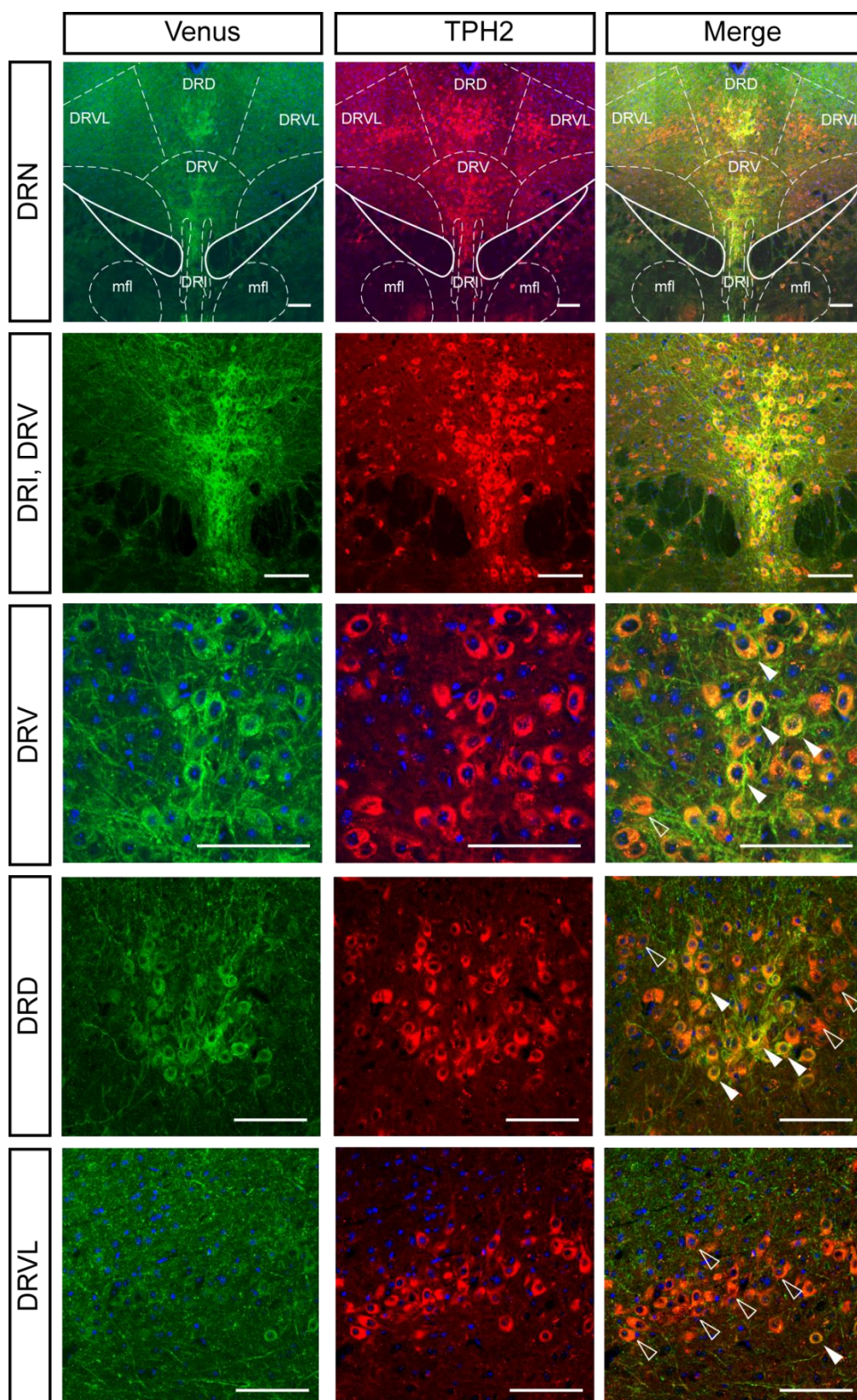
Figure 11. Expression of Venus reporter of CRHR2[Venus] mice in the dorsal raphe nucleus.

CRHR2 reporter was mostly colocalized with TPH2 in the DRV, DRD and DRI subregions. Filled arrows indicate examples of double positive cells, empty arrows, single Venus positive cells. Coronal brain sections. Scale bars 100 µm. DRD dorsal raphe dorsal prart, DRI dorsal raphe intermediate part, DRN dorsal raphe nucleus, DRVL dorsal raphe ventrolateral part, mfl medial longitudal fasciculus.

Also in the median raphe nucleus the majority of the Venus positive neurons were positive for TPH2, confirming expression of CRHR2 in serotonergic neurons (Figure 12).
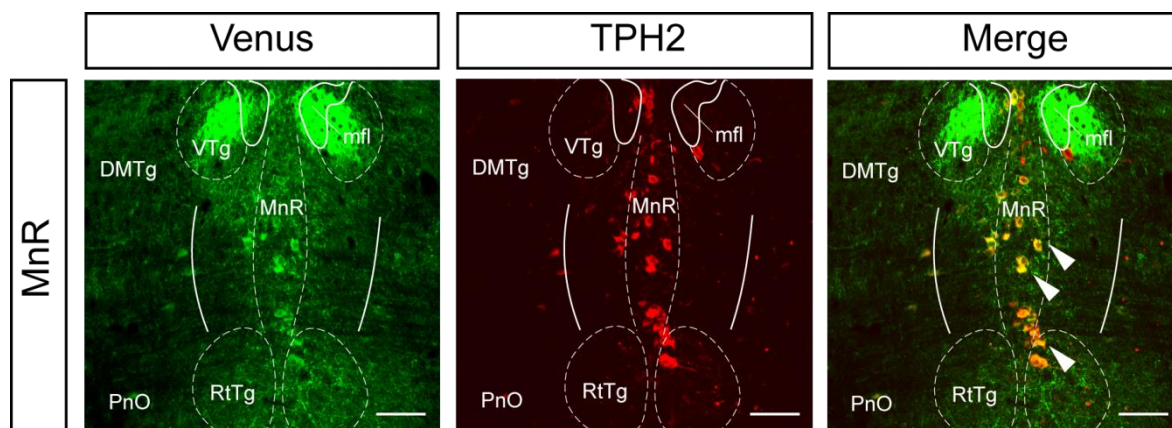
Figure 12. Expression of Venus reporter in the median raphe nucleus of CRHR2^{Venus} mice.

Filled arrows indicate examples of double positive neurons in the MnR.Coronal sections. Scale bars 100 μm. DMTg dorsomedial tegmental area, mfl medial longitudal fasciculus, MnR median raphe nucleus, PnO pontie reticular nucleus oral part, RtTg reticulotegmental nucleus of the pons, VTg ventriculat tegmental nucleus.

Interestingly, in both, i.e. dorsal and median raphe nuclei, a few neurons were found double positive for Venus and calbindin, a GABAergic marker (data not shown).

4.2 Generation of conditional CRHR2 knockout mice (CRHR2^{CKO})

Up to now only constitutive CRHR2 KO mice were established, nevertheless their behavioral phenotype is still a matter of discussion (Bale et al., 2000;Coste et al., 2006;Issler et al., 2014;Kishimoto et al., 2000). In order to address these discrepancies and to overcome the limitations of constitutive gene deletion, mice harbouring a floxed CRHR2 allele were generated using an embryonic stem cell clone with conditional potential which was obtained from the EUCOMM/KOMP consortium. Based on these floxed CRHR2 mice a set of conditional CRHR2 knockout mice was established using the Cre/loxP system. This approach allowed us to spatially and/or temporally control the inactivation of CRHR2 in those neurons which share similar neurochemical identity, while sparing its expression in others. Initially the conditional CRHR2 allele contained a LacZ reporter and selection cassettes which caused disruption of CRHR2 expression throughout all tissues (Figure 13). To restore the expression of CRHR2, these CRHR2 reporter mice (lacZ/LacZ) were bred with Flp-deleter mice (Rodriguez et al., 2000). From the F$_1$ generation animals heterozygous for the conditional CRHR2 allele (+/lox) and carrying gene for Flp were selected and bred to wild-type mice in order to obtain mice heterozygous for the conditional CRHR2 allele and devoid of Flp recombinase. Homozygous offspring for the conditional CRHR2 allele (lox/lox) was selected and used for breeding to mice transgenic for neurotransmitter-specific cre-recombinases. In the following generation obtained offspring which was heterozygotous for the floxed CRHR2 allele (+/lox) and carried the respective cre-recombinase. These mice were bred to double floxed CRHR2 mice (lox/lox). In subsequent experiment mice homozygous for CRHR2 (lox/lox) with (CKO) or without (Ctrl) the cre-recombinase were used for experiments.
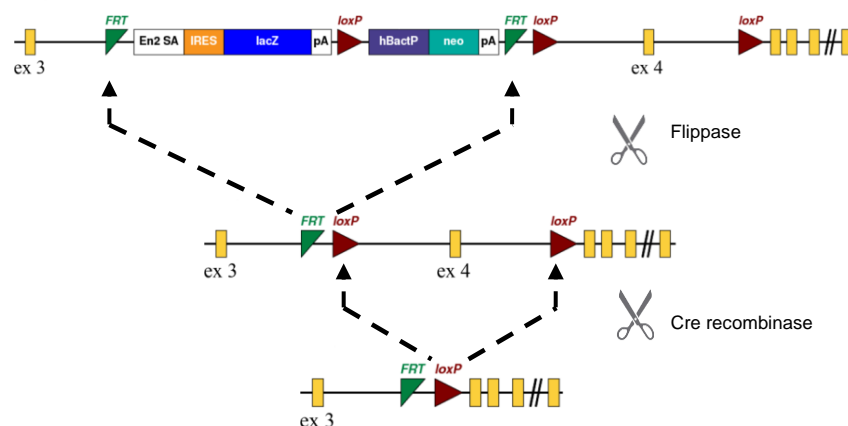
Figure 13. Simplified scheme for generation of conditional CRHR2 knockout mice (CRHR2^{CKO}).

The originally targeted CRHR2 locus contains a LacZ reporter and a Neo selection cassette. Upon Flp mediated recombination both cassettes are deleted and the expression of CRHR2 is restored. Cre-mediated recombination leads to deletion of exon 4 with a downstream premature stop codon which results in the functional inactivation of CRHR2.

In order to establish specific conditional CRHR2 KO mice, homozygous floxed control mice (CRHR2^{Ctrl}) were breed to various cre-recombinase expressing mouse lines (Figure 14). To generate mice lacking CRHR2 in the entire central nervous system, but not in the periphery (CRHR2$^{CKO-CNS}$), CRHR2^{Ctrl} mice were bred to mice expressing cre-recombinase expressed under the control of the Nestin promoter, which is active in the entire central nervous system. To remove CRHR2 from forebrain GABAergic neurons (CRHR2$^{CKO-GABA}$) cre-recombinase active under the Dlx5/6 promoter was introduced. The conditional deletion of CRHR2 KO from serotonergic neurons (CRHR2$^{CKO-5HT}$) was achived by breeding CRHR2^{Ctrl} mice to the ePet-Cre mouse line. Finally, in order to generate CRHR2$^{CKO-Camk2a}$ mice the CRHR2^{Ctrl} mice were bred to mice expressing Camk2a-CreERT2. Homozygous for CRHR2 transgenic allele adult animals carrying gene for Camk2aCreERT2 (CRHR2$^{CKO-Camk2a}$) and their control littermates (CRHR2^{Ctrl}) were fed for two weeks with food pellets containing tamoxifen. This time frame was long enough to induce recombination of CRHR2 floxed gene in the adulthood and produce conditional CRHR2 KO mice.
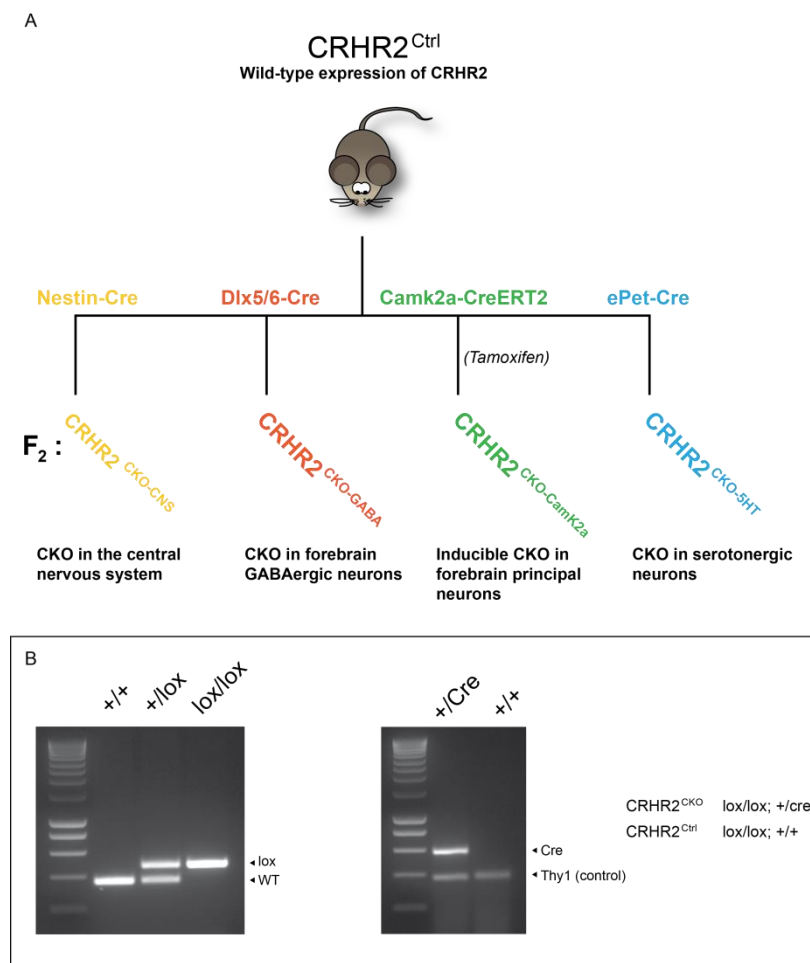
Figure 14. Generation and genotyping of specific CRHR2^{CKO} mice.

A, Homozygous floxed mice were breed to cre-specific mouse lines, in which expression of cre-recombinase is driven by nestin, Dlx 5/6, Camk2a or ePet promoter. B, Genotyping results of CRHR2 transgene and WT alleles and presence of cre recombinase in CRHR2^{CKO} mice.

4.3 Impact of CRHR2 deletion from the central nervous system on animals behavior - CRHR2^{CKO-CNS}

In the first step we generated CRHR2 mice devoid of CRHR2 in the central nervous system (CRHR2^{CKO-CNS}). This strategy would provide us an answer whether the behavioral phenotype observed in CRHR2 KO mice is indeed centrally mediated and not due to secondary effects of CRHR2 deficiency in peripheral tissues. Because of the disruption of CRHR2 in the entire CNS during embryogenesis, CRHR2^{CKO-CNS} mice are the most similar to constitutive CRHR2 KO animals.

4.3.1 Distribution of CRHR2 mRNA expression in CRHR2^{CKO-CNS} mice

As expected CRHR2^{CKO-CNS} mice are devoid of CRHR2 mRNA in neuronal tissue (Figure 15). In brains of CRHR2^{CKO-CNS} mice, a CRHR2 mRNA signal remains in the choroid plexus which consists of non-neuronal cells.
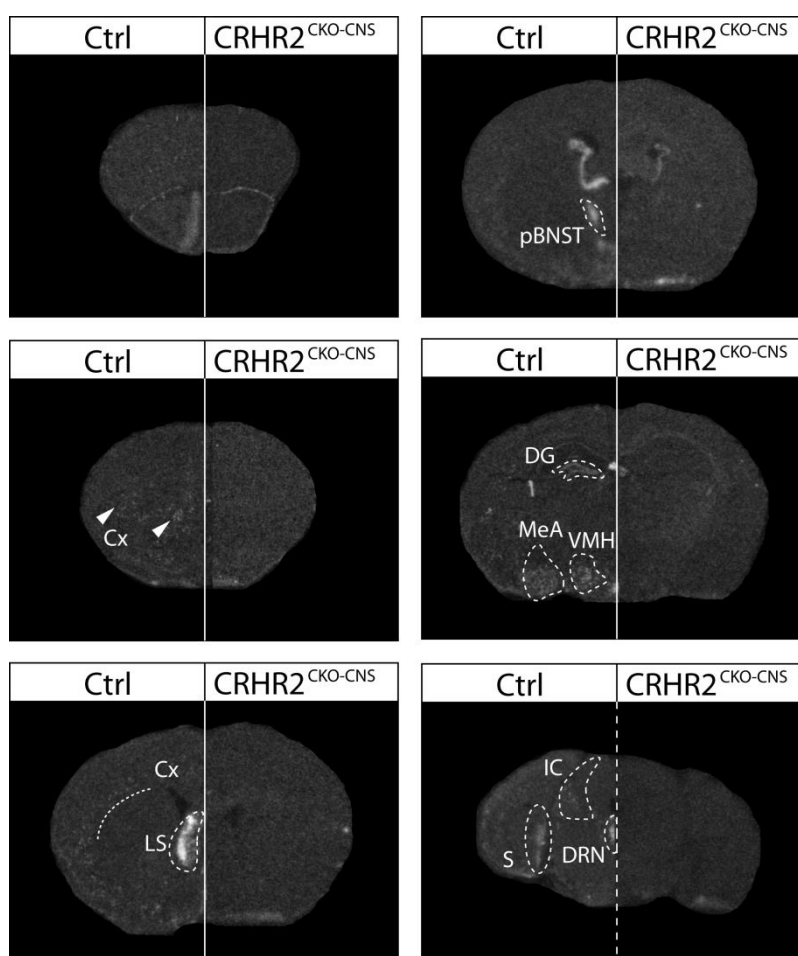
Figure 15. Deletion of CRHR2 mRNA in CRHR2^{CKO-CNS} mice.
In CRHR2^{CKO-CNS} mice no mRNA in the neuronal tissue is present. ISH autoradiographic inverted pictures from coronal brain sections.

4.3.2 Behavioral characterization of CRHR2$^{CKO-CNS}$ mice

4.3.2.1 CRHR2$^{CKO-CNS}$ mice show a mild increase in anxiety-related behavior

In the CRHR2$^{CKO-CNS}$ mouse line CRHR2 is removed from the entire central nervous system, but unlike in the constitutive CRHR2 KO animals the receptor is still present in tissues outside of the CNS. In order to evaluated anxiety-related behavior of CRHR2$^{CKO-CNS}$ mice, animals were tested in the open field test, the dark/light box test. In the open field test (Figure 16) conditional CRHR2$^{CKO-CNS}$ mice did not differ in any of analyzed parameters, i.e. the total distance travelled (p=0.7857), distance travelled in the inner zone (p=0.6444), time spent in the inner zone (p=0.7818), number of entries into the inner zone (p=0.9508), with the exception of a non-significant trend toward longer latency to the first approach into the inner zone (p=0.1227).
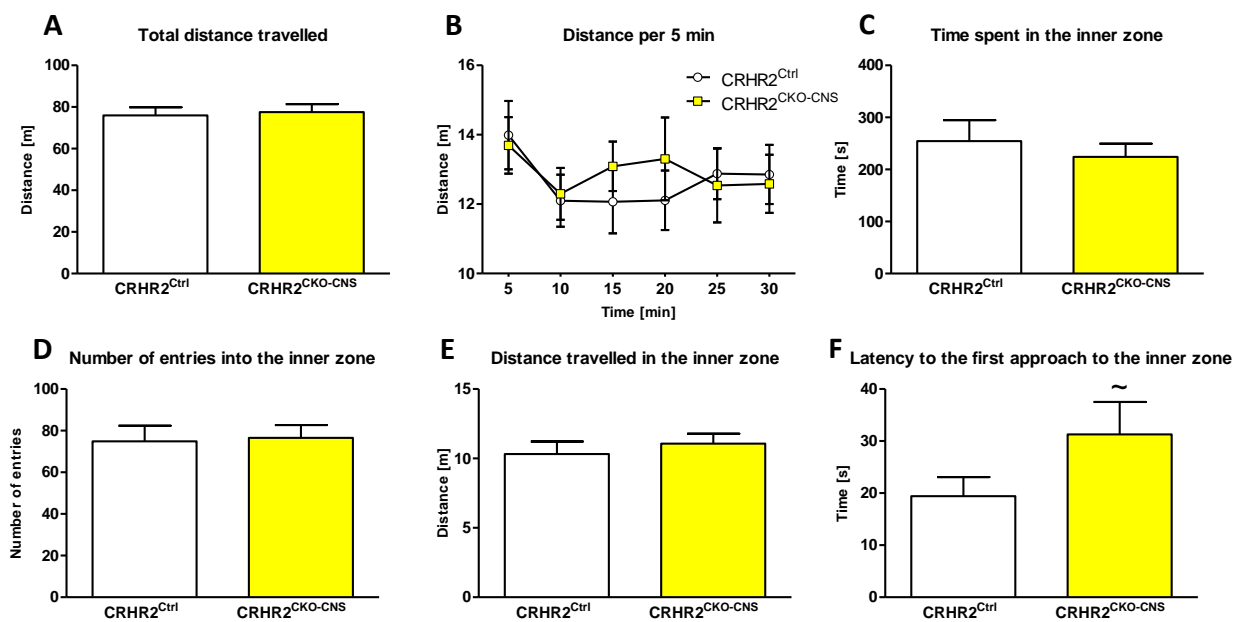
Figure 16. Anxiety-related behavior in CRHR2$^{CKO-CNS}$ mice measured in the open field test.
A, total distance travelled B, distance travelled split in 5 min blocks C, time spent in the inner zone D, number of entries into the inner zone E, distance travelled in the inner zone F, latency to the first entry to inner zone. Data are expressed as means +S.E.M. n=12-11 per group. ~ 0.15>p>0.05 Student's t test.

In the dark/light box test (Figure 17) CRHR2$^{CKO-CNS}$ mice showed increased anxiety-related behavior reflected by a significantly decreased number of entries into the lit compartment (p=0.0114) and an increased latency to the first approach of the lit compartment (p=0.0064). Although the time spent in the lit compartment (p=0.4705) and total distance travelled (p=0.3189) did not differ between genotypes, CRHR2$^{CKO-CNS}$ mice tended to travel less distance in the lit compartment of the apparatus (p=0.0630).
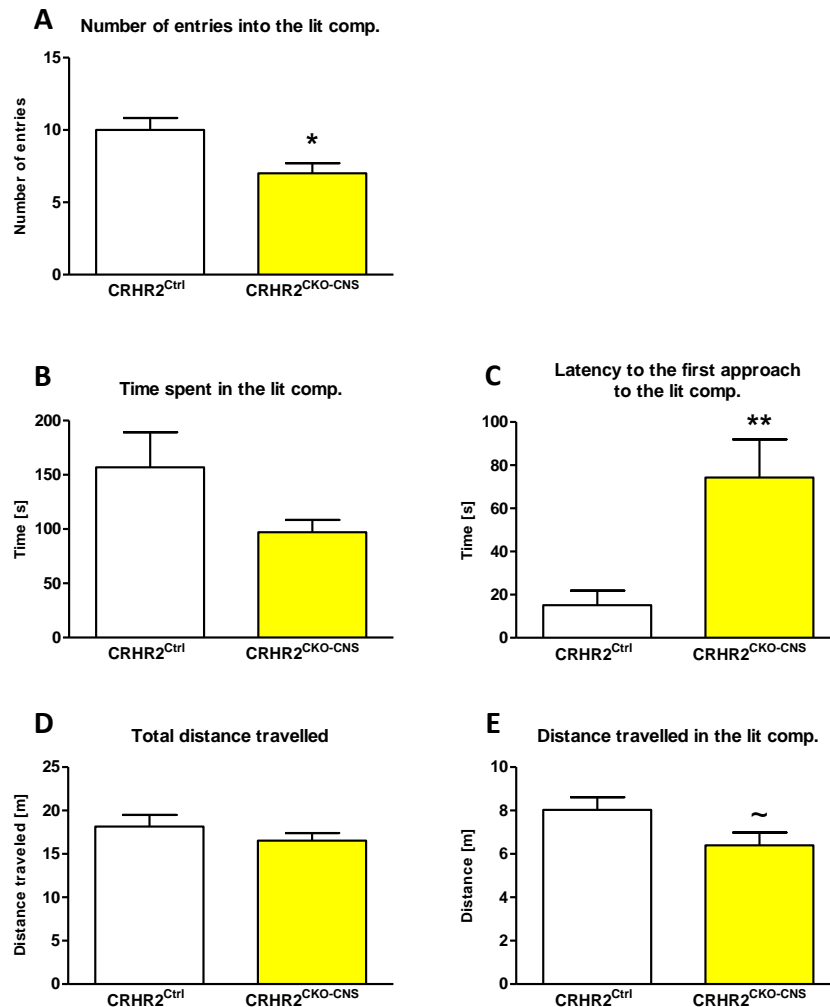
Figure 17. Anxiety-related behavior in CRHR2$^{CKO-CNS}$ mice measured in the dark/light box test.
A, number of entries into the lit compartment B, time spent in the lit compartment C, latency to the first entry to the lit compartment D, total distance travelled E, distance travelled in the lit compartment. Data are expressed as means +S.E.M. n=12 per group. * p<0.05, ** p<0.01, ~ p<0.1. Student's t test.

4.3.2.2 CRHR2$^{CKO-CNS}$ mice show increased passive stress-coping behavior

Despite variable anxiety-related phenotypes of previously CRHR2 KO mice, it has been repeatedly shown that deletion of CRHR2 induces a state of behavioral despair (Bale and Vale, 2003;Coste et al., 2006;Todorovic et al., 2009). In order to evaluate stress coping behavior in mice lacking centrally CRHR2 we performed the forced swim test (Figure 18). In the FST CRHR2$^{CKO-CNS}$ mice showed a significant decrease in time spent swimming along with an increase in time spent floating, indicating the state of behavioral despair.
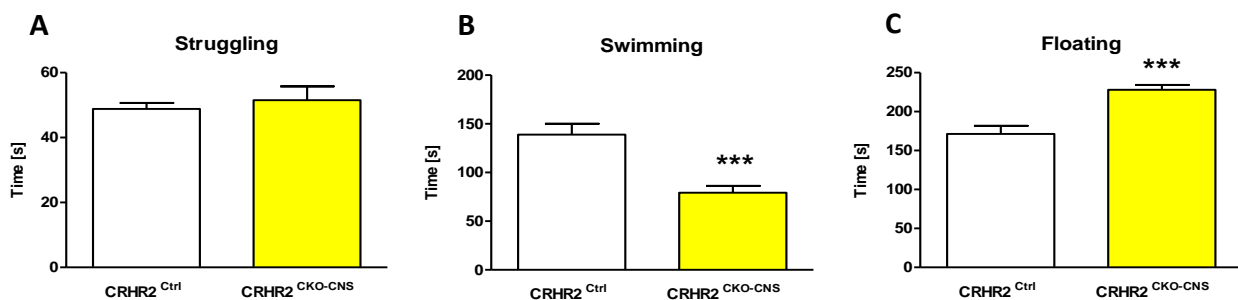
Figure 18. Decreased active stress coping behavior in CRHR2$^{CKO-CNS}$ mice.

A, time spent struggling B, time spent swimming C,time spent floating in the forced swim test. *** *p*<0.001. Student's t-test.

4.4 Assessing the role of CRHR2 in the GABAergic system

We previously confirmed that CRHR2 is mainly expressed in GABAergic neurons of the forebrain, e.g. the LS, BNST, cortex. To specifically address CRHR2 function in these circuits we generated CRHR2$^{CKO-GABA}$ mice.

4.4.1 Conditional deletion of CRHR2 in GABAergic neurons in CRHR2$^{CKO-GABA}$ mice

In order to assess the impact of CRHR2 in GABAergic neurons on behavior and stress response, we generated conditional CRHR2 mice devoid of CRHR2 in forebrain GABAergic neurons. In CRHR2$^{CKO-GABA}$ mice the deletion of CRHR2 in GABAergic neurons is directed by the Dlx5/6-Cre.

4.4.2 Pattern of CRHR2 mRNA deletion in CRHR2$^{CKO-GABA}$ mice

Expression of CRHR2 mRNA in mutant and control mice was evaluated by radioactive in situ hybridization (Figure 19).
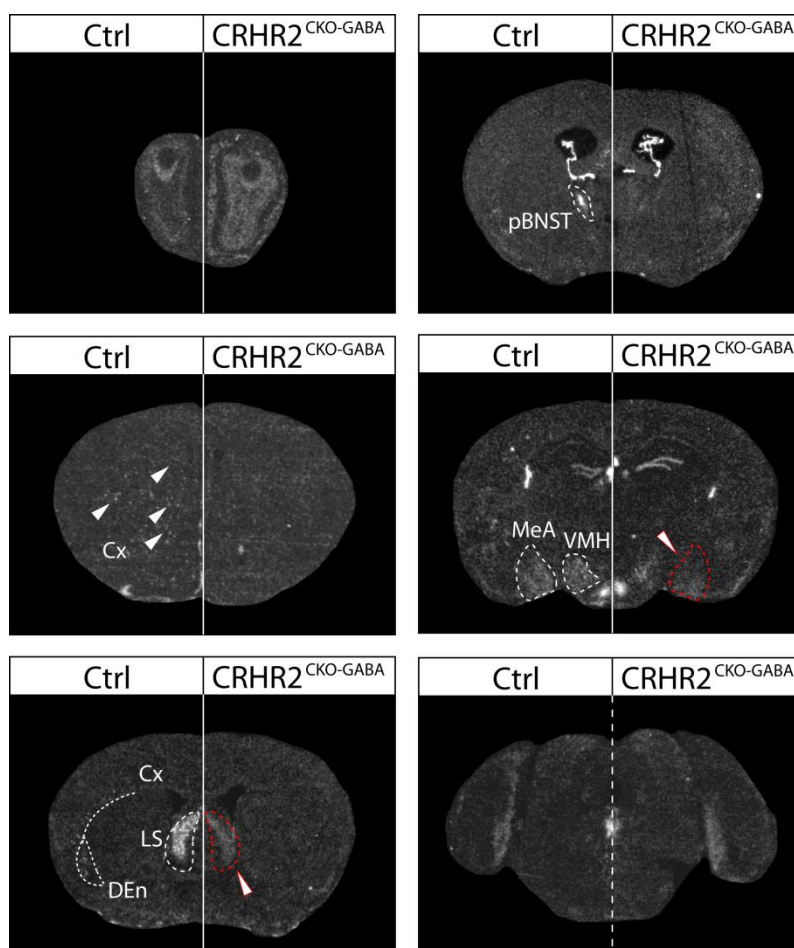
Figure 19. Deletion of CRHR2 mRNA in CRHR2$^{CKO-GABA}$ mice.
Reduction in signal intensity in CRHR2$^{CKO-GABA}$ mice was observed in the LS, pBNST, MeA and the VMH. The signal is fully abolished in cortex and in the endopiriform nucleus. Autoradigraphic inverted ISH pictures of coronal brain sections.

We found a decrease in CRHR2 signal intensity in the LS and MeA of CRHR2$^{CKO-GABA}$ mice. CRHR2 mRNA was entirely gone from the BNST. The majority of CRHR2 mRNA was deleted form the PFC, cortical layers and DEn. Signal intensity in IC, DRN, DG and cp was not changed, confirming the specificity and restriction of the CRHR2 deletion to forebrain GABAergic neurons.

4.4.3 Behavioral characterization of CRHR2$^{CKO-GABA}$ mice.

4.4.3.1 Deletion of CRHR2 in GABAergic neurons increases anxiety-related behavior and affects stress-coping behavior

In terms of anxiety-related behavior the open field test, dark/light box test and the elevated plus maze test were performed. Neither in the open field test (Figure 20) nor in the dark-light box test (Figure 21) any of the anxiety-related parameters, i.e. time spent in the inner zone of the open field (p=0.3423), the number of entries into the inner zone (p=0.5213), the number of entries into the lit compartment (p=0.6668), time (p=0.5144), distance (p=0.6992) or latency to the first approach therein (p=0.8399) were significantly affected by the genotype. There was also no genotype specific difference in the total distance travelled during the open field test (p=0.2752).
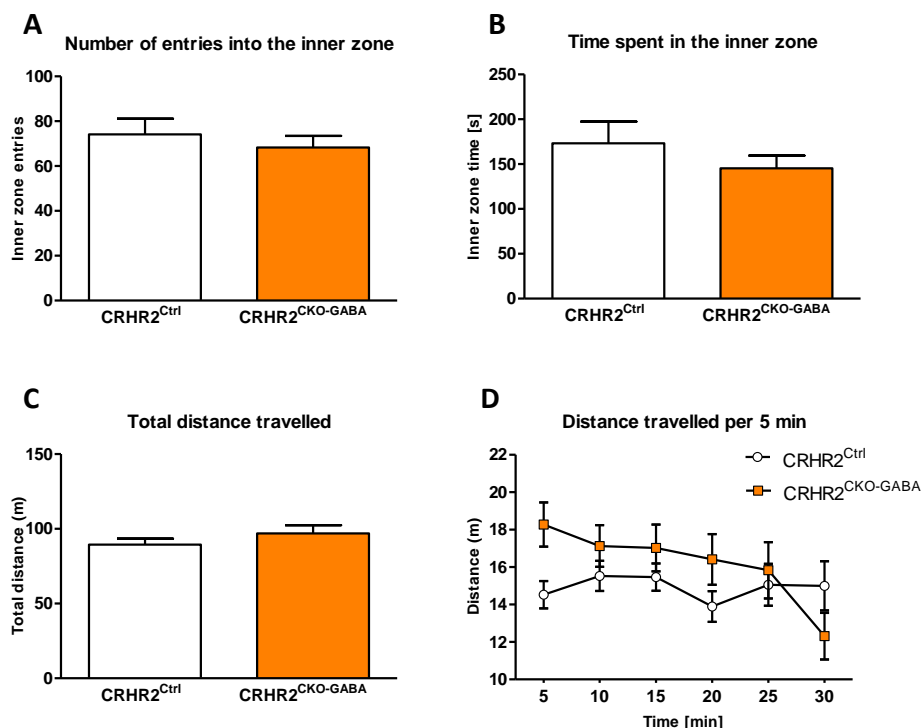
Figure 20. Anxiety-related behavior and locomotion in CRHR2$^{CKO-GABA}$ mice measured in the open field test.
A, number of entries into the inner zone B, time spent in the inner zone C, total distance travelled during 30 min of the test duration or E, split in 5 min intervals. Data are expressed as means +S.E.M. n=12-11 per group. Student's t test.
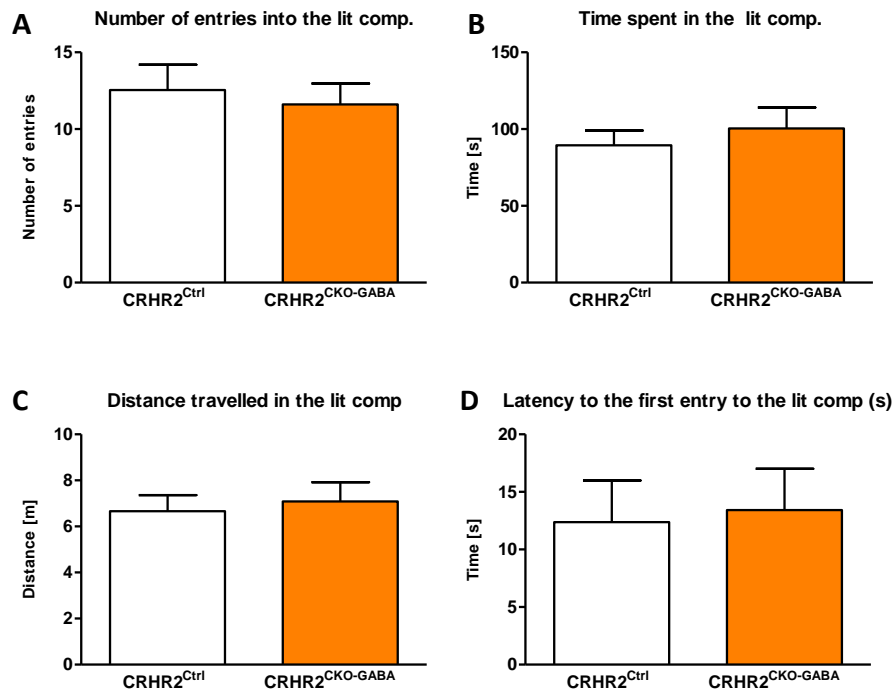
**Figure 21. Anxiety-related behavior in CRHR2^CKO-GABA mice measured in the dark/light box test.**
A, number of entries into the lit compartment B, time spent in the lit compartment C, the distance travelled in the lit compartment D, latency to the first entry to the lit compartment. Data are expressed as means +S.E.M. n=11-10 per group. Student's t test.

In contrast to the open field test and the dark/light box test (Figure 22) conditional CRHR2^CKO-GABA knockout mice spent significantly less percentage of time on the open arms of the elevated plus maze (*p*=0.0481) resulting in a significantly increased percentage of time spent in closed arms (*p*=0.0481). Also the number of entries into the open arms tented to be decreased in CRHR2^CKO-GABA knockout mice compared to control littermates (*p*=0.1378). There was no genotype specific difference in the total distance travelled (*p*=0.3024), the distance travelled on the open arms (*p*=0.1696) or the latency to the first entrance into open arm (*p*=0.8249).
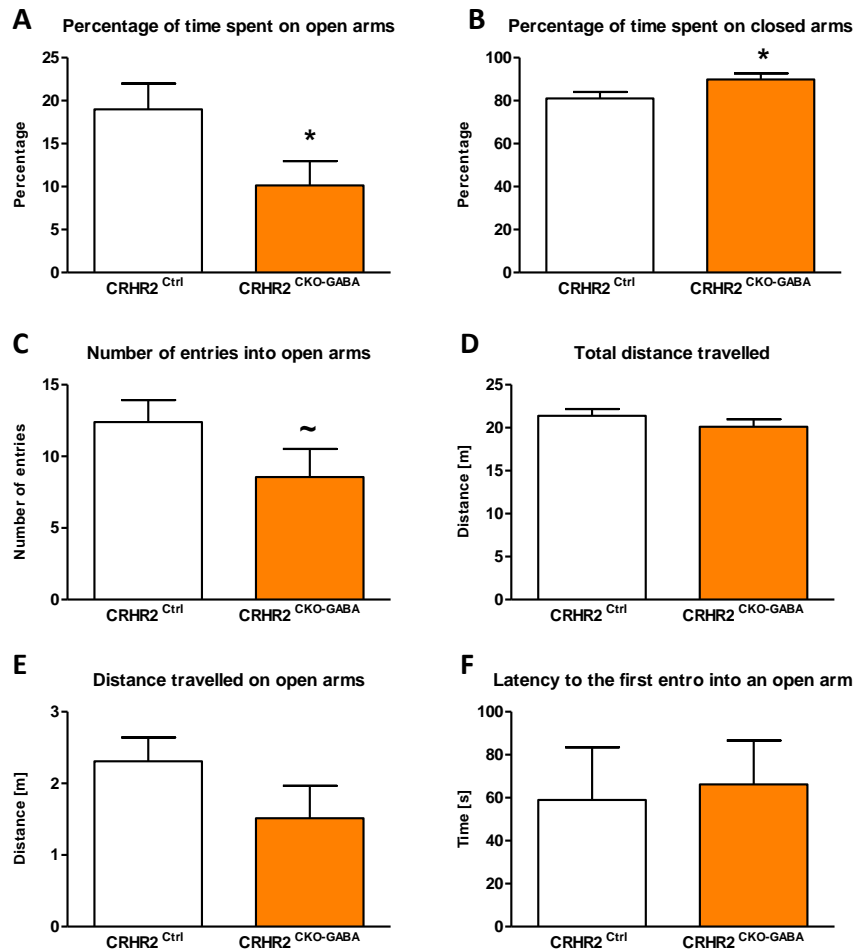
Figure 22. Anxiety-related behaviour in CRHR2$^{CKO-GABA}$ mice measured in the elevated plus maze test.

A, percentage of time spent on the open arms B, percentage of time spent in the closed arms C, number of entries into the open arms D, total distance travelled E, distance travelled on the open arms F, the latency to the first entrance into open arm. Data are expressed as means +S.E.M. n=10-9 per group. * $p<0.05$, ~ $p<0.15$. Student's t test.

CRHR2 KO mice have consistently been shown to display signs of behavioral despair in terms of decreased active stress coping behavior (Bale and Vale, 2003;Todorovic et al., 2009). CRHR2$^{CKO-GABA}$ mice were tested in the forced swim test to evaluate their stress coping behavior (Figure 23). The time spent struggling, swimming and floating was scored. During 6 min of a standard FST session, CRHR2$^{CKO-GABA}$ showed no significant change in time spent struggling ($p=0.1260$) and floating ($p=0.1749$), however the time spent swimming was significantly decreased ($p=0.0464$), implicating a mild decrease in active stress coping behavior, in a similar directionas in CRHR2$^{CKO-CNS}$ mice.
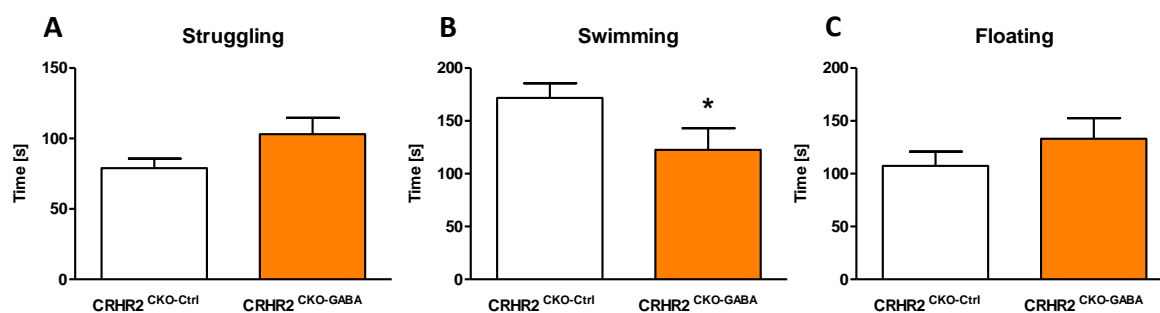
Figure 23. Stress coping behavior measured in the forced swim test in CRHR2$^{CKO-GABA}$ mice.

A, time spent struggling B, time spent swimming and C, time spent floating. Data are expressed as means +S.E.M. n=12-11 per group. * $p<0.05$. Student's t test.

4.4.3.2 Deletion of CRHR2 in GABAergic neurons affects the acoustic startle response but not prepulse inhibition

Aberrations in acoustic startle response (ASR) have been shown to be present in many psychiatric disorders e.g. PTSD and schizophrenia. Interestingly, CRH, UCN2 and UCN3 have been shown to increase the ASR, while UCN1 KO mice display a significant decrease in ASR, which is not due to hearing deficits (Risbrough et al., 2003;Risbrough et al., 2004;Vetter et al., 2002;Wang et al., 2002). In this respect ASR was assessed in CRHR2$^{CKO-GABA}$ mice (Figure 24). The ASR was measured in a soundproof apparatus with the constant background noise level of 55 dB. During a 45 min session animals were presented sound startle stimuli of 75, 90, 105 and 115 dB appearing randomly in time to avoid habituation. CRHR2$^{CKO-GABA}$ mice displayed a significant decrease in ASR to the highest stimulus intensities i.e. 105 and 115 dB.
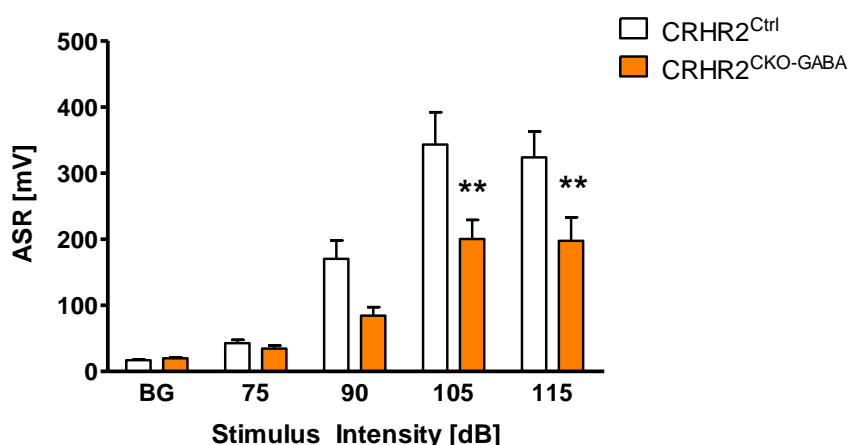
Figure 24. Acoustic startle response in CRHR2$^{CKO-GABA}$ mice.

CRHR2$^{CKO-GABA}$ mice showed significant decrease in acoustic startle response to 105 and 115 dB stimuli. No change was noted in background activity. Data are expressed as means +S.E.M. n=10 per group. ** $p<0.01$. Two way ANOVA repeated measures.

In addition to the ASR also the prepulse inhibition (PPI) was measured (Figure 25). In PPI, the percentage of inhibition of acoustic startle response to low intensity prepulse, preceeding pulse presentation, is measured. In other words presentation of a prepulse before the pulse inhibits the response to the pulse. This inhibition is proportional to the intensity of the prepulse. CRHR2[CKO-GABA] mice did not differ from their littermate controls in the PPI test at any of prepulse intensities, although also here the ASR to a pulse presented without prepulse was significantly decreased (p=0.0146) in line with the data from the direct ASR test. Background activity of animals was not affected by genotype.
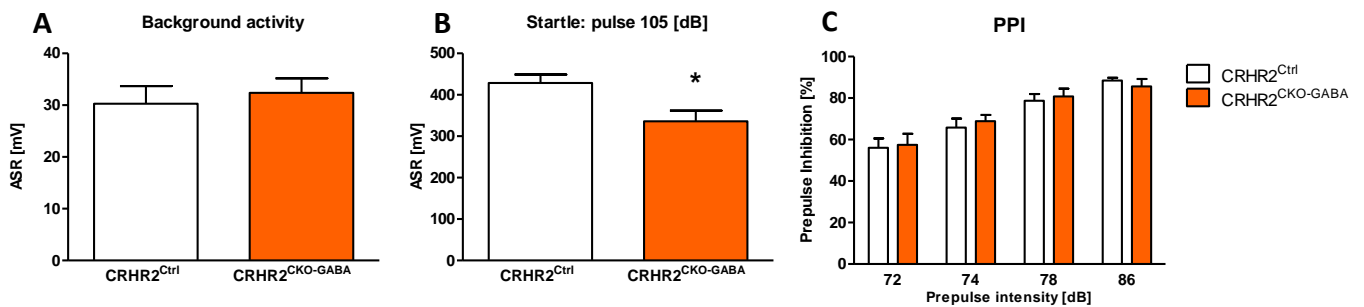
Figure 25. Prepulse inhibition measured in CRHR2[CKO-GABA] mice.
A, background activity B, ASR to pulse alone C, prepulse inhibition. Data are expressed as means +S.E.M. n=11-9 per group.* p<0.05. Student's t-test.

4.4.4 CRHR2$^{CKO-GABA}$ mice exhibit dysregulation of HPA axis similar to CRHR2 KO mice

It has been shown that CRHR2 KO mice are hypersensitive to 2 and 10 min of restraint stress, resulting in excess of released corticosterone (Bale et al., 2000;Coste et al., 2006). In order to recapitulate these outcomes under our laboratory conditions, constitutive CRHR2 KO mice were immobilized for 2 or 10 min and blood samples were collected immediately afterwards. The concentration of corticosterone in the blood of homozygous CRHR2 KO mice was significantly increased upon 2 min (p=0.0043) and 10 min (p=0.0385) of restraint stress (Figure 26). After 90 min of recovery from 10 min of restraint corticosterone concentration did not differ between genotypes (p=0.5664).
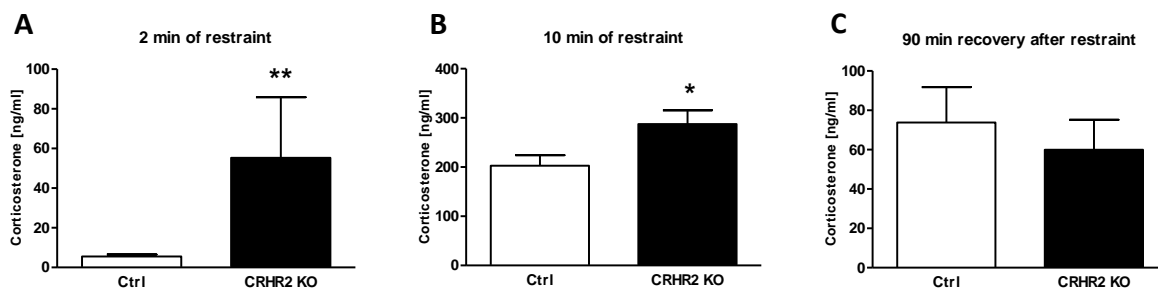
Figure 26. Restraint stress induced corticosterone release in CRHR2 KO mice.
A, plasma corticosterone concentrations after 2 min B, and 10 min of restraint stress C, corticosterone concentration measured 90 min after 10 min of restraint stress. Data are expressed as means +S.E.M. n=7-6 per group, ***** p<0.05, ****** p<0.01. Student's t-test.

The same experiment as described above was repeated in CRHR2$^{CKO-GABA}$ mice, additionally extended for measuring blood corticosterone concentrations during morning trough and evening peak (Figure 27). In CRHR2$^{CKO-GABA}$ mice the plasma corticosterone concentration was significantly increased only after 2 min of restraint (p=0.0003). After 10 min of restraint (p=0.3181) and after the recovery period of 90 min (p=0.3269) CRHR2$^{CKO-GABA}$ mice did not differ from CRHR2^{Ctrl} mice.
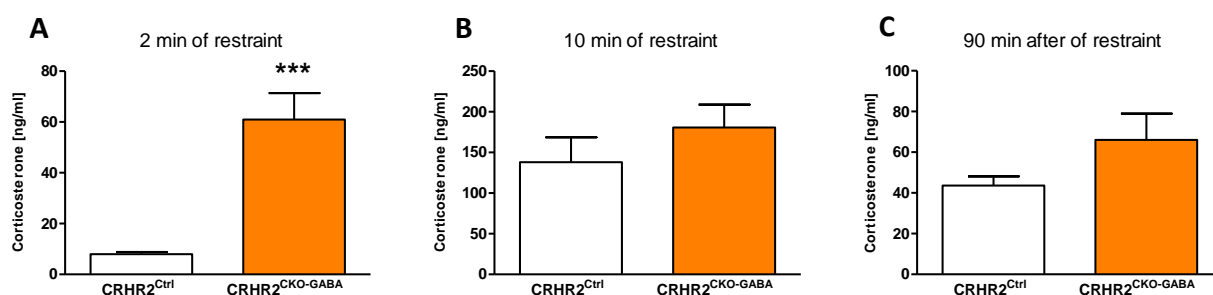
Figure 27. Restraint stress induced corticosterone release in CRHR2$^{CKO-GABA}$ mice.
A, plasma corticosterone concentrations after 2 min and B, 10min of restraint stress. C, corticosterone concentration measured 90 min after 10 min of restraint stress. Data are expressed as means +S.E.M. n=9-22 per group, ******* $p<0.001$. Student's t-test.

There was no genotype difference found in circadian rhythm of corticosterone release between control littermates and CRHR2$^{CKO-GABA}$ knockouts (Figure 28 am nadir: $p=0.3713$; pm peak: $p=0.9402$).
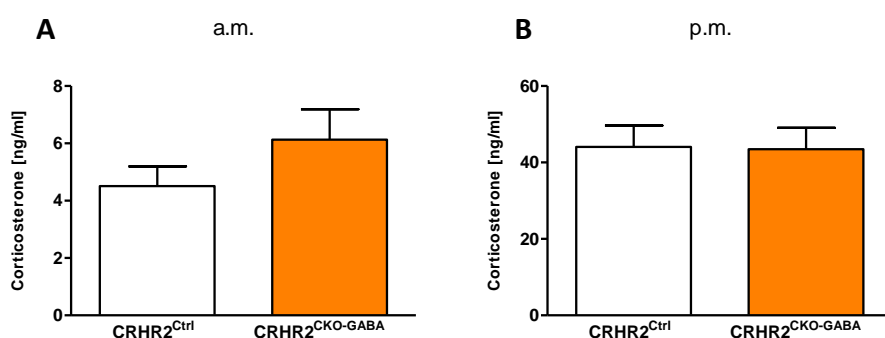
Figure 28. Circadian rhythm of corticosterone release in CRHR2$^{CKO-GABA}$ mice.
A, plasma corticosterone concentrations during morning trough and B, evening peak. Data are expressed as means +S.E.M. n=22-20 per group. Student's t-test.

4.4.4.1 C-fos mRNA expression after 2 min of restraint stress

In order to define the brain sites where CRHR2 controls reactivity of the HPA axis in CRHR2$^{CKO-GABA}$ mice we performed in situ hybridization against the immediete early gene, c-fos.

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Animals were restraint for 2 min and afterwards rapidly sacrificed. Brains were isolated and cut on cryostat. The period of 2 min of restraint did not affect expression of c-fos mRNA in the control group, although it induced strong expression of c-fos mRNA thgoughout the brain in the conditional CRHR2$^{CKO\text{-}GABA}$ mice (Figure 29).
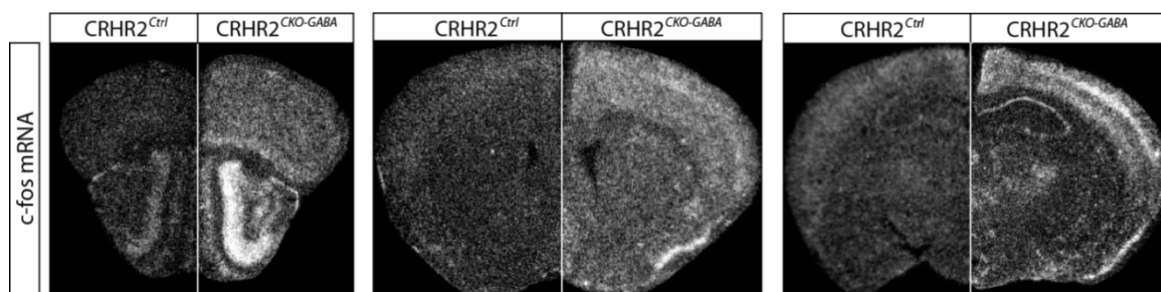
Figure 29. Expression of c-fos mRNA induced by 2 min of restraint stress.
Expression of c-fos mRNA was highest in the cortex. No subcortical structure was particularly affected by 2 min of restraint stress. Inverted utoradiographic pictures of ISH on coronal brain sections.

As the strongest activation was present in the cortex of CRHR2$^{CKO\text{-}GABA}$ mice, for the next experiment only this structure was selected. Animals were immobilized for 2 min and afterwards brains were rapidly isolated. The entire cortex was dissected and used for RNA extraction. Expression of c-fos mRNA was quantified by real-time PCR. In addition, non-stressed control groups of both genotypes underwent the same procedure in order to exclude potential changes in c-fos expression between genotypes existing prior to the restraint. Under non-stress condition no change in cortical c-fos mRNA expression was detected between CRHR2$^{CKO\text{-}GABA}$ and CRHR2^{Ctrl} mice (Figure 30). Nevertheless, stressed CRHR2$^{CKO\text{-}GABA}$ mice showed an almost 2-fold increase in cortical content of c-fos mRNA.
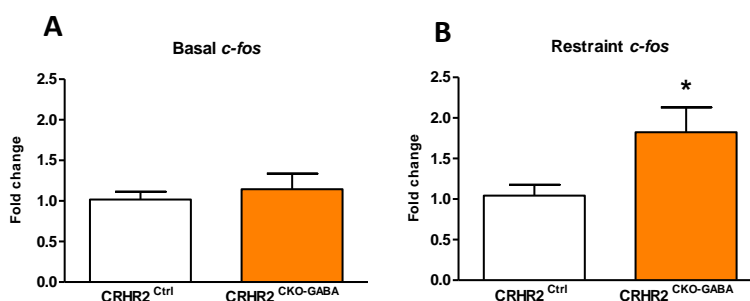
Figure 30. Expression of c-fos mRNA in CRHR2$^{CKO\text{-}GABA}$ mice upon 2 min of immobilization stress and under non-stress conditions.
A, qPCR measured c-fos expression under basal conditions and B, after 2 min of immobilization stress. * $p < 0.05$. Student's t-test.

4.4.4.2 Assesment of the function and expression of the glucocorticoid receptor in CRHR2$^{CKO-GABA}$ mice

Stimulation of glucocorticoid receptors plays an important role in inhibition of the HPA activity. In order to evaluate the expression of glucocorticoid receptors under non-stress conditions, Western blot analysis was performed on protein extracts prepared from isolated cortex and hippocampal tissue from mice of both genotypes. No change in expression of the glucocorticoid receptor was found between genotypes, neither in cortex nor in hippocampus (data not shown).

Cortical glucocorticoid receptor
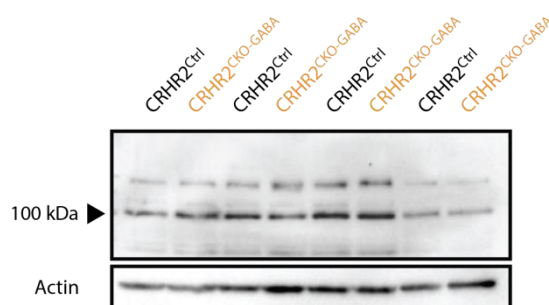
Figure 31. Expression of glucocorticoid receptor protein in the cortex of CRHR2$^{CKO-GABA}$ mice.
The band at 100 kDa corresponds to the glucocorticoid receptor. Actin is provided as a loading control.

Although expression of GR was not changed it was still possible that its activity was decreased. For that reason function of GR was examinated using the dexamethasone suppresion test (Figure 32). Animals of both groups were injected with dexamethasone solution at 12 pm, 6 hours later blood was colected and serum corticosterone concentration was measured. In both groups dexamethasone suppressed the evening peak of corticosterone release, however no genotype specific difference was detected.
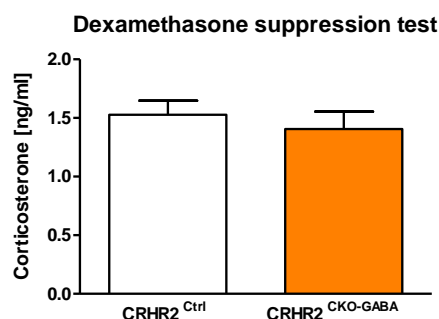
Figure 32. Plasma corticosterone concentration following the dexamethasone suppresion test in CRHR2$^{CKO-GABA}$ mice.
No change in plasma corticosterone levels were detected between CRHR2$^{CKO-GABA}$ and CRHR2^{Ctrl} mice.

As a possible influence of the GR in the cortex of CRHR2$^{CKO\text{-}GABA}$ was excluded on both the expressional and functional levels, we assessed compensatory mechanisms which could occure as a consequense of cortical CRHR2 deficiency. Interestingly, we found significantly increased cortical CRH mRNA expression along with a non-significant increase of CRHR1 mRNA in CRHR2$^{CKO\text{-}GABA}$ mice which could possibly explain observed effects (Figure 33).

A   Basal CRHR1 mRNA   **B**   Basal CRH mRNA
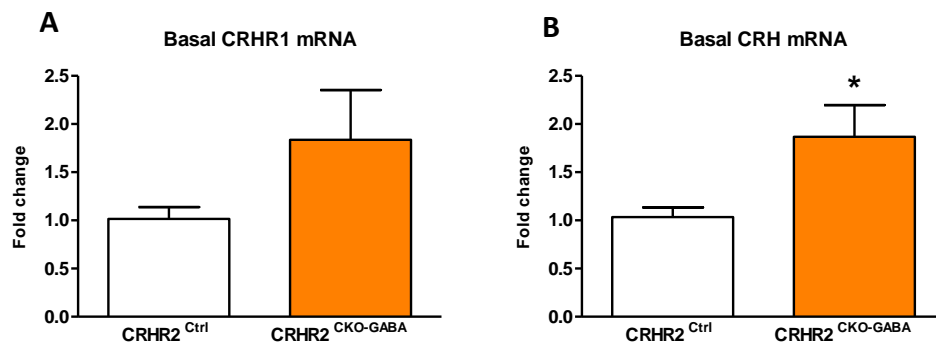
Figure 33. Expression of CRH and CRHR1 genes in the cortex of CRHR2$^{CKO\text{-}GABA}$ mice.
A, basal expression of CRH and B, CRHR1 genes in the cortex of unstressed animals. * $p<0.05$. Student's t-test.

4.4.4.3 Chronic social defeat stress augments the HPA axis dysregulation in CRHR2$^{CKO-GABA}$ mice

Mice of both genotypes were split into 4 groups depending on genotype and chronic stress exposure, i.e. CRHR2^{Ctrl} and CRHR2$^{CKO-GABA}$ stressed groups, which were exposed to every day social defeat stress and CRHR2^{Ctrl} and CRHR2$^{CKO-GABA}$ non-stressed groups, which were left in their home cages undisturbed. After two initial weeks of defeat animals were tested in a battery of anxiety-related tests. Basal and a stress induced concentration of plasma corticosterone were evaluated. Thymus involution along with adrenal gland enlargement were assessed as additional stress markers. As expected chronically stressed animals compared to non-stressed mice, independent of genotype, showed a decreased weight of thymus and an increase in adrenal gland weight. In addition, the weight of thymus was decreased in non-stressed CRHR2$^{CKO-GABA}$ mice compared to non-stressed CRHR2^{Ctrl} mice (Figure 34).
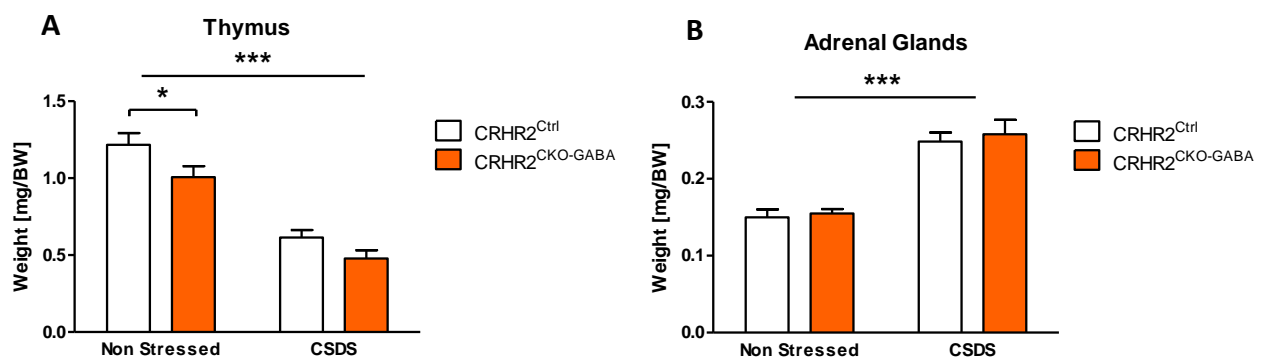
Figure 34. Effect of CSDS on physiological parameters of CRHR2$^{CKO-GABA}$ mice.
A, weight of thymus and B, of adrenal glands of animals exposed to CSDS. Data are expressed as means +S.E.M. n=12-10 per group,* $p<0.05$, *** $p<0.001$

Chronic social defeat stress strongly activates the HPA axis causing increased concentrations of circulating glucocorticoids. In this regard the basal morning corticosterone level was significantly elevated in stressed groups compared to non-stressed groups, yet without any genotype-specific effect. Interestingly, when all animals were challenged with the forced swim test and blood samples were collected 30 min afterwards, the rise in corticosterone concentration in stressed CRHR2$^{CKO-GABA}$ mice was not only significantly increased compared to the non-stressed groups,

but also compared to the stressed CRHR2[Ctrl] group (Figure 35). This interaction between genotype and stress effect was vanished in 60 min after the FST challenge.
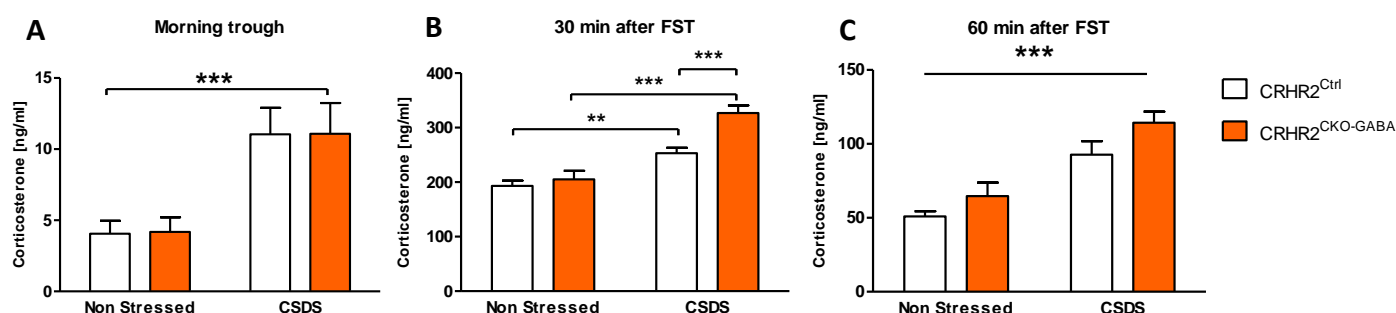
Figure 35. CSDS exaggerates HPA axis regulation upon stress challenge in CRHR2[CKO-GABA] mice.

A, plasma corticosterone measured during the morning peak B, plasma corticosterone measured 30 min after 5 min of FST C, plasma corticosterone measured 60 min after initial 5 min of FST. Two-way ANOVA. Data are expressed as means +S.E.M. n=12-10 per group,** $p<0.01$, *** $p<0.001$

In anxiety-related tests performed following CSDS only a stress effect among CRHR2[Ctrl] and CRHR2[CKO-GABA] was observed but neither a genotype-specific effect nor an interaction of stress and genotype (data not shown).

4.5 CRHR2[CKO-Camk2a] mice: assessing the role of CRHR2 deletion in principal forebrain neurons during adulthood

To study effects of CRHR2 in forebrain projecting neurons we generated CRHR2[CKO-Camk2a] mice. In contrast to other conditional CRHR2 lines in CRHR2[CKO-Camk2a] mice disruption of CRHR2 can be induced at any point of lifetime. This strategy allowed us to delete CRHR2 in adulthood, thus eliminating most of developmental compensatory effects of early life CRHR2 deficiency. In this model a tamoxifen inducible cre recombinase variant (cre fused to ER ligand binding domain) is expressed under control of the Camk2a promoter. Binding of CreERT2 to Hsp90 prevents cre-recombinase from translocation to the nucleus and recombination of DNA. Nevertheless, upon binding exogenously delivered tamoxifen, an estrogen receptor ligand, Hsp90 dissociates from the cre-recombinase enabling its translocation to the nucleus. Recombination and disruption of CRHR2 takes place in Camk2a positive neurons stopping translation of functional CRHR2.

4.5.1 Deletion of CRHR2 mRNA in adult CRHR2$^{CKO\text{-}Camk2a}$ mice

To evaluate the deletion of CRHR2 in CRHR2$^{CKO\text{-}Camk2a}$ mice we performed in situ hybridisation in adult males two weeks after induction of cre recombinase activity. We found a significant decrease of CRHR2 mRNA expression in the LS, BNST, MeA, S and DG (Figure 36).
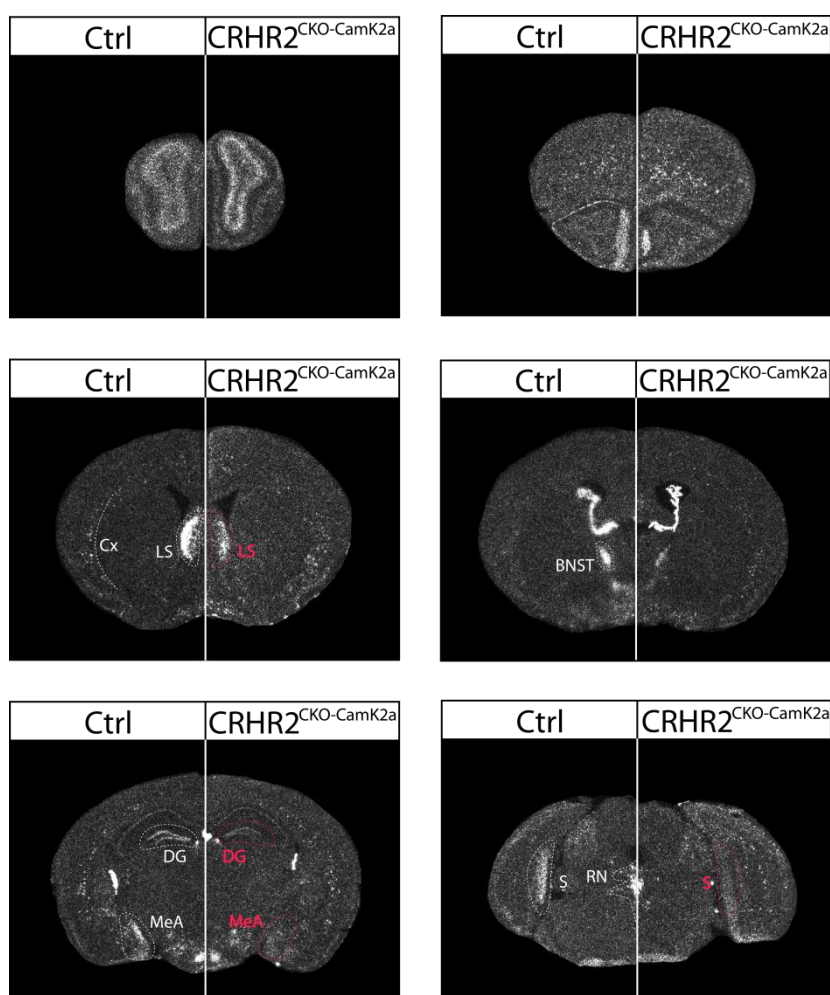
Figure 36. Deletion of CRHR2 mRNA after cre recombinase induction in CRHR2$^{CKO\text{-}Camk2a}$ mice.

In CRHR2$^{CKO\text{-}Camk2a}$ mice the expression CRHR2 mRNA is decreased within the LS, DG, MeA and S. Unexpectedly no change in CRHR2 mRNA was observed in the cortex. Inverted autoradiographic pitures of ISH on coronal brain sections.

In addition, expression of CRHR2 mRNA in CRHR2$^{CKO-Camk2a}$ mice was quantified within structures like LS, pBNST, MeA and DG (Figure 37). The highest decrease in signal intensity was found in LS and pBNST.
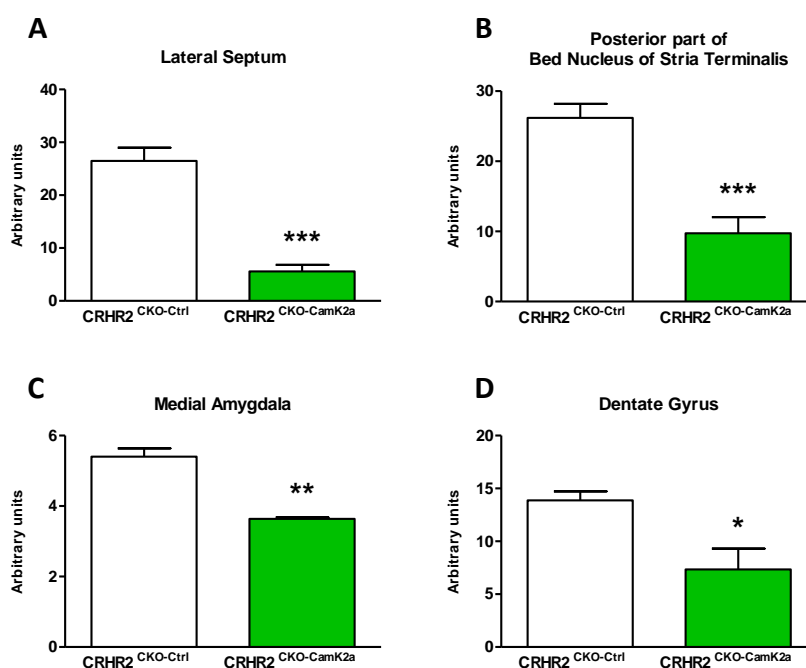
Figure 37. Quantification of CRHR2 mRNA expression in CRHR2$^{CKO-Camk2a}$ mice.

A, Lateral septum B, posterior bed nucleus of stria terminalis C, medial amygdala D, dentate gyrus. Data are expressed as means +S.E.M. n=5-4 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Student's t-test.

4.5.2 Deletion of CRHR2 in principal forebrain neurons in adulthood significantly increases anxiety-like behavior but not stress-coping behavior in CRHR2$^{CKO-Camk2a}$

All tests were performed after 4 weeks from the beginning of treatment of animals with palatable tamoxifen. Adult male mice (8 weeks) had for two weeks *ad libitum* access to manufactured food containing tamoxifen. These two initial weeks were followed by two additional weeks of wash out, when animals received again standard food pellets. Anxiety-related behaviour was assessed in the open field test and in the dark light box test.

In the open field test (Figure 38) CRHR2$^{CKO-Camk2a}$ mice showed increased anxiety-related behavior entering significantly less often the aversive inner zone of the apparatus ($p=0.0142$), spending there significantly less time ($p=0.0276$) and exploring it to lesser extent compared to the control group ($p=0.0144$). The total distance travelled ($p=0.6160$) as well as the latency to the first approach to the inner zone ($p=0.3896$) were not affected by the genotype.
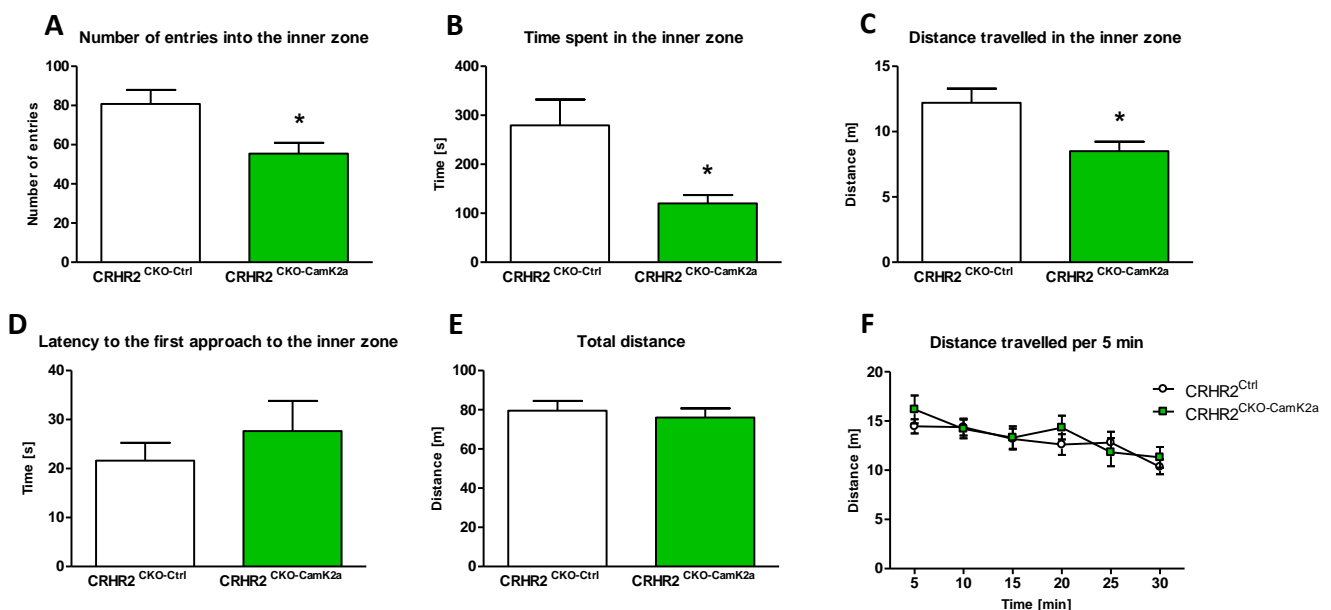
Figure 38. Anxiety-related behavior in CRHR2$^{CKO-Camk2a}$ mice measured in the open field test.

A, number of entries into the inner zone B, time spent in the inner zone C, distance travelled in the inner zone D, the latency to the first approach to the inner zone E, and F, total distance travelled during 30 min of the test duration or E, split in 5 min intervals. Data are expressed as means +S.E.M. n=11-9 per group. * $p<0.05$. Student's t-test.

In the dark/light box test (Figure 39) the entries to the aversive lit compartment were significantly reduced (*p*=0.0128) and the CRHR2$^{CKO-Camk2a}$ mice spent there significantly less time compared to control littermates (*p*=0.0350). In addition, the general locomotion of conditional CRHR2$^{CKO-Camk2a}$ mice was significantly decreased (*p*=0.0279).
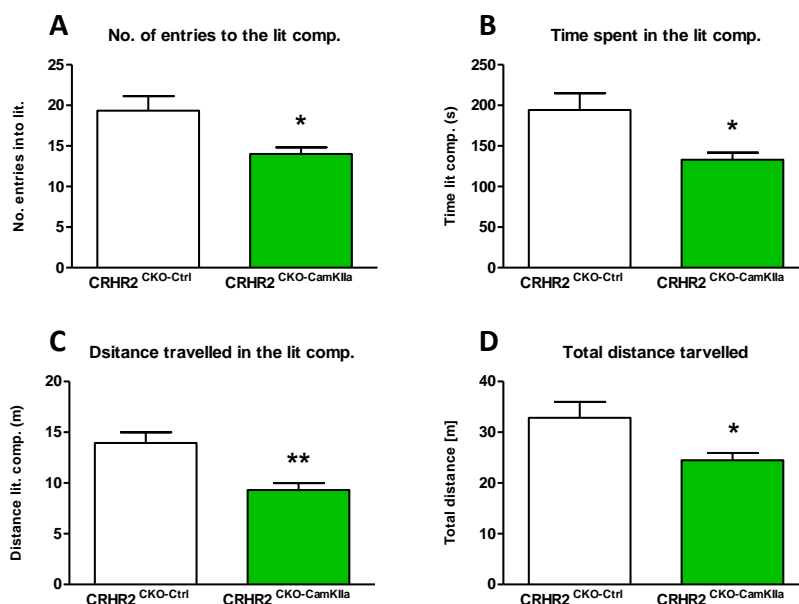
Figure 39. Anxiety-related behavior in CRHR2$^{CKO-Camk2a}$ mice measured in the dark/light box test.

A, number of entries into the lit compartment B, time spent in the lit compartment C, distance travelled in the lit compartment and D, total distance travelled. Data are expressed as means +S.E.M. n=10-9 per group. * *p*<0.05, ** *p*<0.01. Student's t-test.

Stress coping behaviour was evaluated in the forced swim test (Figure 40). None of the assessed parameters, i.e. time spent struggling, swimming, floating or the latency to the first floating episode was significantly affected by genotype.
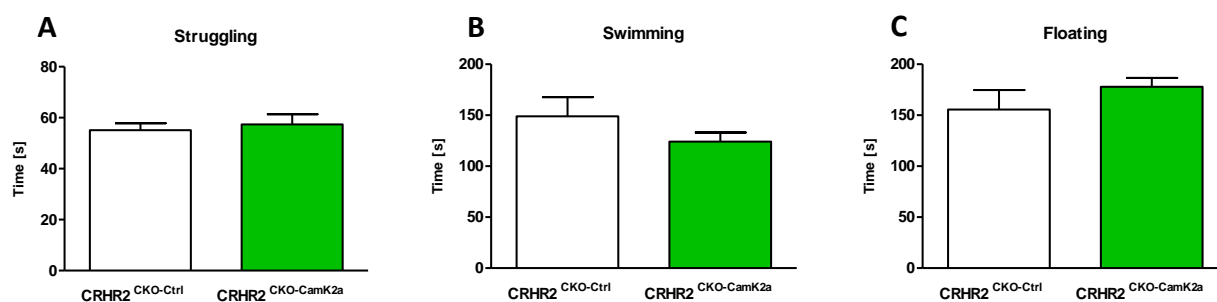
Figure 40. Stress coping behavior in CRHR2$^{CKO-Camk2a}$ mice measured in the forced swim test.

A, time spent struggling B, time spent swimming C, time spent floating. Data are expressed as means +S.E.M. n=10-9 per group.

4.5.3 HPA axis regulation in CRHR2^{CKO-Camk2a} mice

In CRHR2^{CKO-GABA} mice we previously found a hypersensitivity of the HPA axis to restraint stress (Figure 27) similar to that observed in CRHR2 KO mice (Figure 26). As the deletion pattern of CRHR2 mRNA in both lines, i.e. CRHR2^{CKO-GABA} and CRHR2^{CKO-Camk2a} is overlapping, e.g. show a decrease in signal intensity in LS, BNST and MeA, we also we examined the function of the HPA axis in CRHR2^{CKO-Camk2a} mice under basal and stress conditions (Figure 41). Under basal, non-stress conditions during the morning trough both groups did not differ in plasma corticosterone levels, but during the evening peak CRHR2^{CKO-Camk2a} mice showed a significant decrease of plasma corticosterone. We did not find a genotype-specific difference of the corticosterone concentration after 2 or 10 min of immobilization stress nor after a recovery period of 90 min.
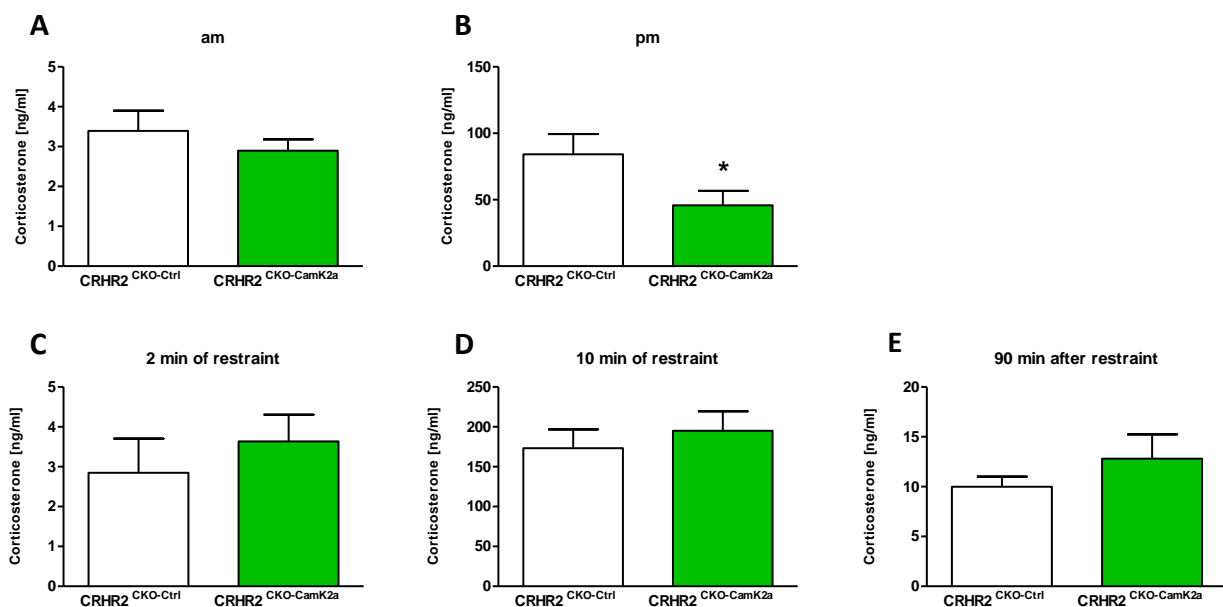
Figure 41. Basal and stress induced plasma corticosterone levels in CRHR2^{CKOCamk2a} mice.
A, plasma corticosterone concentrations during the morning trough and B, the evening peak. Serum corticosterone concentration: C, after 2 min and D, 10 min of restraint stress. E, corticosterone concentration measured 90 min after 10 min of restraint stress. Data are expressed as means +S.E.M. n=12-9 per group, * p<0.05 Student's t-test.

4.6 Assessing the role of CRHR2 in the serotonergic system

We could demonstrate that CRHR2 is mainly expressed in serotonergic neurons of the dorsal and median raphe nuclei. It is of particular interest as these sites provide the main serotonergic input to the central nervous system and their activity is modulated by stress. In this regard expression of CRHR2 in serotonergic neurons might mediate the interplay between the neuropeptidergic CRH/UCN stress system and classical monoaminergic neurotransmission. Up to now a few studies tried to address the role of CRHR2 in modulation of serotonergic neurotransmission. Nevertheless, none of them pointed out its exact functions in respect to anxiety-related and stress coping behavior. To answer this issues we generated CRHR2$^{CKO-5HT}$ mice, devoid of CRHR2 expression in serotonergic neurons of the raphe nuclei, and characterized these mice in terms of behavioral, physiological and neurochemical changes.

4.6.1 Deletion of CRHR2 in serotonergic neurons of the raphe nucleus; CRHR2$^{CKO-5HT}$ mice

In order to study functions of CRHR2 in serotonergic neurons and its impact on behavior and brain neurochemistry, we generated CRHR2$^{CKO-5HT}$ mice. In these mice CRHR2 is depleted only from the DRN and MeR (Figure 43).
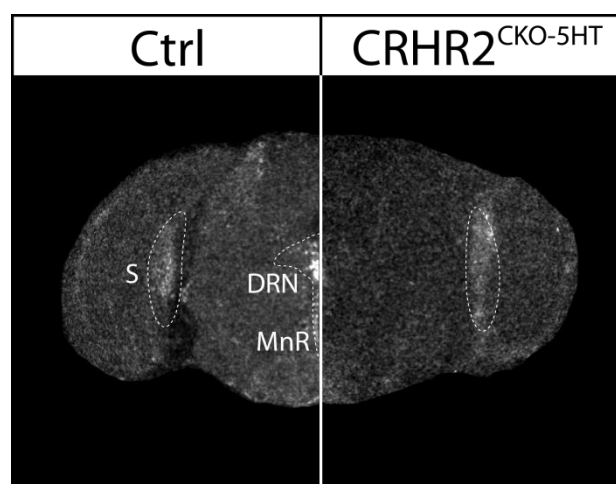
Figure 43. Expression of CRHR2 mRNA in the dorsal and median raphe nuclei of CRHR2$^{CKO-5HT}$ mice.
Inverted autoradiographic picture of a CRHR2-specific ISH on coronal brain sections. DRN dorsal raphe nucleus, MnR median raphe nucleus; S subiculum.

A

B

C

D
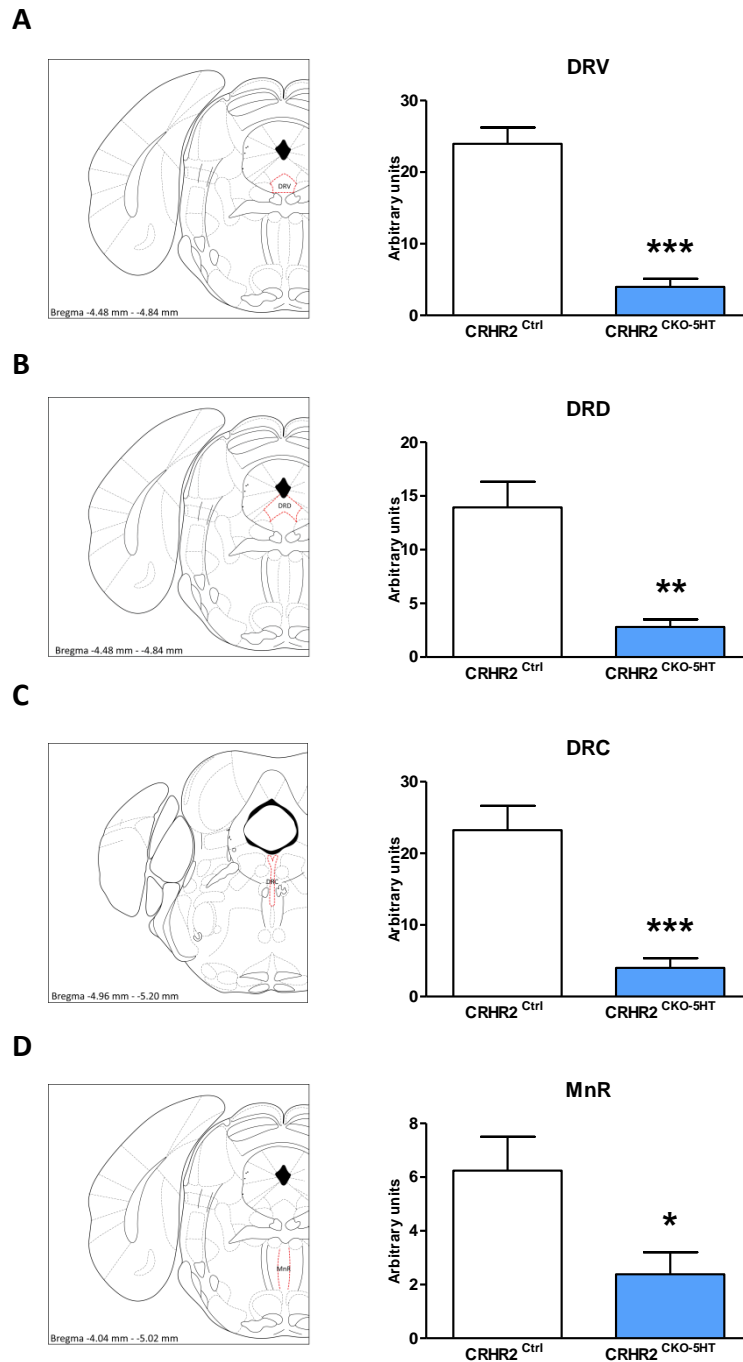
Figure 44. Quantification of CRHR2 mRNA expression in DRN in CRHR2$^{CKO-5HT}$ mice.

Quantification was performed from in situ hybridization autoradiographic film. A, dorsal raphe nucleus, dorsal part (DRD) B, dorsal raphe nucleus ventral part (DRV) C, dorsal raphe nucles caudal part (DRC) D, median raphe (MnR).

Interstingly, a portion of CRHR2 mRNA signal in CRHR2$^{CKO-5HT}$ mice is still remaining in each part of DRN (Figure 44). Most likely CRHR2 in the DRN is expressed not exclusively in serotonergic neurons where the deletion occurs using the ePet-Cre driver.

4.6.2   Behavioral characterization of CRHR2$^{CKO-5HT}$ mice

4.6.2.1 Deficiency of CRHR2 in serotonergic neurons significantly decreases anxiety-related behavior and increases sociability

It was shown that mice with a total deletion of CRHR2 exhibit increased anxiety-related behavior in either sexes (Bale et al., 2000) or in females only (Kishimoto et al., 2000). We also found increased anxiety-related behavior when CRHR2 is depleted from either entire CNS, GABAergic neurons or during adulthood in principal forebrain neurons. In contrast to CRHR2 KO and other CRHR2^{CKO} lines, CRHR2$^{CKO-5HT}$ mice displayed decreased anxiety-related behavior in the open field test (Figure 45), as revealed by an increased amount of entries into the central compartment of the apparatus ($p=0.0283$), increased time spent there ($p=0.0209$) and increased distance travelled inside the inner zone ($p=0.0056$). General locomotion was neither affected throughout duration of the entire test ($p=0.4597$), nor during 5 min intervals of the 30 min test period.
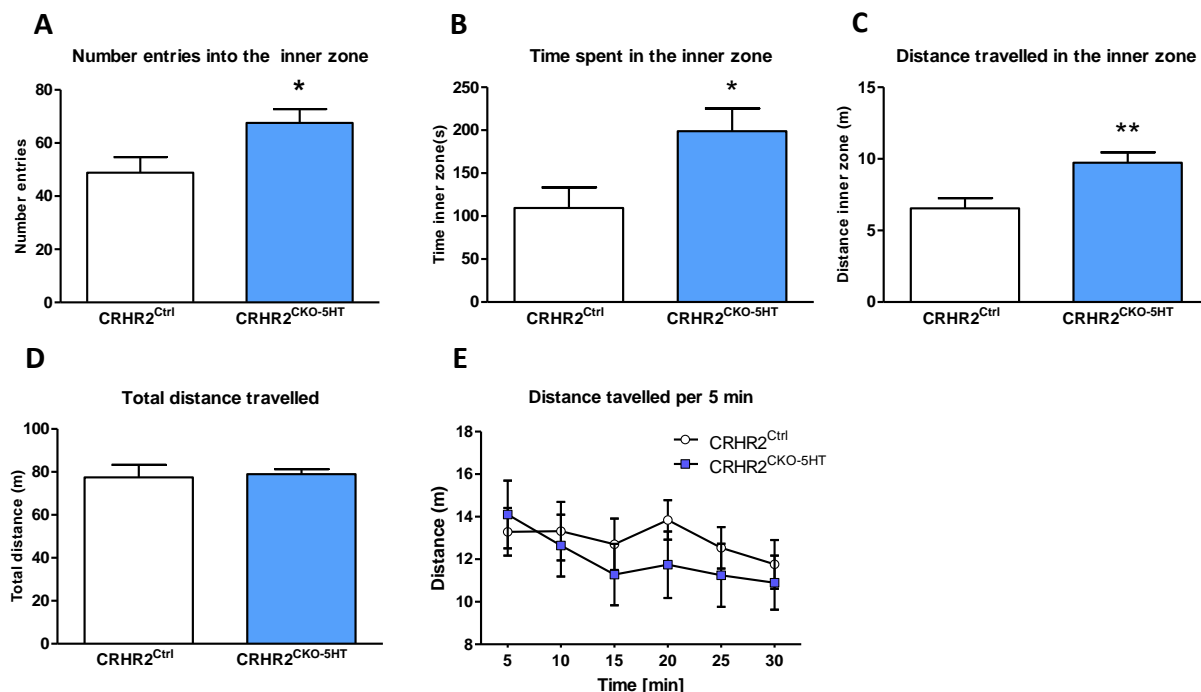
Figure 45. Anxiety-related behavior in CRHR2$^{CKO-5HT}$ mice measured in the open field test.

A, number of entries into the inner zone B, time spent in the inner zone C, distance travelled in the inner zone D, total distance travelled during 30 min of the test duration or E, split in 5 min intervals. Data are expressed as means +S.E.M. n=11-10 per group. * $p<0.05$, ** $p<0.01$. Student's t-test.

In the dark/light box test (Figure 46) CRHR2[CKO-5HT] mutant mice showed decreased anxiety-related behaviour. CRHR2[CKO-5HT] mice entered the lit compartment of the apparatus significantly more times than their control littermates ($p=0.0113$), they tended to enter it sooner ($p<0.05$) and they spent there significantly more time ($p=0.0096$) compared to the control littermates. In addition CRHR2[CKO-5HT] travelled significantly more distance exploring the lit compartment of the apparatus ($p=0.0057$).
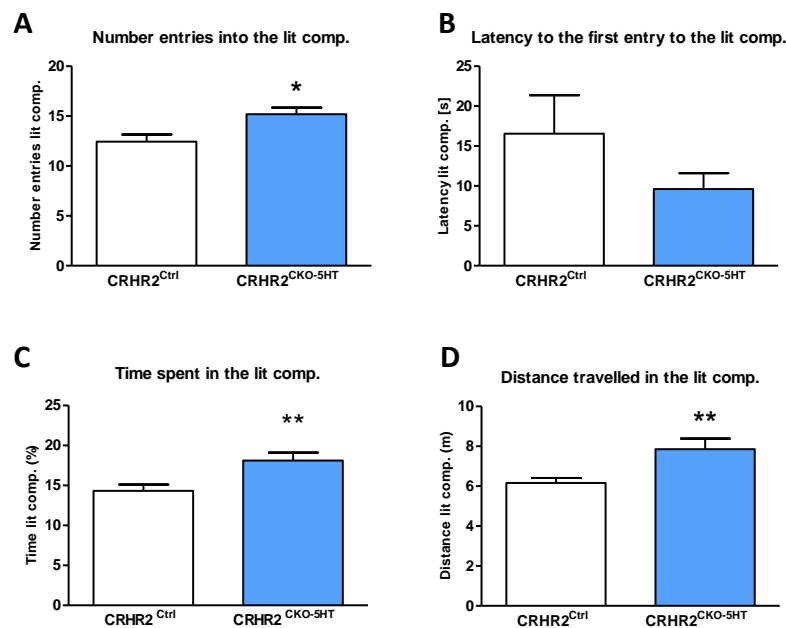
Figure 46. Anxiety-related behavior in CRHR2[CKO-5HT] mice measured in the dark/light box test.
A, number of entries into the lit compartment B, latency to the first entry to the lit compartment C, time spent in the lit compartment D, distance travelled in the lit compartment. Data are expressed as means +S.E.M. n=10-9 per group. * $p<0.05$, ** $p<0.01$. Student's t-test.

In addition to anxiety-related behaviour, also a characterization of basal social behaviour of CRHR2[CKO-5HT] was performed using the sociability test (Figure 47). In the sociability test a previously habituated animal is placed into the middle zone of the three compartment apparatus. From the middle zone animal has the free choice to enter either a compartment with an age-matched male counterpart or to explore a compartment containing a toy similar in colour and shape to a real mouse. The percentage of the total time interacting, without making a difference between the real or toy mouse, did not differ between genotypes ($p=0.2301$). However the percentage of the time spent interacting only with a real counterpart was significantly higher for the CRHR2[CKO-5-HT] mice ($p=0.0012$). In addition, the percentage of the

distance travelled in the compartment with a real mouse tend to be increased in CRHR2[CKO-5HT] mutants (p=0.0782) compared to control littermates.
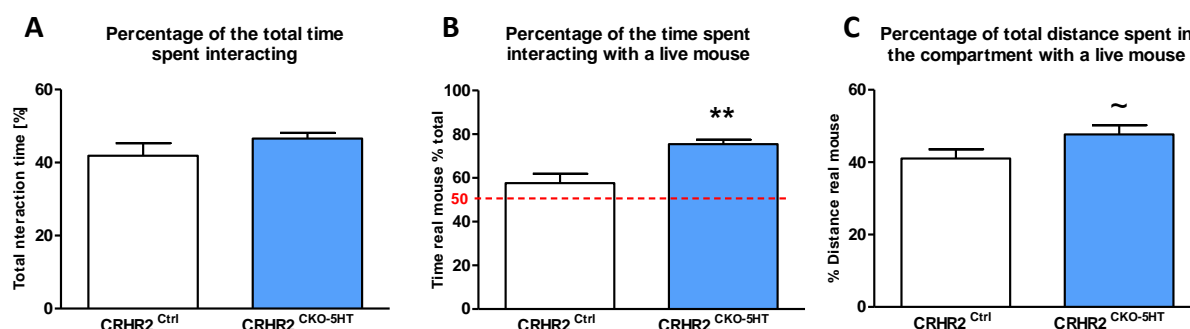
Figure 47. Social behavior in CRHR2[CKO-5HT] mice assessed by the sociability test under basal condition.
A, percentage of total time interacting B, percentage of the time spent interacting with a live mouse C, percentage of total distance travelled in the compartment with a real mouse. Data are expressed as means +S.E.M. n=10-9 per group. ~ p>0.05, ** p<0.01. Student's t-test.

4.6.2.1.1 CRHR2[CKO-5HT] mice are resistant to chronic stress with respect to social behavior

Social behavior was further investigated under chronic social defeat stress conditions (Figure 48). After 14 days of repeated social defeat, mice were tested in the apparatus designed for the sociability test as described before. Chronically stressed CRHR2[CKO-5HT] mice showed a significantly decreased latency to the first approach to a live mouse when compared with stressed control littermates. Also the percentage of time spent interacting with a naïve counterpart was significantly increased for the group of stressed CRHR2[CKO-5HT] mice.
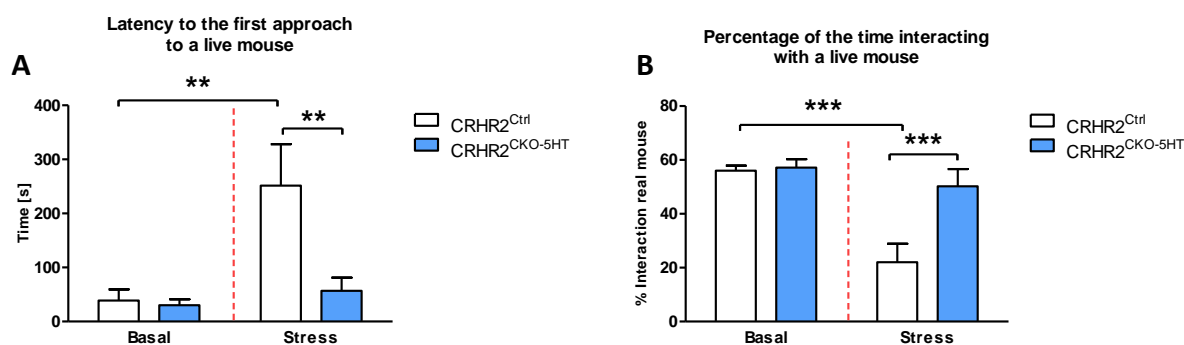
Figure 48. Social behavior in CRHR2[CKO-5HT] mice assessed by the sociability test after chronic social defeat stress.
A, percentage of total time interacting B, percentage of the time spent interacting with a live mouse. Data are expressed as means +S.E.M. n=10-9 per group. ** p<0.01, *** p<0.001. Two way ANOVA.

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4.6.2.2 CRHR2$^{CKO-5HT}$ mice display impaired recall of contextual fear memory

It has been previously shown that blockade of CRHR2 in the MnR of naïve mice decreases the time spent freezing to the same context where animals were fear conditioned (Ohmura et al., 2010). CRHR2$^{CKO-5HT}$ animals and their control littermates were subjected to the fear conditioning paradigm. On day zero animals were conditioned in the apparatus consisting of a dark cabin filled with ethanol scent and equipped with metal grid, connected to the power source, on the bottom. After initial 3 minutes of habituation, animals were presented 20 s of continuous tone (cue) terminated with delivery of an electric shock through the metal grid. Animals were left in the chamber for additional 60 s (Figure 49). On the following days animals were re-exposed either to the cue or to the context associated with the shock and freezing behaviour was assessed. After 4 additional weeks the re-exposure procedure was repeated.
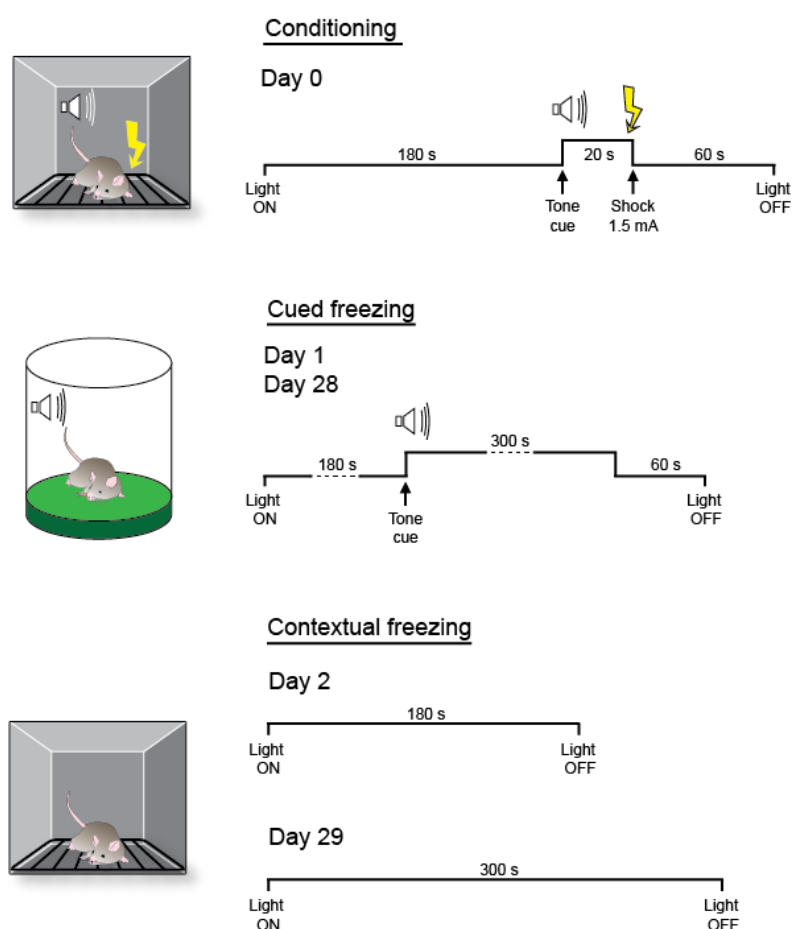
Figure 49. Experimental design of the fear conditioning procedure.

During conditioning animals were exposed to an electric shock in presence of the tone cue. On the following days freezing behavior was scored during either presentation of the tone cue in a separate context or during re-exposure to the same context, where animals received the electric shock but without the tone cue.

No genotype difference in the freezing behavior was detected to the tone cue regardless of the retesting time point (Figure 50 Day 1: p=0.3462 Day 28: p=0.3623).
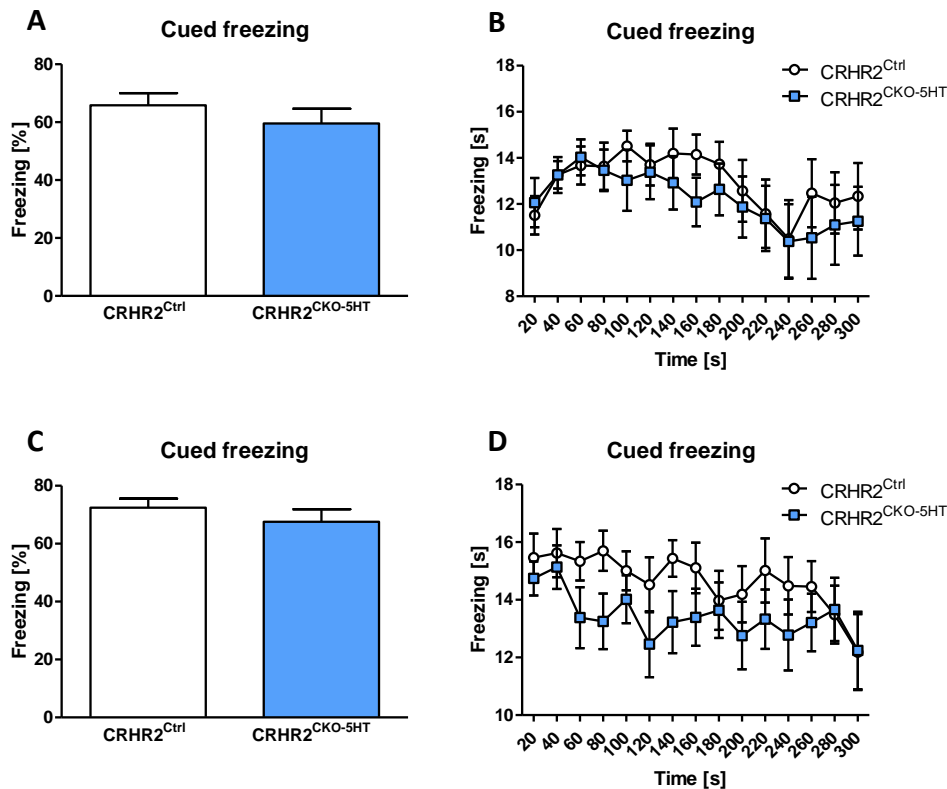
Figure 50. Cued freezing behavior in CRHR2$^{CKO-5HT}$ mice after the fear conditioning procedure.
A and B represent the time spent freezing to the tone cue on the first day after the conditioning procedure, C and D the time spent freezing to the tone cue on the 28th day after conditioning. Data are expressed as means +S.E.M. or ± n=15-14 per group. Student's t-test.

In contrast to cued freezing CRHR2$^{CKO-5HT}$ conditional knockout mice showed significantly decreased contextual freezing (p=0.0098) compared to the control littermates (Figure 51). When the animals were exposed to the same context where they had been previously shocked, after 4 additional weeks of recovery CRHR2$^{CKO-5HT}$ mice still exhibited decreased contextual freezing (p=0.0228).
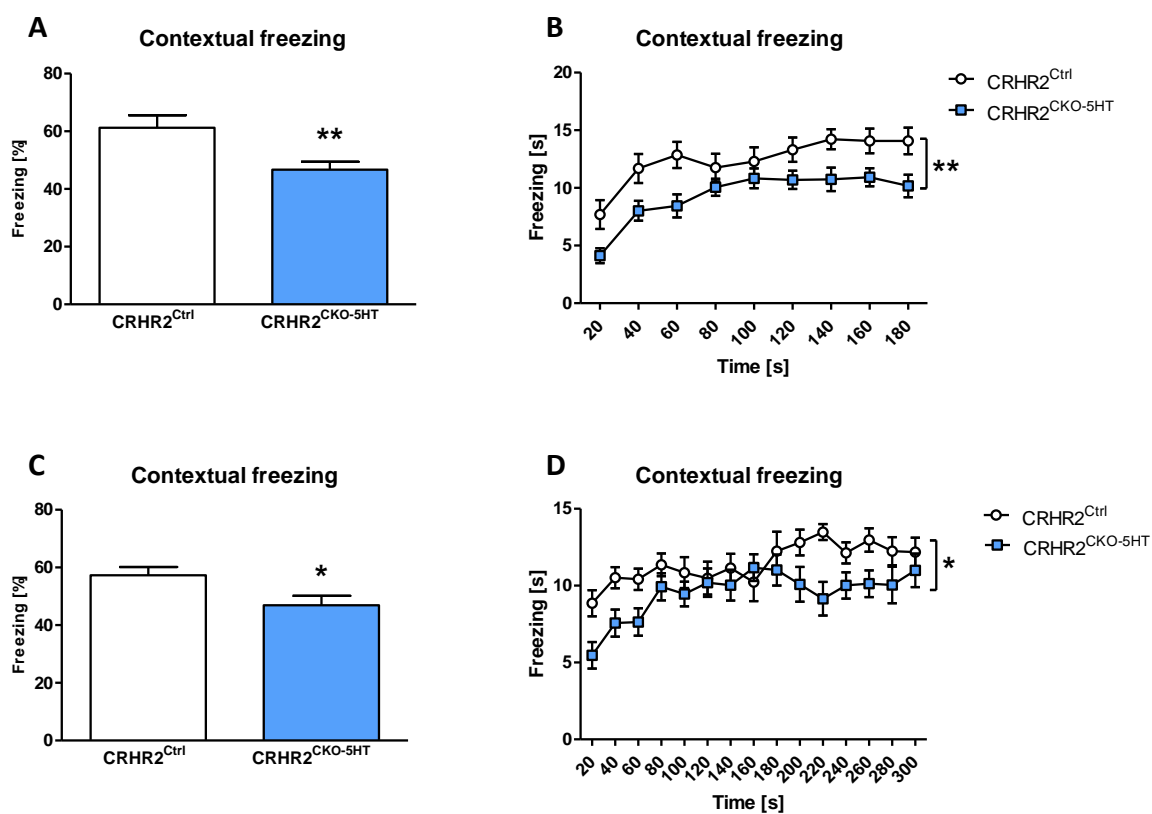
Figure 51. Contextual freezing behavior is decreased in CRHR2^CKO-5HT mice after the fear conditioning procedure.

A and B represent the time spent freezing to the context on the second day after the conditioning procedure, C and D the time spent freezing to the tone cue on the 29th day after conditioning. Data are expressed as means +S.E.M. n=15-14 per group. * $p<0.05$, ** $p<0.01$. Student's t-test.

4.6.2.3 Deletion of CRHR2 in serotonergic neurons significantly increases active stress-coping behavior

Stress-coping behaviour was assessed in the forced swim test (Figure 52). In this test animals are exposed to stressful conditions with no obvious escape option. Active and passive stress coping behavior can be estimated based on the means of amount of time spent struggling, swimming or floating and the latency to the first floating episode. During the FST session CRHR2$^{CKO-5HT}$ mice spent significantly more time struggling (p=0.0101) and swimming (p=0.0013), while the time spent floating was significantly decreased (p=0.0002). The latency to the first floating episode was significantly higher for the CRHR2$^{CKO-5HT}$ mice (p=0.0018) indicating significant increase in active stress coping behavior.
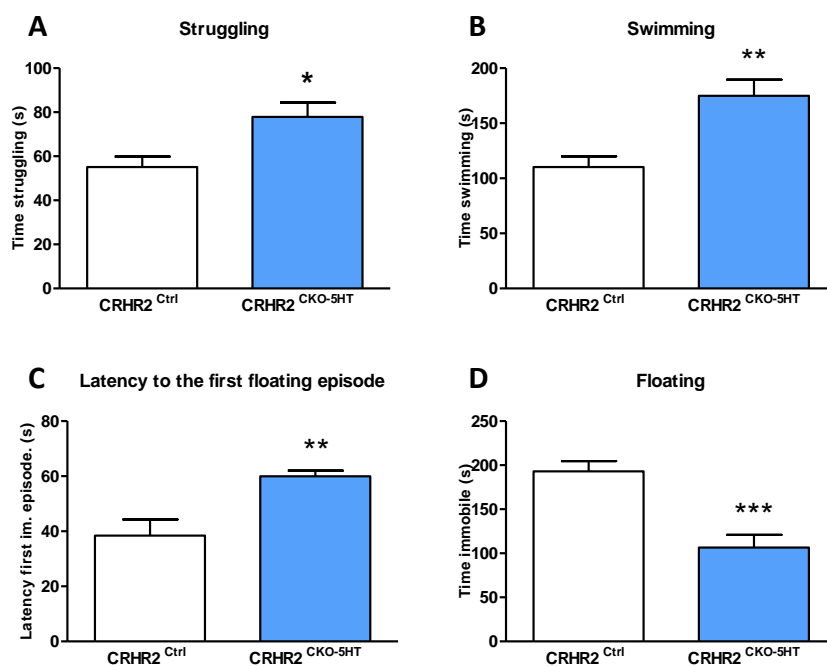
Figure 52. Stress-coping behavior in CRHR2$^{CKO-5HT}$ mice measured in the forced swimming test.
A, time spent struggling (climbing) B, time spent swimming C, the latency to the first floating episode and D, time spent floating. Data are expressed as means +S.E.M. n=11-9 per group. * p<0.05, ** p<0.01, *** p<0.001. Student's t-test.

4.6.3 Behavioral changes observed in CRHR2$^{CKO-5HT}$ mice are not due to disturbed HPA axis activity

It has been shown that the serotonergic system influences the circadian rhythm of corticosterone release and corticosterone itself is able to modulate emotional behavior. In order to exclude possible influences of corticosterone on behavioral changes observed in CRHR2$^{CKO-5HT}$ mice, blood samples were collected during morning nadir and evening peak as well as directly after 10 min of restraint stress and 90 min after 10 min of restraint (Figure 53). Although a.m. values of corticosterone did not differ between genotypes (p=0.3257), p.m. values tended to be decreased in CRHR2$^{CKO-5HT}$ mice (p=0.1288). There was no genotype difference between CRHR2$^{CKO-5HT}$ mice and their control littermates in respect of any time points after immobilization (10 min, p=0.6691; 90 min, p=0.7319)
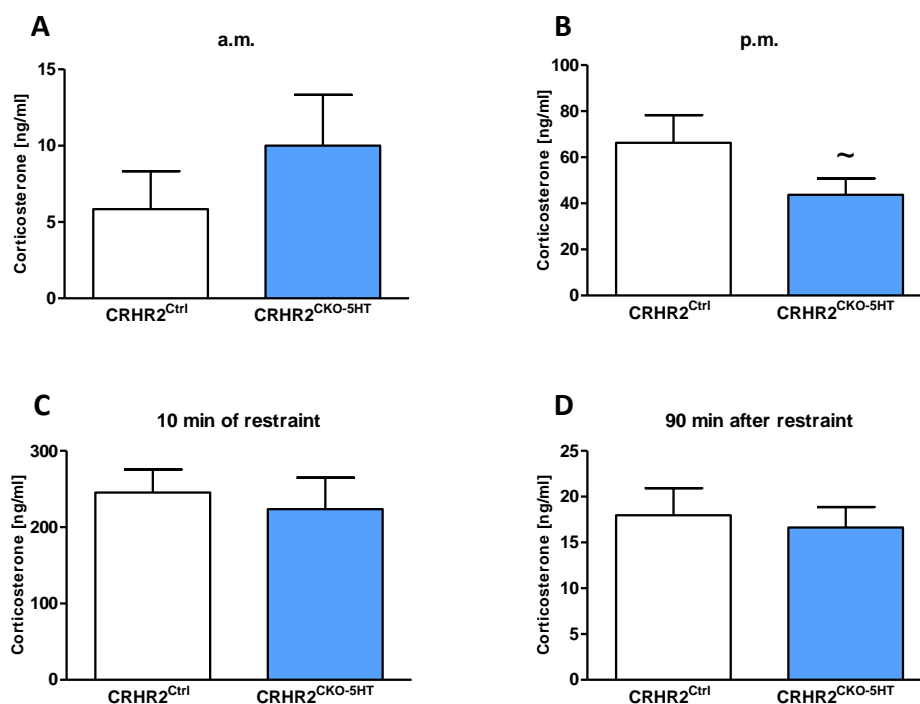
Figure 53. Basal and restraint induced corticosterone level in CRHR2$^{CKO-5HT}$ mice.

Graphs A and B represent basal plasma corticosterone concentrations during morning nadir A or evening peak B. C, Corticosterone concentration directly after 10 min of restraint stress, D Corticosterone concentration 90 min after 10 min of restraint stress. Data are expressed as means +S.E.M. n=12-9 per group, ~ p<0.15. Student's t-test.

4.6.4   CRHR2$^{CKO-5HT}$ mice show an alterated sleep/wake cycle

Serotonin is a wake promoting agent which significantly affects sleep architecture. It is also known that sleep disturbances often precede recurrence of the depressive episode and are sensitive to treatment with antidepressant drugs. Because it was also shown that CRHR2 KO mice display alterations in the sleep pattern, especially to immune challenge (Jakubcakova et al., 2011), sleep architecture was also investigated in CRHR2$^{CKO-5HT}$ mice (Figure 54). During recording, the number of NREM, REM and awake episodes, their length and amount of transitions between them were measured.
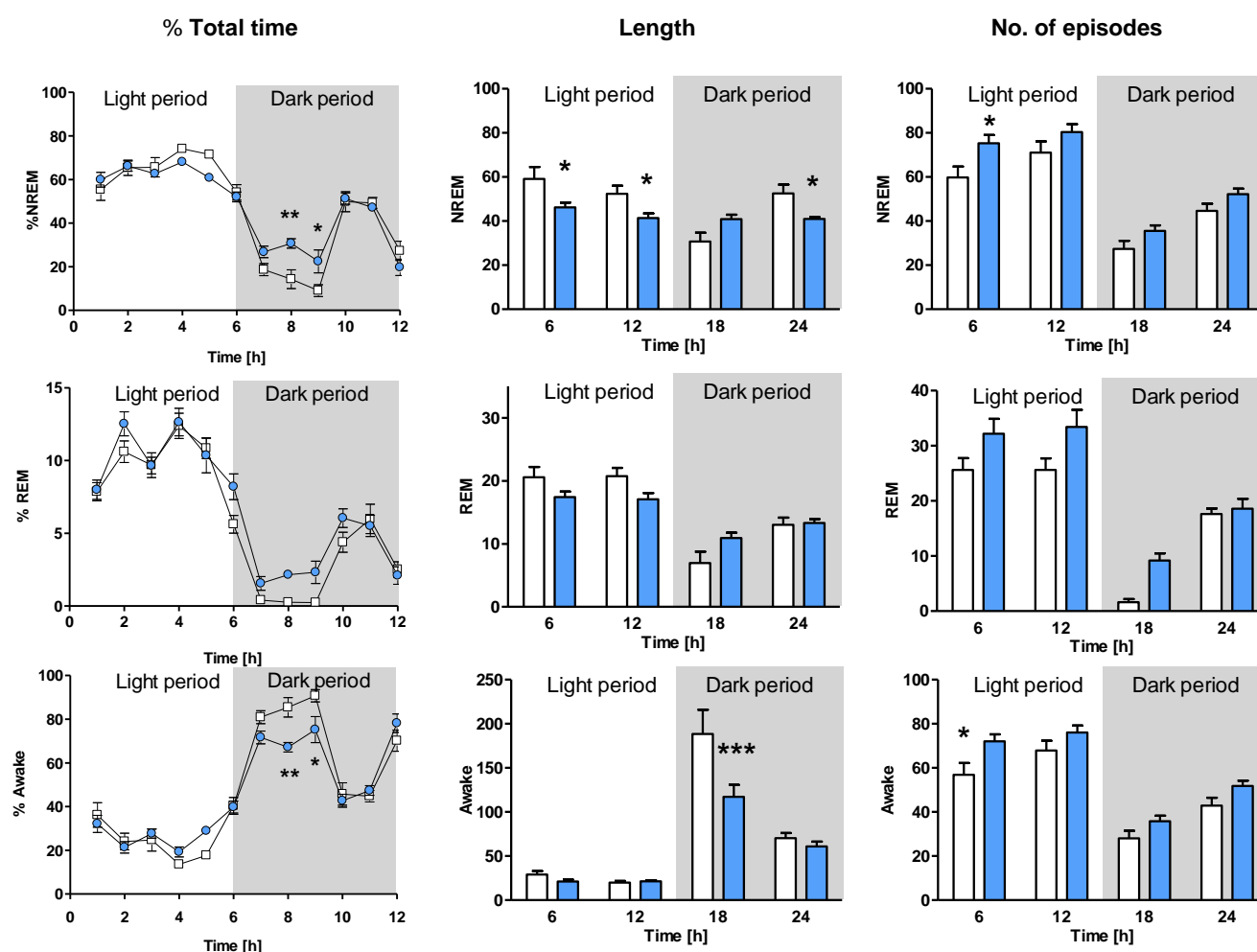
Figure 54. Assessment of sleep architechture of CRHR2$^{CKO-5HT}$ mice.
Data are expressed as means +S.E.M. n=6-5 per group. White bars, CRHR2^{Ctrl} blue bars, CRHR2$^{CKO-5HT}$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Student's t-test. The study was done in collaboration with D. Kumar and M. Kimura.

During the first part of the dark period CRHR2$^{CKO-5HT}$ mice dispayed a decreased portion of time spent awake along with decreased length of awake episodes, although their number was not affected. Interestingly, the number of awake episodes at the beginning of the light period was significantly decreased without an effect on the length of episodes or the time spent awake. In contrast, the portion of total time spent in the NREM phase during sleep at the beginning of the dark period was significantly increased in CRHR2$^{CKO-5HT}$ mice. The length of the NREM phase was generally decreased throughout the 24 h cycle, with the exception of the beginning of the dark phase. The number of NREM episodes differed significantly between genotypes only in the beginning of the light period. There was no genotype effect on percentage of time, length or number of REM episodes. During the 24 h sleep recording the number of transitions between wake, NREM and REM episodes was also counted with significant genotype difference for wake to NREM and REM to wake transitions (Figure 55).
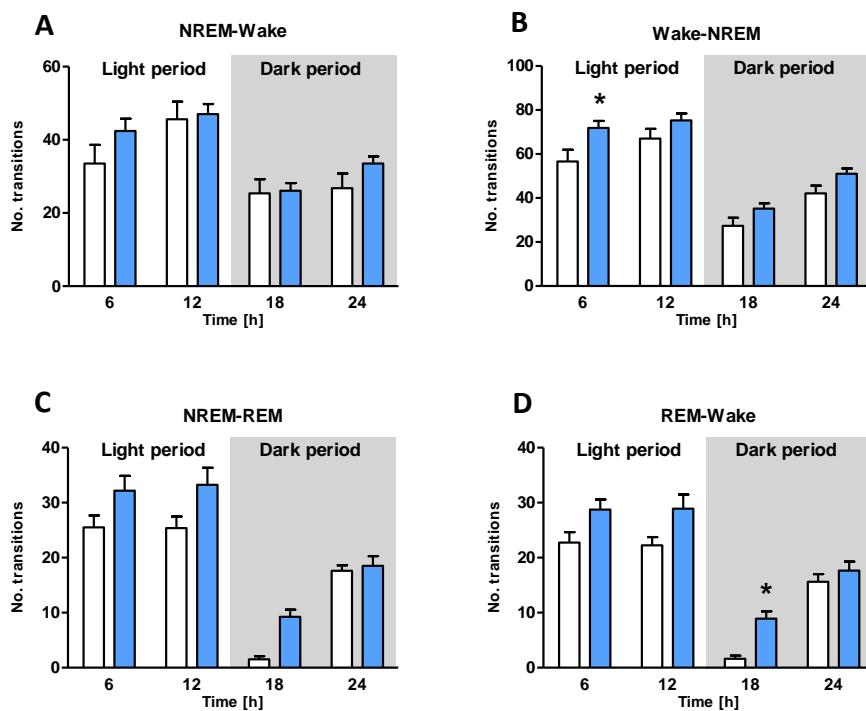
Figure 55. Number of transitions between Wake, NREM and REM phases assessed in CRHR2$^{CKO-5HT}$ mice.
A, the number of NREM-wake transitions B, the number of Wake-NREM transitions C, the number of NREM-REM transitions D, the number of REM-Wake transitions. Data are expressed as means +S.E.M. n=6-5 per group. * $p<0.05$. The study was done in collaboration with D. Kumar and M. Kimura.

4.6.5   Phenotypic changes in CRHR2$^{CKO-5HT}$ mice correlate with disrupted function of the serotonergic system

Activation of CRHR2 in serotonergic neurons is known to change their electrophysiological properties. Stimulation of CRHR2 in the raphe nucleus increases the firing rate of serotonergic neurons along with the amount of released serotonin. To estimate the impact of conditional CRHR2 deletion in CRHR2$^{CKO-5HT}$ mice on serotonergic neurotransmission, tissue content as well as release of 5HT and 5HIAA was measured.

4.6.6   Basal 5HT and 5HIAA tissue content in CRHR2$^{CKO-5HT}$ mice

Brains of CRHR2$^{CKO-5HT}$ and CRHR2^{Ctrl} mice were removed, cut in coronal sections and micropunched to isolate stress-related brain regions. Monoamine content was measured by high pressure liquid chromatography (HPLC).

The raphe complex was divided into subregions of DRN and MnR. In the DRC part of DRN of CRHR2$^{CKO-5HT}$ mice 5HT content was found to be particularly increased. In contrast 5HT/5HIAA ratio was decreased in DRVL/VLPAG, DRC and MnR (Figure 56).

In forebrain structures of CRHR2$^{CKO-5HT}$ mice the 5HT and 5HIAA content was significantly increased in the LS. A slight but significant increase of 5HT was also found in the PVN. The calculated 5HT/5HIAA ratio was shown to be decreased in mPFC and BNST (Figure 57).

In contrast to LS, the tissue content of 5HT and 5HIAA was decreased in the CA1 region of ventral hippocampus of CRHR2$^{CKO-5HT}$ mice. Also 5HIAA was decreased in the CA1 region of dorsal hippocampus and DMH. Additionally, the 5HT/5HIAA ratio was found to be decreased in VMH of CRHR2$^{CKO-5HT}$ mice (Figure 58).
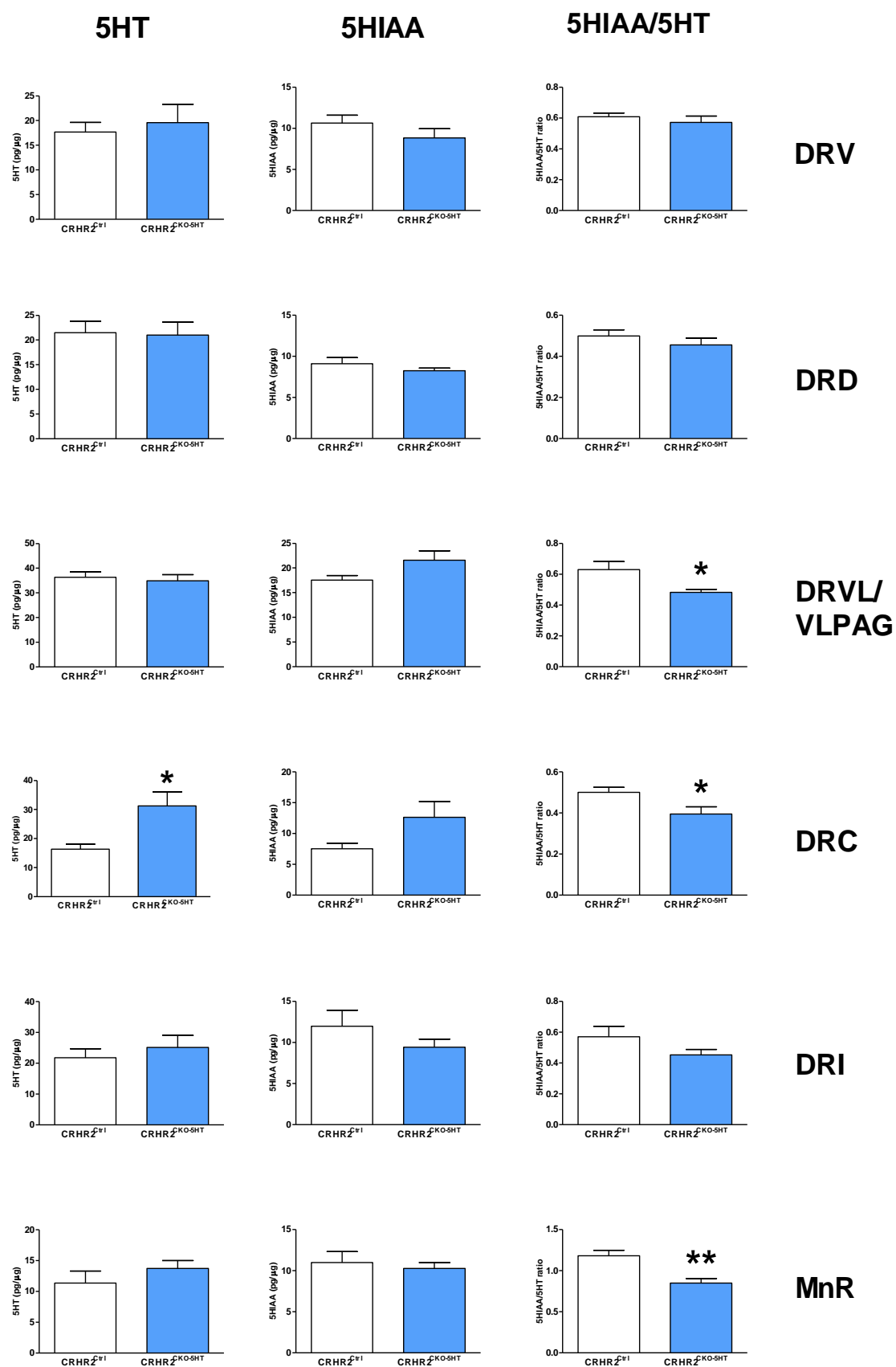
Figure 56. Basal 5HT and 5HIAA tissue content in subdivisions of DRN and MnR of CRHR2^CKO-5HT mice.

Data are expressed as means +S.E.M. n=8-5 per group, * $p<0.05$, ** $p<0.01$. Student's t-test. The study was done in collaboration with N. Donner and C. Lowry.
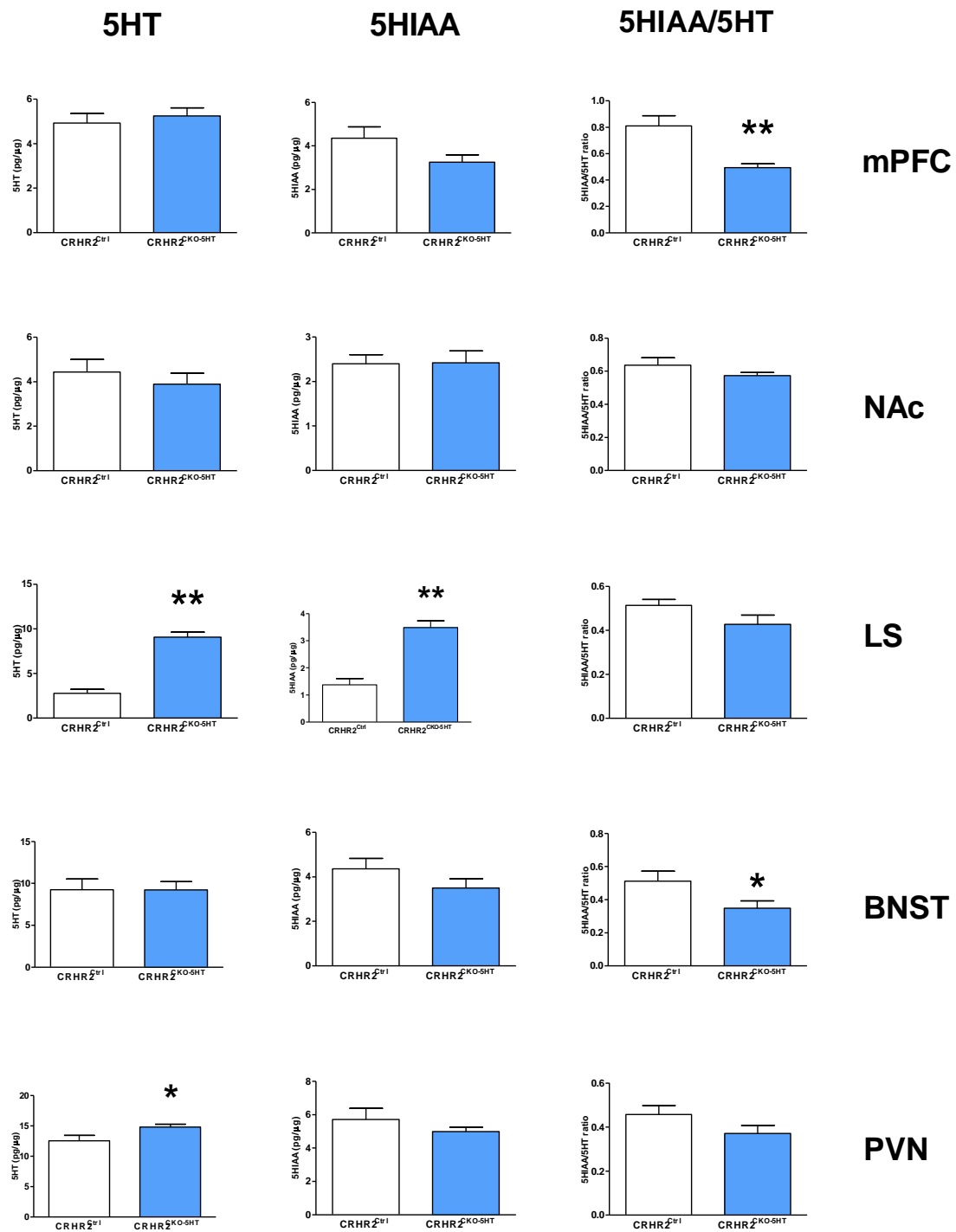
Figure 57. Basal tissue 5HT and 5HIAA content in forebrain structures of CRHR2$^{CKO-5HT}$ mice (part 1).

Data are expressed as means +S.E.M. n=8-6 per group, * $p<0.05$, ** $p<0.01$. Student's t-test. The study was done in collaboration with N. Donner and C. Lowry.
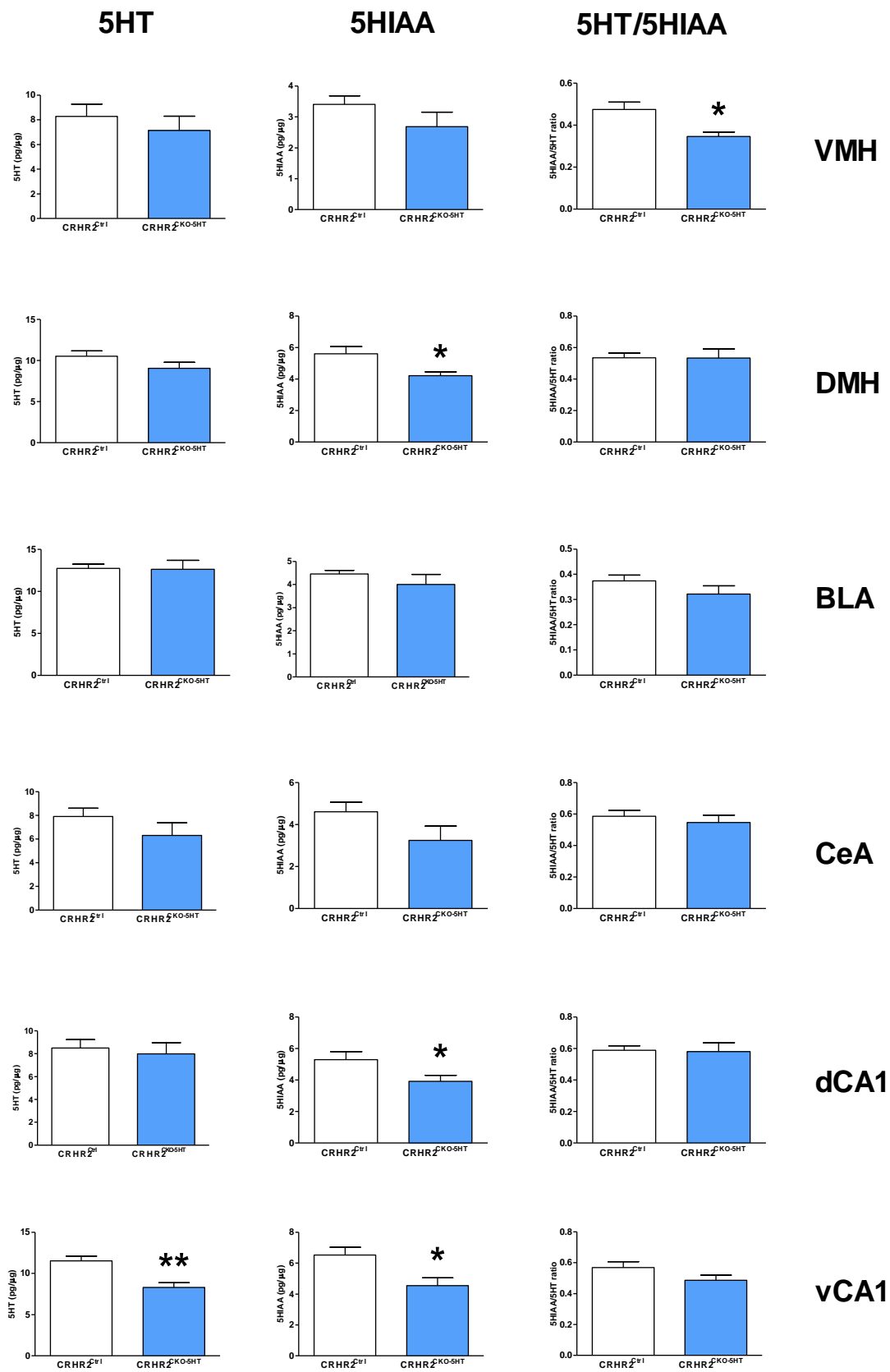
Figure 58. Basal tissue content of 5HT and 5HIAA in forebrain structures of CRHR2^CKO-5HT mice (part 2)

Data are expressed as means +S.E.M. n=8-6 per group, * $p<0.05$, ** $p<0.01$. Student's t-test. The study was done in collaboration with N. Donner and C. Lowry.

4.7 Studying effects of CRHR2 activation in the dorsal raphe nucleus

We were able to show that the deletion of CRHR2 exclusively from serotonergic neurons of the raphe nuclei in CRHR2$^{CKO-5HT}$ mice profoundly decreases anxiety-related behavior and increases active stress coping behavior. Furthermore, these behavioral changes correspond to disrupted physiology of the serotonergic system. Thus, we wondered if it is possible to induce a phenotype opposite to the phenotype of CRHR2$^{CKO-5HT}$ mice by activation of CRHR2 in the raphe complex.

4.7.1 Pharmacological activation of CRHR2 in naïve mice decreases active stress-coping behavior

We found that inactivation of CRHR2 in serotonergic neurons increases active stress coping behaviour in CRHR2$^{CKO-5HT}$ mice (Figure 52). In order to study opposite effect, i.e. CRHR2 activation in DRN, naïve C57Bl/6 mice were implanted with a metal guide cannula targeting the ventral part of the dorsal raphe nucleus. One week later mice were injected with 0.5 µl either of 100 ng of mouse urocortin 2 (mUCN2) or vehicle. Following 25 min of recovery animals were subjected to the forced swim test and subsequently sacrificed in order to confirm the injection site. Only animals with the correct placement of the injection needle were included into the analysis (Figure 59).
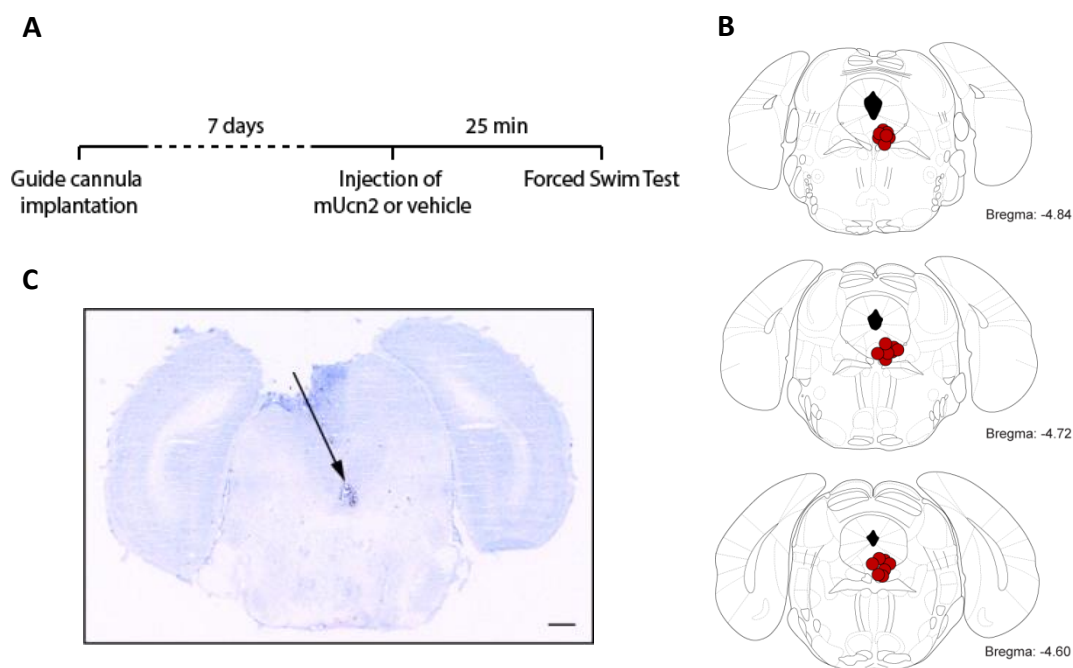
Figure 59. Experimental design of pharmacological CRHR2 activation in DRV.

A, the experimental schedule B, presentation of injection sites of (one dot/animal) animals taken into statistical analysis C, an example of correct placement of the injection needle. The arrow points out the injection site and the placement of guide cannula.

Although there was no difference in the time spent struggling between mice injected with mUCN2 and vehicle (*p*=0.1703), animals treated with mUCN2 spent significantly less time swimming (*p*=0.0091) and more time floating (*p*=0.0021) showing signs of behavioral despair (Figure 60).
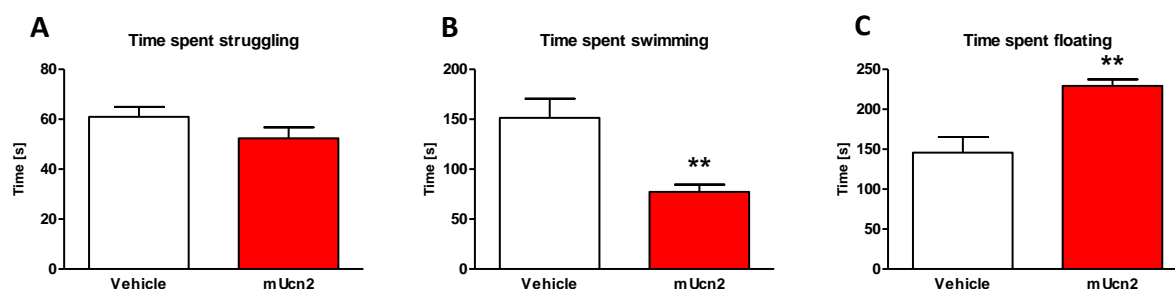
Figure 60. Effect of mUCN2 injection into the DRV nucleus on stress-coping behaviour measured in the forced swim test.

A, time spent struggling B, time spent swimming and C, time spent floating. Data are expressed as means +S.E.M. n=10 per group. ** *p*<0.01. Student's t-test.

4.7.2 The locus coeruleus, endogenously expresses UCN2 and projects to the dorsal raphe nucleus

In the next step we wanted to find out which structure could provide endogenous agonist to CRHR2 in the raphe nucleus. Available literature suggests that the locus coerules (LC) projects to the dorsal raphe nucleus and shows expression of UCN2. In order to confirm such LC-DRN connections, we traced anterograde noradrenergic projections of the LC. A cre-inducible viral construct containing a green fluorescent protein fussed to synaptophysin (Syn-GFP) was bilaterally injected in the LC of Nat-Cre mice. Syn-GFP protein is expressed only in transduced noradrenergic neurons expressing cre recombinase. Due to fusion of GFP to synaptophysin the GFP signal is mostly visible in axon terminals of those neurons. Two weeks after the injection, brains were extracted and the signal traced. Native signal from GFP protein was clearly visible already after two weeks of virus expression. On the level of the injection site green fluorescence of GFP was labelling the locus coerules stained with an anti-tyrosine hydroxylase (TH) antibody. Within the same virus injected brain, green dots of noradrenergic presynaptic terminals and axonal fibres were localized in the region of the dorsal raphe nucleus confirming this structure as a projection target of the locus coeruleus (Figure 61).
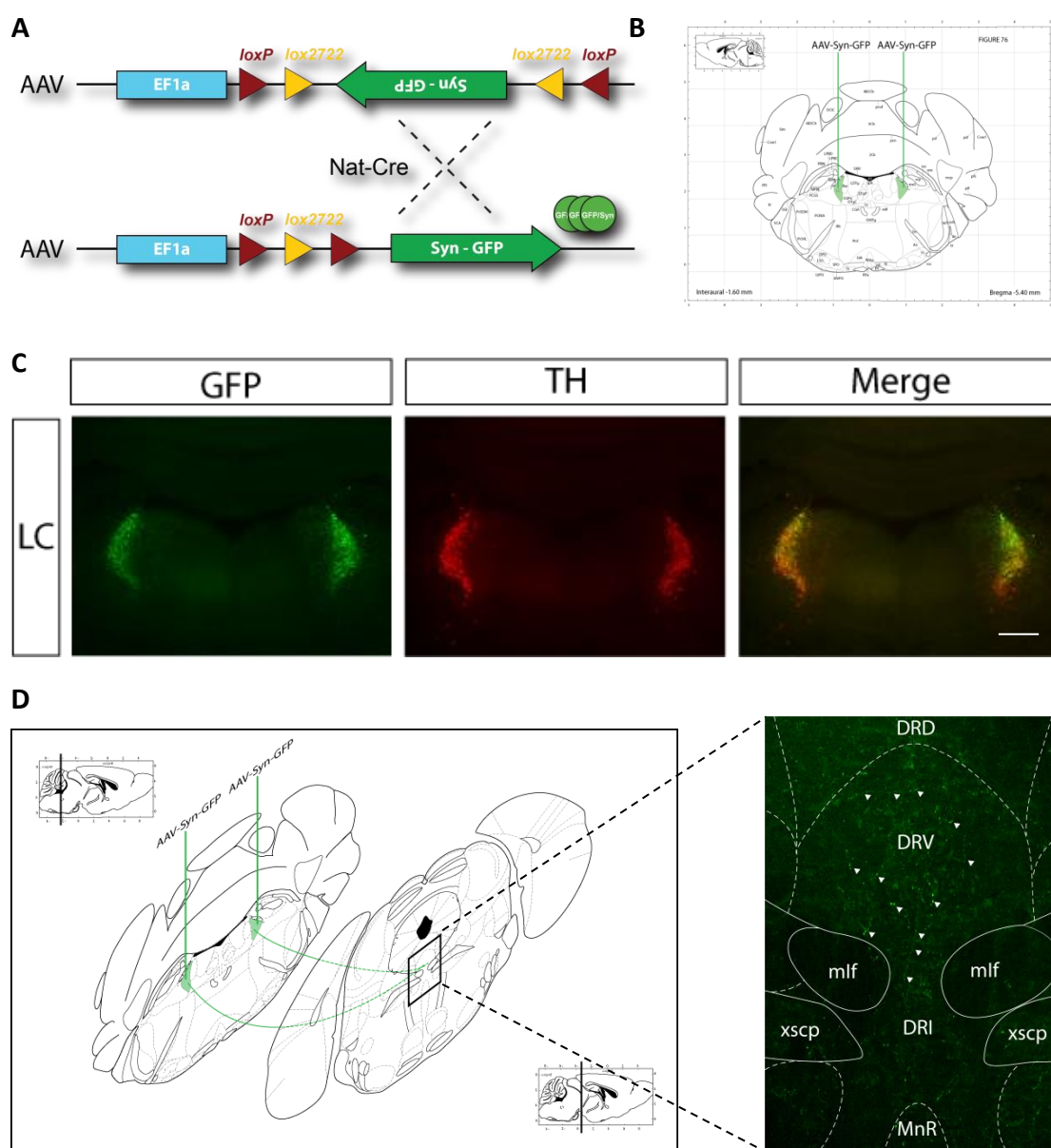
Figure 61. The locues coeruleus projects to the dorsal raphe nucleus.

A, AAV construct for cre-inducible expression of a Syn-GFP fusion protein. Syn-GFP is expressed following activation by cre-mediated recombination. B, injection site of the viral Syn-GFP construct. C, expression of Syn-GFP protein is restricted to the locus coerules and co-localized with the expression of tyrosin hydroxylase a marker of noradrenergic neurons. D, native signal of Syn-GFP can be detected in the dorsal raphe nucleus of injected Nat-Cremouse. Scale bar 250 µm. DRD dorsal raphe dorsal prart, DRI dorsal raphe intermediate part, DRN dorsal raphe nucleus, MnR medial raphe dorsal raphe, mfl medial longtufinal fasciculus. Arrowheads, Syn-GFP signal.

4.7.3 Generation of mice overexpressing UCN2 in the locus coeruleus

In order to investigate the function of UCN2 in the LC with respect to the modulation of the

serotonergic system and emotional behavior, we generated mice conditionally overexpressing UCN2 in noradrenergic neurons of the LC. Mice with a modified Rosa26 allele allowing for conditional overexpression of mUCN2 were generated by Ailing Lu. The Rosa26 locus was modified by gene targeting in embryonic stem cells. The conditional allele contains a transcriptional unit consisting of the murine UCN2 cDNA followed by an IRES site connected to a tau-LacZ reporter gene. This expression unit is preceded by a loxP flanked (floxed) transcriptional termination sequence which prohibits expression of UCN2 and tau-LacZ. Only upon cre recombinase mediated removal of the transcriptional terminator sequence expression of UCN2 and tau-LacZ is activated. These mice were bred to Nat-Cre mice, in which cre is driven by the promoter of the noradrenaline transporter (Slc6a2), restricting its expression to noradrenergic neurons. Animals overexpressing UCN2 (UCN2-COE^{NE}) and control littermates (UCN2-COE^{Ctrl}) were used for all further experiments. We confirmed expression of β-galactosidase by X-Gal staining and concomitant overexpression of UCN2 by in situ hybridization (Figure 62).
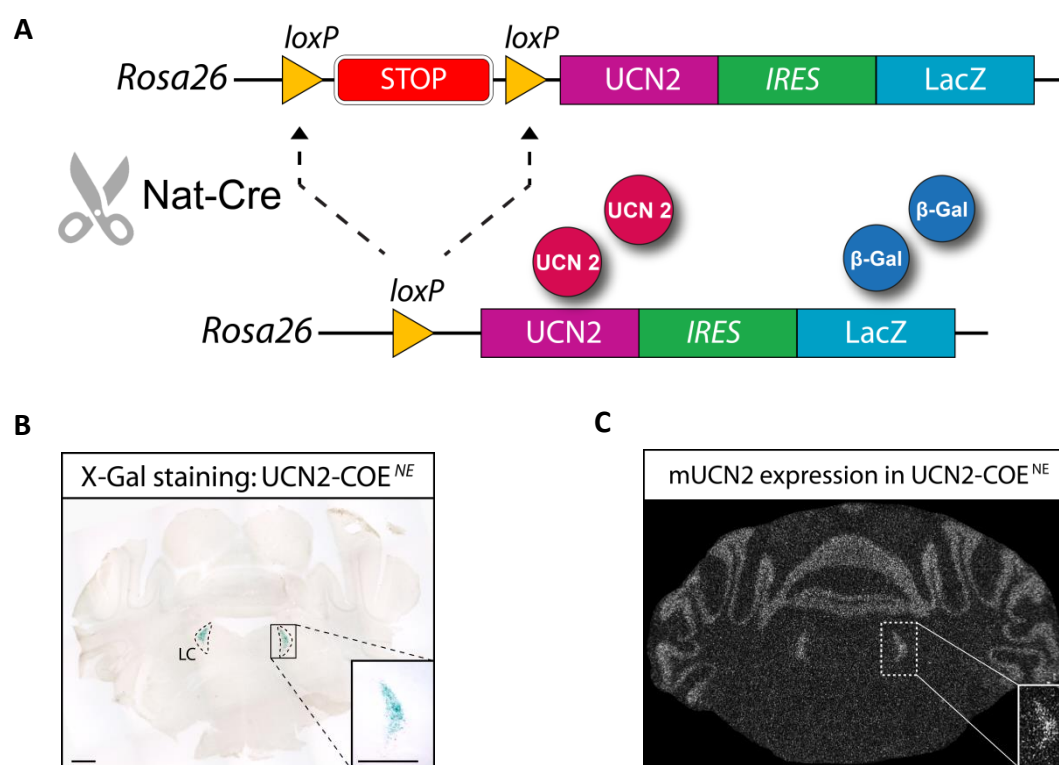
Figure 62. Generation and validation of UCN2-COE^{NE} mice.

A, Schematic representation of the modified Rosa 26 locus allowing cre recombinase mediated overexpression of UCN2 B, β-Gal expression in UCN2-COE^{NE} mice visualized by X-Gal staining, scale bars equal to 500 µm C, expression of mUCN2 mRNA in UCN2-COE^{NE} mice detected by ISH using a riboprobe against mUCN2. B and C coronal brain sections.

To evaluate effects of conditional UCN2 overexpression in the locus coeruleus on emotional behavior we performed standard anxiety-related test and the forced swim test.

4.7.4.1 Ucn2-COE^{NE} mice show no change in anxiety-related behavior under basal condition

Under basal conditions UCN2-COE^{NE} mice did not differ from their control littermates in any of the analyzed anxiety-related parameters measured in the open field test (Figure 63). Nevertheless, the general locomotion, measured as a total distance travelled, was significantly decreased in UCN2-COE^{NE} mice (p=0.0206).
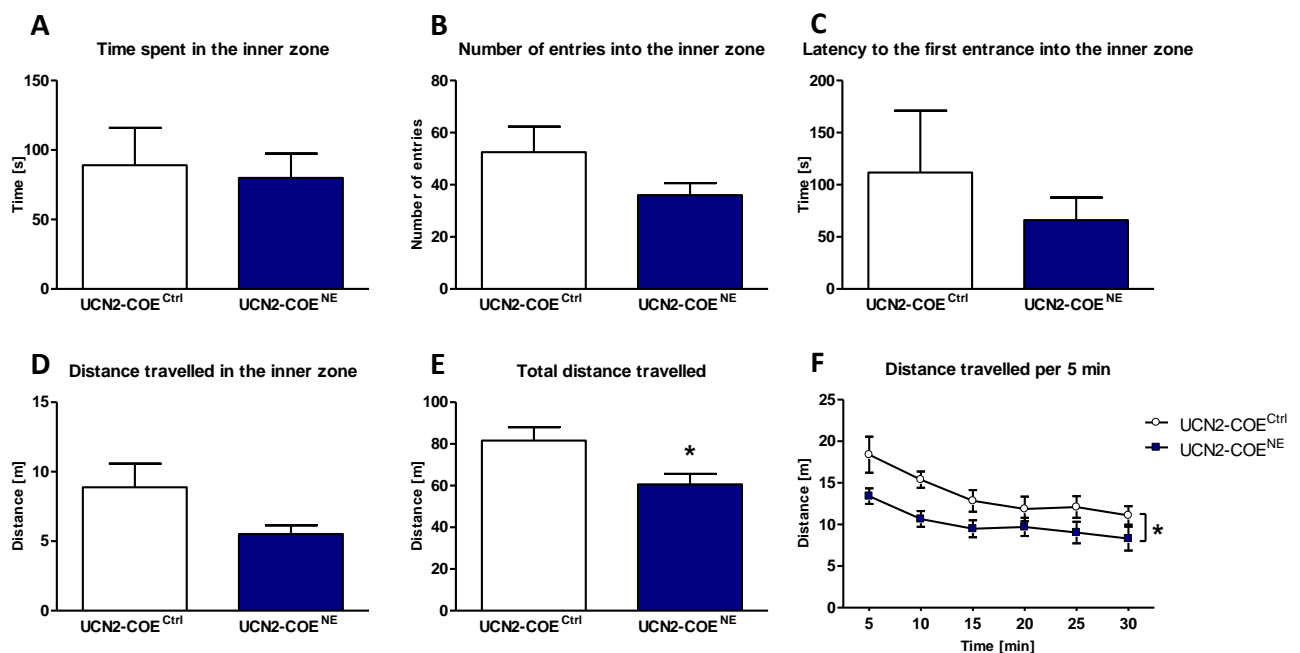
Figure 63. Anxiety-related behavior in UCN2-COE^{NE} mice measured in the open field test.

A, time spent in the inner zone B, number of entries into the inner zone C, the latency to the first approach to the inner zone D, distance travelled in the inner zone E, total distance travelled during 30 min of the test duration or F, split in 5 min intervals. Data are expressed as means +S.E.M. n=10-9 per group. * p<0.05, ~ p<0.1. Student's t-test.

There was also no genotype dependent difference between UCN2-COE[NE] and UCN2-COE[Ctrl] mice in any anxiety-related parameters of the dark/light box test (Figure 64). There was a trend towards decreased the total distance travelled in UCN2-COE[NE] mice (p=0.0864).
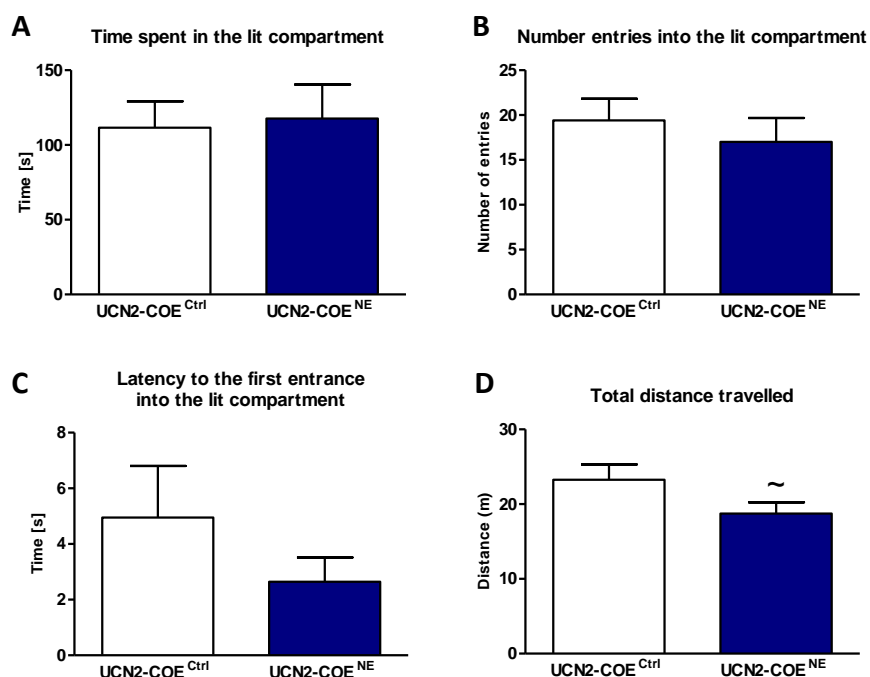
Figure 64. Anxiety-related behavior in UCN2-COE[NE] mice measured in the dark light box test.

A, time spent in the lit compartment B, number of entries into the lit compartment C, the latency to the first approach to the lit compartment D, total distance travelled. Data are expressed as means +S.E.M. n=10-9 per group., ~ 0.15>p>0.05. Student's t-test.

4.7.4.2 Increased passive stress coping behavior in UCN2-COE[Nat] mice is further enhanced 24 h afterwards

It has been shown that mice deficient in UCN2 display decreased passive stress coping, despite lack of changes in anxiety-related behavior. Therefore, effects of UCN2 overexpression in the locus coeruleus on stress coping behavior were assessed using the forced swim test. To further investigate behavioral stress recovery capacities, the test was repeated 24 hours later. Already during the first day of the FST UCN2-COE[NE] mice showed significantly decreased swimming and

increased floating behavior. On the second day the same effect was present in all three measured parameters. Moreover, UCN2-COE^{NE} mice still displayed significantly increased floating behavior compared to the control group (Figure 65).
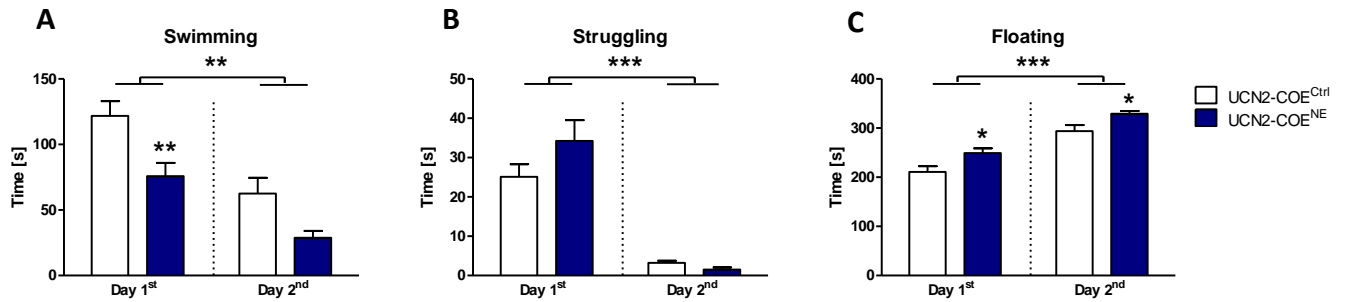
Figure 65. Stress coping behavior in UCN2-COE^{NE} measured in the forced swim test.

A, time spent swimming B, time spent struggling and C, time spent floating. Data are expressed as means +S.E.M. n=12-11 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Two-way ANOVA repeated measures.

4.7.4.3 Effects stress on the behavior of UCN2-COE^{NE} mice

As we did not see any anxiety-related changes in behavior of UCN2-COE^{NE} mice, but decreased active stress coping which was aggravated by the re-exposure to the FST, we thought that UCN2 overexpression in the LC, might impair proper stress recovery. Indeed such disturbances in stress recovery have been previously shown in triple UCN1/2/3 knockout mice. In the second round of behavioral experiments an independent cohort of animals was exposed to the stressor shortly before testing (Figure 66). With the exception of the elevated plus maze test, anxiety-related tests, i.e. the dark/light box test and the open field test, were preceded by 10 min of physical restraint stress followed by 30 min of recovery. Each test was performed on a separate day. The sucrose preference test was performed one week after the last behavioral test and the home cage locomotion was assessed 4 weeks after the sucrose preference test.
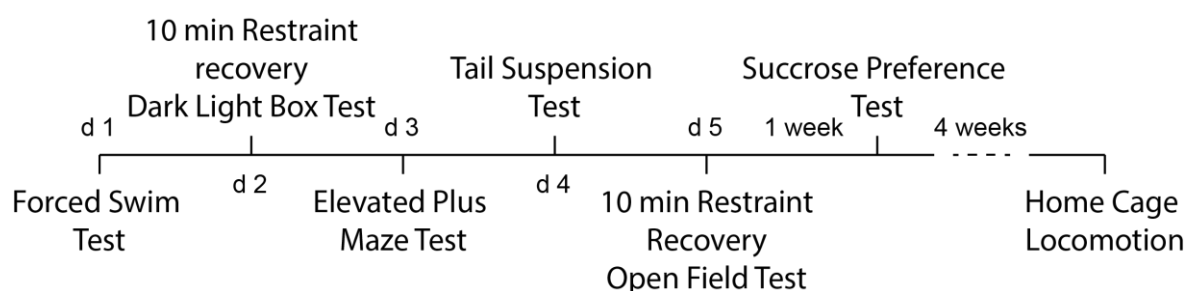
Figure 66. Experimental design of testing UCN2-COE^{NE} mice following previous stress exposure.

4.7.4.3.1 UCN2-COE^{NE} mice show consistent increase in passive stress-coping behavior

The forced swim test was performed as the first behavioral task to confirm previous results. It was also used as a primary stressor. Therefore, the standard session of 6 min of FST was prolonged to 10 min. Behavior of animals was scored for both, the initial 6 minutes and the entire period of 10 minutes (Figure 67). During the first 6 minutes of the test duration UCN2-COE^{NE} mice spent significantly more time floating (p=0.0004), less time struggling (p=0.0044) and less time swimming (p=0.0004) compared to the control littermates. Additionally, the time period to the first floating episode was significantly shorter in UCN2-COE^{NE}, than for the UCN2^{Ctrl} mice (p=0.0052).
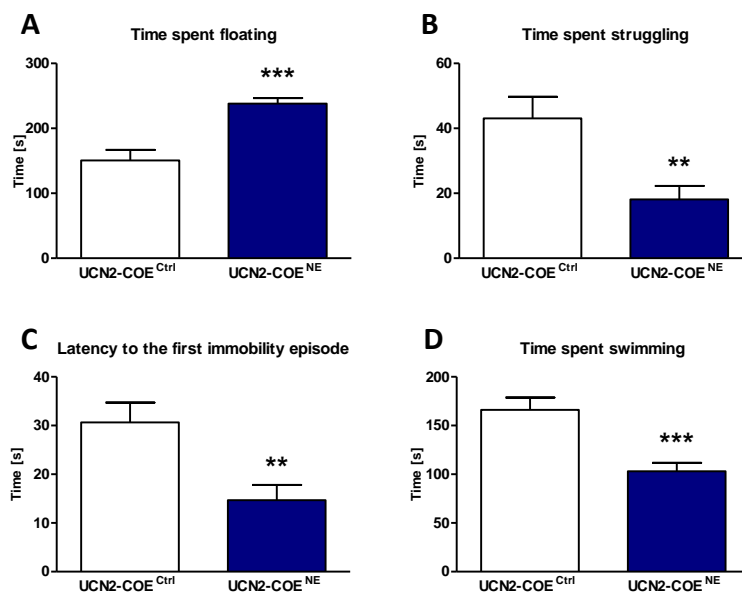
Figure 67. Stress-coping behavior in UCN2-COE^{NE} mice measured during first 6 minutes of the forced swim test.

A, time spent floating B, time spent struggling C, time spent swimming and D, the latency to the first floating episode. Data are expressed as means +S.E.M. n=14-13 per group. ** p<0.01, *** p<0.001. Student's t-test.

The tail suspension test was performed without additional restraint stress preceeding the testing as the test itself is a stressor. Here again the test was performed for a prolonged time of 10 min, but both, i.e. the standard period of 6 minutes and the entire testing time of 10 minutes were scored (Figure 68). During both, 6 (p=0.0008) and 10 minutes (p=0.0022) of the test UCN2-COE^{NE} mice showed significantly increased time being immobile with concomitantly decreased time period to the first immobile episode (p=0.0028).
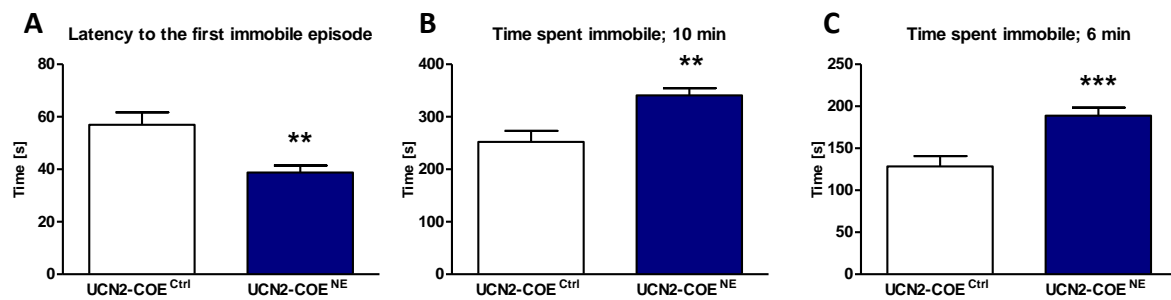
Figure 68. Stress-coping behavior in UCN2-COENE mice measured in the tail suspension test.

A and B represent time spent immobile A, during the first 6 minutes of the test B, during the entire testing period of 10 minutes, C the latency to the first immobile episode. Data are expressed as means +S.E.M. n=15-13 per group. ** p<0.01, *** p<0.001. Student's t-test.

4.7.4.3.2 UCN2-COE^{NE} mice show increased anxiety-related behavior after acute stress exposure

When animals of both genotypes were exposed to the restraint stress preceeding testing, UCN2-COE^{NE} mice showed increased anxiety-related behavior in the dark/light box test (Figure 69). The time spent in the lit compartment (p=0.0327) and the number of entries (p=0.0099) therein were significantly reduced in UCN2-COE^{NE} mice compared to their control littermates. In addition, the distance travelled in the lit compartment (p=0.0220) was significantly decreased along with a tendency towards decreased total distance travelled (p=0.0770). Although the latency to the first approach to the lit compartment was not significantly affected by genotype, it tended to be increased in UCN2-COE^{NE} mice compared to the controls (p=0.0603).
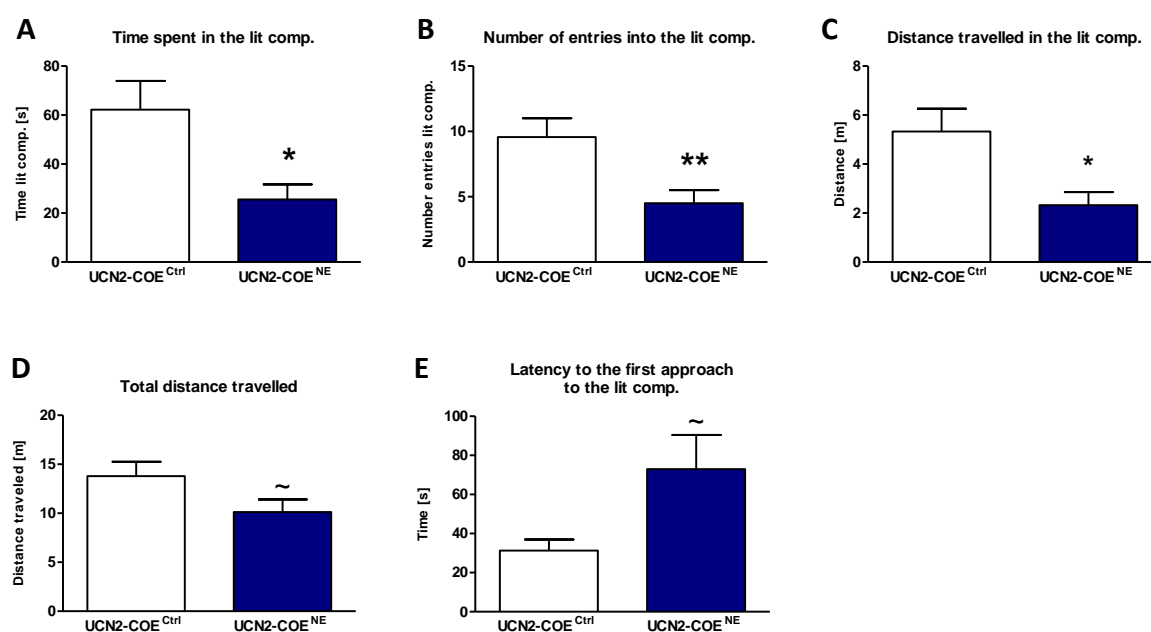
Figure 69. Anxiety-related behavior in UCN2-COE^{NE} mice measured in the dark/light box test following the exposure to restraint stress.

A, time spent in the lit compartment B, number of entries into the lit compartment C, distance travelled in the lit compartment D, total distance travelled E, latency to the first entry to the lit compartment. Data are expressed as means +S.E.M. n=14-12 per group. * p<0.05, ** p<0.01 ~ p<0.1. Student's t-test.

Also in the open field test stressed UCN2-COE[NE] mice exhibited a significant increase in anxiety-related behavior compared to control littermates (Figure 70). The time spent in the inner zone of the apparatus ($p=0.0477$) along with the number of entries into it ($p=0.0147$) and the distance travelled therein ($p=0.0770$), were significantly decreased in UCN2-COE[NE] mice. In addition the latency to the first entrance into the inner zone ($p=0.0849$) tended to be increased in UCN2-COE[NE] animals. UCN2-COE[NE] mice also showed a decreased total distance travelled ($p=0.0406$).
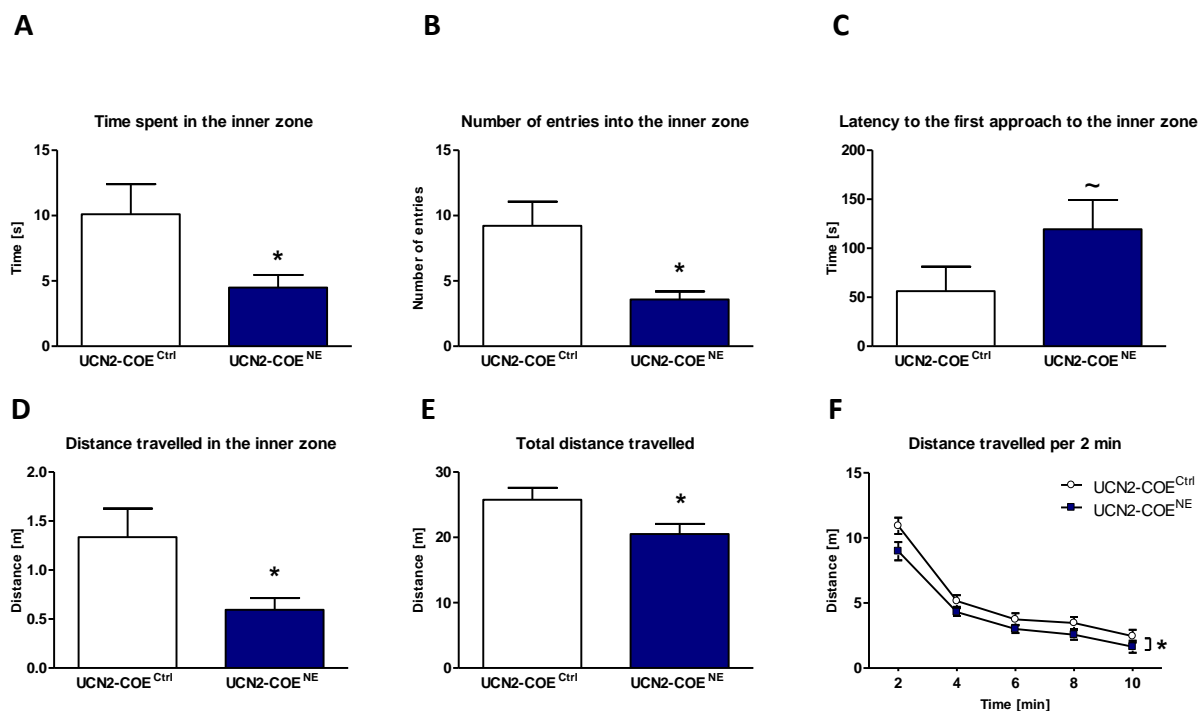
Figure 70. Anxiety-related behavior in UCN2-COE[NE] mice measured in the open field test following exposure to restraint stress

A, time spent in the inner zone B, number of entries into the inner zone C, the latency to the first approach to the inner zone D, distance travelled in the inner zone E and F total distance travelled during 30 min of the test duration or F, split in 5 min intervals. Data are expressed as means +S.E.M. n=14-12 per group. * $p<0.05$, ~ $p<0.1$. Student's t-test.

4.7.4.4 UCN2-COE^{Nat} mice show anhedonic behavior and decreased home cage locomotion

As all previously conducted behavioral tests were depending on locomotor abilities of animals it was unclear whether the increase in anxiety-related behavior and passive stress coping are secondary effects of decreased locomotion observed in UCN2-COE^{NE} mice. To solve this issue we performed the sucrose preference test.

Animals had unlimited access to two identical bottles containing either 1% sucrose or water. We found that after a period of one week UCN2-COE^{NE} mice consumed less amount of 1% sucrose compared to the control group. Nevertheless, this outcome did not reach significance (Figure 71).

Sucrose preference test

p=0.0781

%consumed sucrose

UCN2-COE^{Ctrl}   UCN2-COE^{NE}
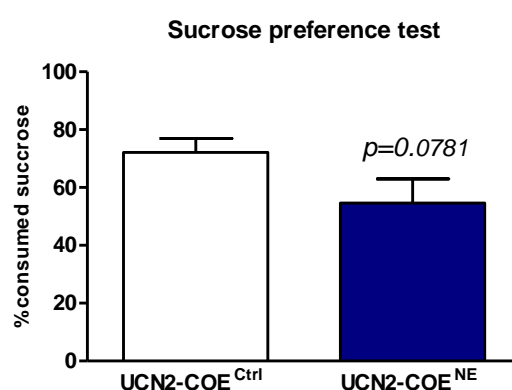
Figure 71. Sucrose preference test in UCN2-COE^{NE} mice.
UCN2-COE^{NE} show a tendency towards anhedonic behavior. Data are expressed as means +S.E.M. n=13-12 per group. Student's t-test.

Additionally, four weeks after the sucrose preference test, the total locomotion of animals during 24 h was recorded in their native environment, i.e. home cage. Mice of both genotypes showed a significant change in activity between the dark and light period (Figure 72). However, UCN2-COE^{NE} mice showed significantly decreased activity throughout the entire dark period with the strongest reduction during the first 6 hours of darkness. During the light period total activity of conditionally overexpressing animals and their control littermates was not significantly affected by the genotype.
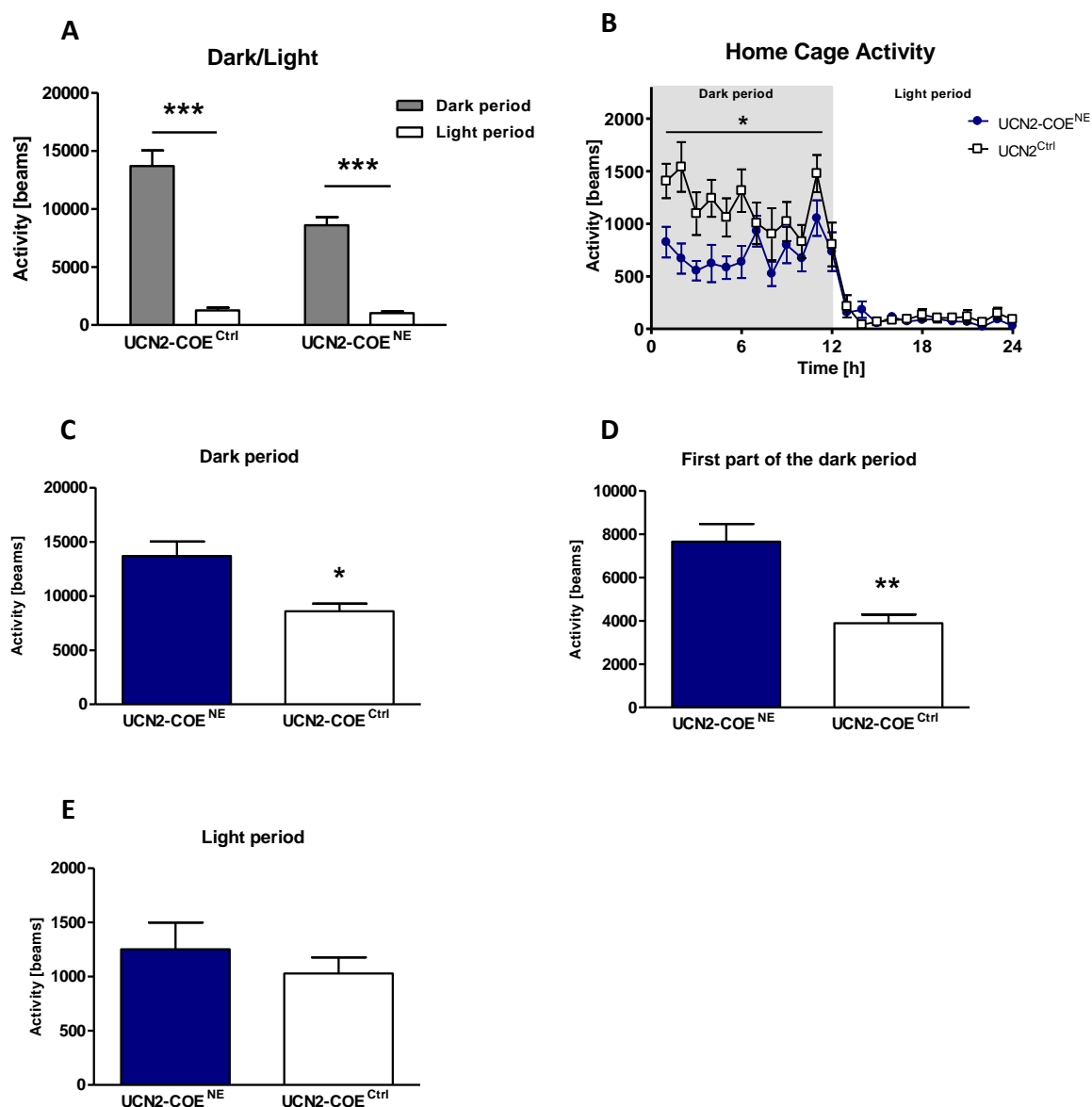
Figure 72. Home cage activity of UCN2-COE[NE] mice measured during a 24 hour period of time.

A, Total activity of animals during the dark and light period B, animals' activity in home cage over 24 h of surveillance C, Activity during 12 h of dark period D, activity during the first 6 h of the dark period, E activity during the 12 h of the light period. Data are expressed as means +S.E.M. n=14-11 per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Student's t-test.

5 Discussion

5.1 CRHR2^{Venus} mice as a reporter of CRHR2 expression.

CRHR2 is a protein which cannot be reliably detected using traditional immunohistochemistry methods. Unfortunately, commercially available antibodies are not specific and sensitive enough thus do not serve a tool to determine exact places where CRHR2 protein is expressed. In order to pass by this obstacle, we took advantage a BAC transgenic CRHR2 reporter mouse line (CRHR2^{Venus}) in which the expression of the GFP variant, i.e. Venus, is driven by the CRHR2 promoter. By characterization of Venus expression we solved expression of CRHR2 with high resolution and, in some instances, were able to determine the neurochemical identity of CRHR2 positive neurons. Comparing expression pattern and strength of the reporter signal with endogenous CRHR2 mRNA expression, we clearly see a complete alignment between them, proving the versatility of the CRHR2^{Venus} mouse line. Additionally, the GFP variant expressed in CRHR2^{Venus} reporter compromises a membrane localization signal, due to this it can be visualized also in neurons' processes, i.e. axons and dendrites. This additional feature carries the possibility of further tracking projection sites and targets of CRHR2 positive neurons, especially when combined with modern microscopy and techniques like CLARITY (Chung and Deisseroth, 2013).

5.2 Conditional CRHR2 knockout mouse lines

In order to study effects of CRHR2 deletion in the central nervous system and in separate neurotransmitter systems we generated a set of conditional CRHR2^{CKO} mouse lines. For the first time we successfully established mouse models in which the deletion of CRHR2 is restricted to either the entire central nervous system or its components, i.e. the GABAergic system, forebrain principal neurons or the serotonergic system and at the same time is preserved in peripheral tissues. With the help of CRHR2$^{CKO-CNS}$, CRHR2$^{CKO-GABA}$, CRHR2$^{CKO-Camk2a}$ and CRHR2$^{CKO-5HT}$ mouse lines we pointed out similarities and differences between behavioral and physiological effects, which resulted from precisely restricted inactivation of CRHR2. Interestingly, in some of our lines i.e. CRHR2$^{CKO-CNS}$, CRHR2$^{CKO-GABA}$ and CRHR2$^{CKO-Camk2a}$ we found increased anxiety-related and/or decreased active stress-coping behavior, which resemble features of the phenotype of CRHR2 KO mice. On the other hand when the deletion of CRHR2 was restricted to serotonergic neurons of the DRN and MnR, i.e. in CRHR2$^{CKO-5HT}$ mice, animals displayed significantly decreased anxiety-related behavior along with increased stress coping behavior. Moreover, deletion of CRHR2 from

serotonergic neurons changed neurophysiology of serotonergic system producing a variety of emotionally, socially and physiologically-related changes. These strikingly contrasting effects of CRHR2 deletion in CRHR2[CKO-CNS], CRHR2[CKO-GABA] and CRHR2[CKO-Camk2a] mice versus CRHR2[CKO-5HT] mouse line could only be reveled due to the used conditional deletion approach.

5.2.1 Generation and characterization of CRHR2[CKO-CNS] mice

To study effects of central deletion of CRHR2 we generated CRHR2[CKO-CNS] mice. By performing in situ hybridization we confirmed the complete disruption of CRHR2 expression in the central nervous system and further showed that CRHR2[CKO-CNS] mice display a mild increase in anxiety-related behavior and significant decrease in active stress coping behavior. It has been shown that the total deletion of CRHR2 might increase anxiety-related behavior measured under non-stress conditions (Bale et al., 2000;Kishimoto et al., 2000). Nevertheless, in two other studies, the enhanced anxiety-related phenotype of CRHR2 KO mice was either not evident (Coste et al., 2006) or appeared only after acute immobilization (Issler et al., 2014). In contrast to the variable anxiety-related behavior, all available studies equally showed a significant impairment of active stress coping behavior of CRHR2 KO mice (Bale and Vale, 2003;Coste et al., 2006;Todorovic et al., 2009). Furthermore and as expected, central stimulation of CRHR2 by i.c.v. infusion of CRHR2 agonists produces an opposite effect, i.e. increases active stress coping behavior (Tanaka and Telegdy, 2008b;Tanaka and Telegdy, 2008a;Tanaka et al., 2011c). In CRHR2[CKO-CNS] mice CRHR2 is depleted only from the CNS in contrast to CRHR2 KO, in which expression of the receptor is abolished in all tissues. Thus our results support the hypothesis that CRHR2 is involved in modulation of anxiety-related and stress coping behaviors and explicitly point towards a neuronal origin of these effects. Increased anxiety-related behavior along with signs of behavioral despair observed in CRHR2[CKO-CNS] indeed underscore a general anxiolytic role of the receptor. In addition, decreased active stress coping behavior in the FST might indicate changes in physiology of neurotransmitter systems, e.g. serotonergic or noradrenergic circuits. Deletion of CRHR2 from the entire CNS does not allow to speculate about the neurocircuits or particular brain sites mediating observed phenotypes. To narrow down possible neurocircuits we proceeded with generation of more restricted CRHR2[CKO] lines, i.e. CRHR2[CKO-GABA], CRHR2[CKO-Camk2a] and CRHR2[CKO-5HT] mouse lines.

5.2.2 Characterization of CRHR2 in the GABAergic system

5.2.2.1 Expression of CRHR2 in GABAergic neurons and establishing the CRHR2$^{CKO-GABA}$ model

As expected, we confirmed that in the CNS, CRHR2 is mostly expressed in GABAergic neurons positive for calbindin D-28K. Neurons double labeled for Venus and calbindin in CRHR2^{Venus} reporter mice were found in the cortex, lateral septum, the posterior part of bed nucleus of stria terminalis, dorsal endopiriform nucleus and to some degree also in the medial amygdala and dorsal and median raphe nuclei. In the cortex no double labeling for Venus and Camk2a was found, meaning that all CRHR2 GABAergic neurons are most likely local double-bouquet interneurons (Baimbridge et al., 1992). This hypothesis is additionally supported by our data concerning the distribution of residence CRHR2 mRNA signal in our conditional CRHR2$^{CKO-GABA}$ and CRHR2$^{CKO-Camk2a}$ mice. In CRHR2$^{CKO-GABA}$ mice the entire CRHR2 signal is abolished from cortex, but remains unaffected in CRHR2$^{CKO-Camk2a}$ mice. Interestingly, expression of CRHR2 in the lateral septum is not exclusively restricted to GABAergic neurons. In CRHR2^{Venus} reporter mice, only a subpopulation of CRHR2 positive neurons was double labeled for calbindin D-28K and Venus. Based on the fact that calbindin is present only in a subpopulation (approximately 60%) of all GABAergic neurons, remaining CRHR2 positive cells still might be GABAergic (Zhao et al., 2013). Nevertheless, this scenario does not seem to be probable as the CRHR2 mRNA signal in the LS of CRHR2$^{CKO-GABA}$ mice is only decreased (Anthony et al., 2014), but not entirely removed, and the Dlx5/6 promoter is believed to be expressed in almost all forebrain GABAergic neurons. These data support results obtained so far by Anthony and colleges, who found that colocalization between CRHR2 and Gad2 (glutamate decarboxylase type 2) in the LS reaches 85%. We also found that some but not all neurons of the medial amygdala are double positive for calbindin and GFP, meaning that also here CRHR2 is present at least partially in GABAergic neurons.

5.2.2.2 CRHR2$^{CKO-GABA}$ mice show increased anxiety-related phenotype and passive stress coping behavior

Anxiety-related behavior was evaluated in adult male mice using standard anxiety paradigms i.e. the open field, the dark/light box and elevated plus maze test. We found that under non stress conditions CRHR2$^{CKO-GABA}$ mice show a mild increase in anxiety-related behavior in the elevated plus maze test. These results point out that CRHR2 activation in GABAergic system might promote an increased anxiety-related response under non-stress conditions. In the light of available literature indicating a rather anxiogenic role of septal CRHR2 activation it might

appears surprising that we observed increased anxiety-related behavior in CRHR2$^{CKO-GABA}$ mice, in which most of CRHR2 in the LS is deleted (Anthony et al., 2014;Bakshi et al., 2007b;Henry et al., 2006). Nevertheless, in contrast to pharmacological or optogenetic studies, we deactivated the majority of CRHR2 present in the GABAergic system, i.e. in the LS, BNST, MeA and cortex, where CRHR2 is expressed. It still might be that mentioned structures, for instance the LS or BNST, exert opposite effects on behavior upon selective and structure-specific CRHR2 stimulation. One should also remember that septal application of CRHR2 agonists, activates CRHR2 in vicinity of the injection site, but does not distinguish between different subpopulations of CRHR2 expressing neurons, i.e. GABAergic and those of other identity. Even sophisticated optogenetic studies proposed by Anthony and colleagues, do not provide unequivocal evidence whether observed effects are mediated by activation of CRHR2 on GABAergic neurons. In this study the authors took advantage of light stimulation or inhibition of cre-expressing neurons within the lateral septum. In this design cre-recombinase is driven by the CRHR2 promoter, though as the authors mention, only 34% of cre positive neurons are positive for CRHR2 mRNA what unfortunately was not further evaluated with respect to how many of cre-positive neurons are positive for GABAergic markers. Another fact differentiating our genetic model from pharmacological or optogenetic experiments is the possibility of compensation taking place in other systems of CRHR2$^{CKO-GABA}$ mice caused by life-long CRHR2 deficiency. Such compensatory mechanisms were shown to occur in CRHR2 KO mice (Bale et al., 2000) and are a general caveat of mouse mutants with early gene deletion. It is worth to note, that stress during behavioral experiments with transgenic CRHR2^{CKO} lines is reduced to a level which is unachievable in experiments involving central application of pharmacological agents. Importantly, CRHR2$^{CKO-GABA}$ mice also show hypersensitivity of the HPA axis. As we found, already 2 min of physical restraint-stress induced a 4-fold increase in released plasma corticosterone in CRHR2$^{CKO-GABA}$ mice compared to control littermates. In addition, we showed that this effect is correlated with significant activation of cortical areas and, most likely, compensatory overproduction of CRH in the cortex. Although the animals were not stressed, e.g. restrained before any of the behavioral tests for these hyperactive mice the testing situation per se might have been stressful enough to burst a corticosterone release and thereby enhance anxiety-related responses. An additional factor, which might bare possible impact on the observed behavior is an increased expression of cortical CRH along with a non-significant increase of CRHR1 expression. It has been shown that conditional removal of CRHR1 from forebrain principal GABAergic and glutamatergic neurons but also from glutamatergic neurons alone, decreases anxiety-related behavior (Muller et al.,

2003;Refojo et al., 2011). In our model the slight increase in the anxiety-response to the elevated plus maze might represent the opposite situation of cortical CRHR1 overactivation. We also found that CRHR2[CKO-GABA] mice show decreased active stress coping behavior. This finding resembles the phenotype of CRHR2 KO and CRHR2[CKO-CNS] mice, which were also shown to display features of behavioral despair (Bale and Vale, 2003;Todorovic et al., 2009). A large body of literature points towards the lateral septum in modulation of anxiety-related and stress coping behavior. Singewald and colleagues showed that excitoxic lesions of the LS neurons significantly impair active stress coping behavior in the FST and hypersensitises the HPA axis response to 10 min of swimming (Singewald et al., 2011). Nevertheless, the role of septal CRHR2 involvement in modulation of behavior in the FST still requires further investigation.

5.2.2.3 Decreased acoustic startle response of CRHR2[CKO-GABA] mice

Startle abnormalities measured in humans appear in anxiety disorders like PTSD (Grillon and Baas, 2003). The acoustic startle response is a reaction of the animal's entire body to a randomly occurring acoustic stimulus of determined intensity (Koch, 1999). We found that CRHR2[CKO-GABA] mice showed decreased ASR to the high intensity stimuli in two independent tests, i.e. ASR and prepulse inhibition, despite no significant changes in PPI were noted. It has been shown that i.c.v. infusions of CRH potentiate the ASR (Liang et al., 1992b). Moreover, this potentiation is blocked by central pretreatment either with CRHR1 selective antagonist NBI-30775 or CRHR2 selective antagonist, ASV-30 (Risbrough et al., 2003;Risbrough et al., 2004). Furthermore, also selective CRHR2 activation, by i.c.v. UCN2 injection, was shown to increase ASR in naïve animals albeit less efficient than CRH (Risbrough et al., 2003). In our model CRHR2[CKO-GABA] mice display a reduced ASR without any stimulation by exogenous ligand, i.e. UCN2 or CRH. Most likely this effect is mediated by deletion of CRHR2 from GABAergic neurons of the BNST. First, lesions of the BNST block CRH potentiated startle. Second, blockade of CRH receptors in the BNST, alleviates effects of CRH on the ASR (Lee and Davis, 1997b). Third, selective antagonism of CRHR2 in the BNST was recently shown to reduce ASR in animals which underwent water avoidance stress (Tran et al., 2014). In contrast to the BNST lesions, lesions of the LS, the other prominent site of CRHR2 expression, were shown not to affect CRH potentiate startle (Lee and Davis, 1997a). Interestingly, in subjects suffering from PTSD as well as in animal models of PTSD, startle reactivity is increased (Elharrar et al., 2013;Lebow et al., 2012). An increase in the ASR is thought to correspond to an increased aversive avoidance response, which results from a state of enhanced anxiety. In this regard, decreased ASR stays in opposite to increased anxiety-related

behavior observed in CRHR2$^{CKO-GABA}$ mice. Nevertheless, such phenomenon is also observed in mice and rats inbred for high anxiety-related behavior (Yen et al., 2013;Landgraf and Wigger, 2002;Yen et al., 2013), and suggests that changes in ASR do not reflect states of anxiety, although in some cases might be intertwined with it.

5.2.2.4 CRHR2$^{CKO-GABA}$ mice show a dysregulation of HPA axis and are hypersensitive to immobilization stress

In order to examine functionality of the HPA axis, CRHR2$^{CKO-GABA}$ mice were subjected to 2 or 10 min of immobilization stress and released plasma corticosterone was measured. We found a significant increase in plasma corticosterone concentration after 2 min of restraint stress, although after 10 min of immobilization and during recovery, this genotype specific effect disappeared. A similar increase in corticosterone upon 2 min of restraint stress was found in CRHR2 KO mice. Furthermore, 2 min of immobilization induced a significant increase in cortical expression of the immediate response gene c-fos in CRHR2$^{CKO-GABA}$ mice. Under non-stress conditions expression of cortical c-fos was indistinguishable between CRHR2$^{CKO-GABA}$ and CRHR2^{Ctrl} mice, yet expression of cortical Crh mRNA was found to be almost 2-fold up-regulated in CRHR2$^{CKO-GABA}$ mice. Accordingly, we found a non-significant increase in cortical expression of Crhr1 mRNA in CRHR2$^{CKO-GABA}$ mice. Additionally, the dysregulation of the HPA axis in response to stress was further enhanced in CRHR2$^{CKO-GABA}$ mice when animals were exposed to CSDS. As it was previously shown, we also confirmed a significant hypersensitivity of the HPA axis in CRHR2 KO mice to immobilization stress (Bale et al., 2000;Coste et al., 2000). Moreover, we extended these results to our model of CRHR2$^{CKO-GABA}$ mice, suggesting that the hypersensitivity of the HPA axis is due to deletion of CRHR2 in the GABAergic system. The current literature points towards the BNST and LS as possible structures modulating the HPA activity in context of the CRHR2 deletion from the GABAergic system (Anthony et al., 2014;Singewald et al., 2011). We found that CRHR2$^{CKO-GABA}$ mice lack CRHR2 in GABAergic neurons of the BNST, which is thought to be one of relay nuclei through which information is passed to CRH containing cells of PVN. Taking into account that activation of CRHR2 on GABAergic neurons of the BNST should provide neuronal inhibition to the PVN and prevent thereby activation of the HPA axis, lack of CRHR2 on GABAergic neurons of the BNST should exert an opposite effect, i.e. enhanced stimulation of the HPA axis. Indeed our in situ hybridisation analysis of c-fos expression following 2 min of restraint stress suggests that such scenario might be possible although does not provide a direct proof (as in control animals, despite basal levels of corticosterone, the BNST does not seem to be

activated either). Instead it points to the hypothesis that these hormonal changes could be linked to general cortical hyperactivity. Such hyperactivity might be due to the lack of GABAergic brake on neurons indirectly projecting from cortex to PVN (Herman et al., 2005). It is important to emphasize that cortical GABAergic neurons missing CRHR2 expression in CRHR2$^{CKO\text{-}GABA}$ mice are most likely local interneurons. Two arguments support this hypothesis. First, we found almost exclusive co-localization of our Venus reporter with calbindin D-28K, which is known to be expressed in double bouquet cortical interneurons (Baimbridge et al., 1992), while no co-localization with parvalbumin and calretinin was observed. Second, CRHR2 mRNA expression is preserved in the cortex of CRHR2$^{CKO\text{-}Camk2a}$ mice after induction of conditional knockout. These conditional mutants do not show a hypersensitivity of the HPA axis, which is characteristic for CRHR2 inactivation in CRHR2$^{CKO\text{-}GABA}$ and CRHR2 KO mice (Bale et al., 2000;Coste et al., 2006). One feasible explanation assumes that at the beginning of the stress response CRH activates CRHR2 on GABAergic cortical interneurons, which in turn inhibit stimulatory input to NST or raphe nuclei (Herman et al., 2005). As a consequence the activation of the PVN increases step-wise. When CRHR2 is absent from cortical GABAergic interneurons stimulatory input to the PVN is enhanced inducing the increased release of corticosterone. Another possible explanation would assume increased expression of CRH on the level of the PVN, which leads to an increase of the hormonal stress response. Both situations are possible and both might occur at the same time, contributing to the hypersensitivity of the HPA axis. Although we did not find any increase in corticosterone concentrations during the morning nadir or the evening peak in CRHR2$^{CKO\text{-}GABA}$ mice compared to the CRHR2^{Ctrl} group, we noted a reduction in the thymus size which might be caused by excess of released corticosterone to irrelevant situations which normally do not induce an activation of the HPA axis. Interestingly, when animals of both genotypes were subjected to CSDS, the stressed groups still do not differ between each other with respect to the corticosterone level during the morning peak, meaning that the basal control of HPA axis is undisturbed. Nevertheless, after stress, i.e. FST, we still observed a significant increase in serum corticosterone of stressed CRHR2$^{CKO\text{-}GABA}$ mice compared to stressed CRHR2^{Ctrl} mice and non-stressed controls, but not between non-stressed CRHR2$^{CKO\text{-}GABA}$ mice compared to non-stressed CRHR2^{Ctrl} mice. Most likely, under acute stress condition, such as 30 min after FST, corticosterone levels in the non-stressed groups reach a ceiling effect where any further increase in the CSDS group is not possible. Such serious deregulation of the HPA axis might lead to sever systemic consequences resulting from excess of corticosterone, i.e. increased risk of infectious diseases, overweight, insulin resistance and diabetes, hypertension, edemas, neuropathies, decreased

bone density etc. Indeed we see some of these features in our CRHR2$^{CKO-GABA}$ mice, i.e. increased body weight and decreased quality of coat. It would be of great interest to track systemic and physiological changes occurring with aging of CRHR2$^{CKO-GABA}$ mice in the future.

5.2.3   Deletion of CRHR2 from forebrain principal neurons in adulthood

5.2.3.1 Expression of CRHR2 in adult CRHR2$^{CKO-Camk2a}$ mice

To study effects of CRHR2 deletion beginning in adulthood we took advantage of the tamoxifen inducible CreERT2 recombinase. Although this kind of cre-recombinase is expressed in all Camk2a positive forebrain neurons, its activity is suppressed by presence of Hsp90 protein forming Cre-Hsp90 complex. Upon tamoxifen binding to the Cre-Hsp90 complex, Hsp90 dissociates and enables translocation of cre to the cell nucleus and recombination. In this system, recombination begins with delivery of exogenous tamoxifen at the any time point in life. We found that the Venus reporter is expressed in Camk2a positive neurons in the LS, MeA and subiculum. Accordingly, induction of cre-recombination in CRHR2$^{CKO-Camk2a}$ mice, revealed a significant reduction of CRHR2 mRNA in the LS, MeA and subiculum which extended to the DG and BNST. It is known that Camk2a is expressed in projecting principal neurons of the forebrain, i.e. GABAergic and glutamatergic neurons. In our conditional CRHR2$^{CKO-GABA}$ and CRHR2$^{CKO-Camk2a}$ mice the deletion pattern of CRHR2 is similar. Nevertheless, we see in CRHR2$^{CKO-Camk2a}$ mice a more prominent reduction of septal CRHR2. Most likely the Camk2a promoter covers more of CRHR2 positive neuronal population than, the Dlx5/6 promoter. It is rather unlikely that enhanced deletion of CRHR2 in the septum was caused by deletion in septal glutamatergic neurons. We were not able to find any glutamatergic neurons in the LS which would be positive for the Venus reporter in CRHR2^{Venus} mice. Similar lack of co-localzation between CRHR2 mRNA and glutamatergic markers was found by Anthony and colleagues (Anthony et al., 2014). Interestingly, we also confirmed that CRHR2 in the cortex is expressed in GABAergic interneurons, as these were not affected by Camk2a-cre recombinase in CRHR2$^{CKO-Camk2a}$ mice.

5.2.3.2 Increased anxiety-related behavior in CRHR2$^{CKO-Camk2a}$ mice and changes in HPA axis function

In order to assess the phenotype of CRHR2$^{CKO-Camk2a}$ mice we performed the standard anxiety-related tests and evaluated stress coping behavior using the FST. We found that deletion of CRHR2 in Camk2a positive neurons of adult mice, increases anxiety-related behavior in the open field and the dark/light box tests. Interestingly, although we were not able to completely remove

CRHR2 from any particular brain structure we observed a significant decrease of its expression in the BNST, LS, MeA and subiculum, but it was enough to promote anxiety-like responses. Taking into account that in CRHR2$^{\text{CKO-Camk2a}}$ mice, CRHR2 is depleted from the cre-expressing forebrain GABAergic and glutamatergic projection neurons we assume that indeed those projecting GABAergic neurons from the BNST, LS, MeA or subiculum are mediating the observed increase in anxiety-related behavior. We excluded any possible involvement of glutamatergic neurons since those neurons do not express CRHR2 (unpublished observations). Unfortunately, not much is known about the role of CRHR2 in medial amygdala or subiculum, since most previous studies were focusing on the BNST and LS. In contrast to our results Anthony and colleagues showed that activation of a subpopulation of CRHR2 positive septal projections to AHA induces anxiety-related behavior (Anthony et al., 2014). Also Henry et al. indicated that UCN2 infused into the lateral septum increases anxiety-like behavior (Henry et al., 2006). Based on that information, it is most likely that the deletion of CRHR2 from LS neurons is not responsible for the observed increase in anxiety-related behavior in CRHR2$^{\text{CKO-Camk2a}}$ mice under basal conditions. The BNST consists of two populations of CRHR2 expressing GABAergic neurons, one positive and the other negative for Camk2a. We assume that double positive neurons, i.e. CRHR2+Camk2a, are projecting GABAergic cells, and observed reduction of CRHR2 mRNA expression in pBNST of CRHR2$^{\text{CKO-Camk2a}}$ mice corresponds to deletion of the receptor from those neurons, although future investigations are necessary to confirm this hypothesis.

Importantly, we did not find any changes in the HPA axis response to restraint stress, what excludes a possible involvement of glucocorticoids in mediation of observed effects. Also a possible involvement of stable compensatory mechanisms can be excluded due to the short period of time from introducing tamoxifen, and thereby inducing the conditional knockout, to testing, i.e. 4 weeks. Although expression of CRHR2 in CRHR2$^{\text{CKO-Camk2a}}$ mice is strongly diminished throughout many subcortical sites, CRHR2$^{\text{CKO-Camk2a}}$ mice do not show any changes in active or passive stress coping behavior in the FST, what is in contrast to CRHR2$^{\text{CKO-CNS}}$ and CRHR2$^{\text{CKO-GABA}}$ lines.

5.2.4 Characterization of expression of CRHR2 in the serotonergic system and generation of CRHR2$^{CKO-5HT}$ mice

Before we started studying the role of CRHR2 in the raphe complex we proofed that indeed CRHR2 is expressed in serotonergic neurons. For this purpose we characterized the expression of the Venus reporter in the raphe complex of CRHR2^{Venus} mice. We found that the majority of CRHR2 reporter is present in the midline or the DRN. The highest number of GFP positive neurons was found in the DRD, DRV and DRI. Additionally, we also localized a Venus signal in the MnR and other groups of serotonergic neurons which are typically not included in raphe complex, i.e. PnR. By performing double immunostaining we found that the majority of Venus positive neurons in the dorsal raphe nucleus co-express TPH2, a marker of serotonin neurons. The majority of double positive neurons resided in the DRI, DRV and DRD divisions of the DRN. In contrast, the DRVL part, despite the fact that it contains serotonergic neurons, was almost completely devoid of double labeled neurons. In addition, the MnR showed similar to the DRN, a high number of double positive neurons. Our results are in full agreement with studies showing expression of CRHR2 mRNA in the DRN and MnR. Van Pett and colleagues first showed high expression of CRHR2 in the DRN and MnR (Van Pett K. et al., 2000). Furthermore, these results were extended by Day and colleagues showing that despite the fact that CRHR2 is expressed in the DRN and MnR only a portion of CRHR2 positive neurons is indeed serotonergic (Day et al., 2004). In rostal to mid-caudal parts of the DRN, CRHR2 mRNA was found exclusively or almost exclusively in serotonin reuptake transporter (SERT) positive neurons. In caudal parts the number of the CRHR2/SERT double positive neurons droped down in favor of GAD65/67-CRHR2 mRNA double labeled cells (Day et al., 2004). A recent study from Lukkes and colleagues provides additional detailed information about the expression of CRHR2 in TPH2 positive neurons (Lukkes et al., 2011). In contrast to Day et al. they found that the number of CRHR2/TPH2 positive neurons in the DRV does not exceeds 44.9%. A high percentage of double labeled neurons was found in the DRD, DRC and in PnR. Interestingly, only a sparse number of CRHR2/TPH2 positive cells was localized in the DRVL part (Lukkes et al., 2011). Unfortunately, the authors did not address the question of the neurochemical identity of non-serotonergic neurons expressing CRHR2. Most likely CRHR2 in the DRN is also present on a subpopulation of GABAergic neurons. This was also suggested based on electrophysiological recordings from DRN neurons (Pernar et al., 2004). This suspicion finds confirmation in our colocalization experiments where we saw sporadic GFP positive neurons expressing calbindin D-28K, a marker for GABA double bouquet interneurons (Baimbridge et al., 1992). In contrast to the midline DRN which is

dominated by expression of CRHR2, CRHR1 is almost not present on serotonergic cells (Refojo et al., 2011). It has been shown that the CRHR1 in the DRN is predominantly expressed in GABAergic neurons. Their activation might indirectly influence the activity of serotonergic neurons and due to that counterbalance effects of CRHR2 activation (Kirby et al., 2008;Pernar et al., 2004;Price et al., 1998). One should not forget that the DRN was shown to express CRH and UCN1, but up to now it is not known whether these neurons are serotonergic or positive for CRHR2.

To study functions of CRHR2 in the raphe complex we generated a mouse line, CRHR2$^{CKO-5HT}$, devoid of CRHR2 expression in serotonergic neurons. In line with our colocalization studies CRHR2$^{CKO-5HT}$ mice lack the majority of CRHR2 mRNA in the DRN. In situ quantification analysis showed reduction of CRHR2 signal ranging from 80% to 87%. Our data indicate that most of CRHR2 in the dorsal raphe is present in serotonergic neurons with a sparse population being expressed in non-serotonergic cells. At the moment we are trying to solve the neurochemical identity of remaining CRHR2 positive neurons in the DRN. This is of particular importance as it was claimed that the promoter used to drive the cre-recombinase specifically in serotonergic neurons, i.e. Pet-1, is not active in all serotonergic neurons (Kiyasova et al., 2011). Moreover, this Pet-1 independent serotonergic subpopulation projects to stress-related brain sites like the PVN and BLA (Kiyasova et al., 2011). Double in situ hybridization analysis of residual CRHR2 mRNA signal with possible serotonergic and GABAergic markers in CRHR2$^{CKO-5HT}$ mice will ultimately answer this question.

5.2.4.1 Effect of CRHR2 deletion from 5HT neurons on anxiety-related response and social behavior

To investigate effects of CRHR2 deletion from serotonergic neurons under non-stress conditions we performed standard anxiety-related tests, i.e. the open field and dark-light box test. We found that CRHR2$^{CKO-5HT}$ mice displayed significantly decreased anxiety-related behavior in both tests. These results are in sharp contrast to all previously investigated CRHR2^{CKO} lines as well as to behavior of the constitutive CRHR2 KO (Bale et al., 2000;Coste et al., 2000;Issler et al., 2014;Kishimoto et al., 2000). While it has been shown in some studies that life-long deficiency of CRHR2 increases anxiety-related behavior under non-stress conditions (Bale et al., 2000;Kishimoto et al., 2000), others observed increased anxiety after stress exposure (Henry et al., 2006;Issler et al., 2014). No study reported similar to CRHR2$^{CKO-5HT}$ an anxiolytic-like phenotype as a consequence of genetic CRHR2 deletion. It is of particular interest that despite

the fact that both CRHR2 KO mice and CRHR2[CKO-CNS] mice do not show any expression of CRHR2 in the raphe complex, similarly to CRHR2[CKO-5HT] mice, they display an opposite anxiety-related behavioral phenotype. It has been shown that complete deletion of CRHR2 produces compensatory effects, like upregulated expression of CRH and UCN1, what could hinder proper interpretation of those data from CRHR2 KO mice and thus complicate pinpointing the primary source of the observed phenotype (Bale et al., 2000). Additionally, it has been shown that the deletion of CRHR2 might impair the mother's behavior so that the offspring of a homozygous or heterozygous CRHR2 knockout dam displayed increased anxiety-related behavior (Bale et al., 2002b). Despite the fact that the majority of genetic studies claims that central activation of CRHR2 decreases anxiety-related behavior and that it is required for the proper stress-recovery, some hints about a possible anxiogenic role of CRHR2, in particular in the dorsal and median raphe, can also be found (Hammack et al., 2003b;Hammack et al., 2003a;Ohmura et al., 2010). For instance Hammack and colleagues found that behavioral changes normally associated with inescapable tailshock, i.e. increased time freezing and latency to escape the compartment of the apparatus where animals were previously shock-stressed, is mimicked in non-stressed rats by activation of CRHR2 in the dorsal raphe nucleus (Hammack et al., 2003b;Hammack et al., 2003a). Furthermore, these behavioral changes present in shock-stressed rats are prone to reversal by intra-DRN infusion of ASV-30, a selective CRHR2 antagonist (Hammack et al., 2003b;Hammack et al., 2003a). Similar to these results it has been found in the fear conditioning paradigm that the selective inhibition of CRHR2 in the MnR decreases the time spent freezing to the context where animals were shock-stressed before (Ohmura et al., 2010).

To further examine the role of CRHR2 in the serotonergic system with regards to social behavior we performed a sociability test under non-stress conditions and following chronic social defeat stress conditions. First, we found that under non-stress conditions CRHR2[CKO-5HT] mice showed increased social preference measured by the time spent interacting with a real mouse compared to the control group. Second, following chronic social defeat stress, CRHR2[CKO-5HT] mice did not show any increase in the latency to the first interaction with a real counter partner what would be typical for defeated animals but insead still spent significantly more time interacting with an alive mouse when compared to the stressed CRHR2[Ctrl] group. These results indicate that deletion of CRHR2 from serotonergic neurons protects CRHR2[CKO-5HT] mice from development of social aversion. Mice are highly social animals, thus it is not surprising that two unfamiliar male animals, when paired, start interacting almost immediately. Such interaction might lead to a new hierarchy in a group, defensive or aggressive behavior when an intruder is placed to previously

occupied space. Nevertheless, in the sociability test a conflict situation that might lead to a fight for dominance does not occur. The sociability test was proposed as an indicator of autistic-like behavior, depicted here as decreased interaction time with a real mouse as counter partner. An increase in time spent interacting with a real mouse under non-stress conditions is a rather uncommon phenomenon. Because of the fact that CRHR2$^{CKO-5HT}$ mice show a decreased anxiety-related phenotype it is possible that they are naturally less afraid of a novel animal as a possible threat. Moreover, a such hypothesis seems to be valid and supported by the case when animals are being chronically defeated by a bigger and stronger aggressor mouse, as it take place in the chronic social defeat paradigm. Unfortunately, we were not able to reproduce a similar increase in interaction time in the group of non-stressed CRHR2$^{CKO-5HT}$ mice as we have seen in the previous experiment. Still, we found that stressed CRHR2$^{CKO-5HT}$ mice behaved as non-stressed animals, while stressed CRHR2^{Ctrl} mice show a significant reduction in time spent interacting with an unfamiliar counter partner. Activity of the serotonergic system has been linked to social and aggressive behaviors in animals and humans. For instance SSRIs change social perception in healthy subjects. Moreover the activity of serotonergic neurons can be modulated by stress and CRHR2 activation, thus it would be of a great interest to further elaborate these outcomes from CRHR2$^{CKO-5HT}$ mice by performing social avoidance and social recognition tests the under stress and non-stress conditions. In this regard, targeting raphe CRHR2 might represent and alternative strategy for treatment of autism-spectrum disorders.

5.2.4.2 Decreased contextual freezing in CRHR2$^{CKO-5HT}$ mice

It has previously been shown that the selective CRHR2 inhibition especially in the median raphe nucleus decreases the time spent freezing to the context where the animals were previously shock-stressed (Ohmura et al., 2010). Thus we assumed that we should observe a similar phenotype in mice lacking CRHR2 in the serotonergic system, i.e. the CRHR2$^{CKO-5HT}$ line. In the fear conditioning protocol animals received an electric shock in a cued, clearly defined context and subsequently were retested in presence of the conditional cue alone in an unfamiliar surrounding or were re-exposed to the familiar context. We found that CRHR2$^{CKO-5HT}$ mice display decreased freezing behavior but only to the context where they were shock-stressed. Interestingly, when the re-exposure procedure was repeated 4 weeks afterwards, animals showed similar behavior to the previous phenotype, i.e. decreased freezing to the context, but not to the cue alone. An increase in freezing behavior is interpreted as heightened fear response. During conditioning animals connect the appearance of the conditioning cue and context with an

aversive experience, i.e. electric shock. It has been shown that consecutive re-exposure to the cue preceding shock in a neutral context activates amygladala-dependent neurocircuitries, while re-exposure to the same context activates hippocampus-dependent memory mechanisms (Phillips and LeDoux, 1992). It is known that the hippocampus plays an important role in acquisition, expression and fear memory consolidation especially to the contextual cues as well as emotional regulation (Bannerman et al., 2004). It has been proposed that especially the ventral part of the hippocampus might contribute to proper anxiety expression as animals with leasioned hippocampi display decreased anxiety-related behavior. Interestingly the ventral hippocampus receives dense serotonergic innervation from the dorsal and median raphe and expresses several subtypes of serotonergic receptors (Berumen et al., 2012). An increase in hippocampal serotonin is observed during conflict behavioral tasks, the elevated plus maze or during re-exporuse to the fear conditioning context (Matsuo et al., 1996;Ohmura et al., 2010;Rex et al., 1999;Wright et al., 1992). Interestingly, rats characterized by an anxiolytic-like phenotype do not show the typical increase in hippocampal serotonin (Rex et al., 1999). Similarly anxiolytic agents such as benzodiazepines prevent serotonergic rise in hippocampus what correlates with anxiolytic-like behavior (Matsuo et al., 1996;Wright et al., 1992). Recently Ohmura and colleagues showed that selective inhibition of CRHR2 in MnR prevents serotonin increase in ventral hippocampus and decreases time spent freezing to the shock-context (Ohmura et al., 2010). Also Hammack and colleagues showed that activity of CRHR2 in the DRN exerts reciprocal control over stressor processing. Inhibition of CRHR2 in the DRN hamper development of behavioral despair while its stimulation mimics its effects (Hammack et al., 2003b). Thus we assume that deletion of CRHR2 in serotonergic neurons of the DRN and MnR prevents stimulation of serotonergic output to the hippocampus and therefore decreases freezing time and improves the anxiety-related profile in CRHR2$^{CKO-5HT}$ mice.

5.2.4.3 CRHR2$^{CKO-5HT}$ mice show increased active stress coping behavior

Constitutive CRHR2 KO mice and previously discussed conditional CRHR2$^{CKO-CNS}$ and CRHR2$^{CKO-GABA}$ mice displayed signs of behavioral despair in the forced swim test (Bale and Vale, 2003;Coste et al., 2006;Todorovic et al., 2009). In line with genetic studies, pharmacological activation of CRHR2 increases active stress coping behavior (Tanaka and Telegdy, 2008b;Tanaka and Telegdy, 2008a;Tanaka et al., 2011c). In contrast to mouse models, where CRHR2 is removed from the entire organism, exclusively from the central nervous system or GABAergic neurons respectively, we found that CRHR2$^{CKO-5HT}$ mice display significantly increased active stress coping

behavior by spending more time struggling and swimming and less time floating. Such behavior could be an indicator of changed catecholaminergic neurotransmission (Detke et al., 1995;Page et al., 1999). Indeed by comparison with noradrenergic and serotonergic antidepressant agents it is assumed that increased struggling corresponds to altered noradrenergic while increased swimming to changes in serotonergic neurotransmission (Detke et al., 1995;Page et al., 1999). Interestingly, in CRHR2$^{CKO-5HT}$ mice both swimming and struggling parameters are increased suggesting changes in both neurotransmitter systems, despite the fact that CRHR2 is selectively depleted only in serotonergic neurons. It might be explained on the basis that the serotonergic system is known to modulate activity of noradrenergic output (Matsumoto et al., 1995;Fink and Gothert, 2007). Indeed the DRN sends projections to the LC and noradrenergic neurons express serotonergic receptors (Vertes and Kocsis, 1994). It has been presented that chronic treatment with paroxetine, a selective serotonin reuptake inhibitor, decreases the firing rate of the locus coeruleus, what is thought to contribute to its antidepressant and anxiolytic properties (Szabo et al., 1999). Thus it is also possible that the anxiolytic and antidepressant-like phenotype of CRHR2$^{CKO-5HT}$ mice is at least partially mediated by secondary effects within the noradrenergic system. Significantly increased active stress coping behavior in CRHR2$^{CKO-5HT}$ mice resembles the phenotype of naïve animals treated with antidepressant agents (Detke et al., 1995;Page et al., 1999). Along with the anxiolytic-related profile, the mouse model of CRHR2 deletion in serotonergic neurons phenotypically mimics many effects of currently available and clinically active antidepressants. Different mechanism of action, i.e. deactivation of CRHR2 in the DRN, might represent an alternative for developing new mood improving and anxiolytic drugs.

5.2.4.4 HPA axis regulation and sleep architecture in CRHR2$^{CKO-5HT}$ mice

The dorsal raphe nucleus expresses high levels of glucocorticoid receptors and its projections to the PVN are known to promote corticosterone release (Jorgensen et al., 1998). Moreover, mice deficient in CRHR2 display a dysregulation of the HPA axis in response to restraint stress (Bale et al., 2000;Coste et al., 2000). In contrast, we did not find any alterations in HPA axis function in CRHR2$^{CKO-5HT}$ mice, besides a non-significant decrease in corticosterone concentration during the evening release peak. Importantly we detected no differences in the HPA axis response to acute immobilization measured both directly after cessation of 10 min restraint and after 90 min of recovery. These outcomes exclude possible influences of the HPA axis dysregulation on the observed emotionality-related phenotype of CRHR2$^{CKO-5HT}$ mice.

Sleep disturbances often precede the occurrence of depression and are comorbid with anxiety-disorders (Armitage, 2007;Steiger and Kimura, 2010). In addition they are alleviated with successful medication of psychiatric conditions (Armitage, 2000). Serotonin plays an important role in regulation of sleep-wake cycle. It is believed that serotonin promotes wakefulness and increases the latency to falling asleep (Monti, 2011). Taking into account the importance of CRHR2 in regulation of the serotonergic system we investigated the impact of selective deletion of CRHR2 on sleep architecture in CRHR2$^{CKO-5HT}$ mice. It is important to mention that mice are nocturnal animals, what means that the peak of their behavioral activity is referred to the dark period. Interestingly, we found that that sleep-wake pattern in CRHR2$^{CKO-5HT}$ mice differ from the pattern observed in control mice. CRHR2$^{CKO-5HT}$ mice showed a decreased length of NREM sleep during the light period, but increased number of NREM sleep episodes. In addition, the proportion of total time spent in the NREM phase was increased at the beginning of the dark period. Increased time spent in REM and NREM phases during the first 6 hours of the dark period corresponded to a decrease in the portion of being awake as well as decreased length of awake episodes, but not their number. As it has been shown that CRHR2 activation in serotonergic neurons correlates with serotonin release, one could expect decreased serotonin activity in the absence of CRHR2. Such decreased activity of the serotonergic system would then prolong sleeping time, preventing wakefulness, a situation observed in CRHR2$^{CKO-5HT}$ mice. Although during the light period we did not find significant difference in the total time asleep and awake, we observed a decreased length of NREM and REM episodes, but increased number of respective episodes, including transitions between particular phases. Thus we assume that despite the absence of changes in the proportion of NREMs, REMs and awake phases during the light period, the speed of entire sleep cycle is increased in CRHR2$^{CKO-5HT}$ mice. Due to the high expression of one of the stress component systems, i.e. CRHR2, in serotonergic neurons, CRHR2$^{CKO-5HT}$ mice could serve a valuable tool to investigate effects of stress on sleep architecture and its possible role in precipitating depression and anxiety-related disorder.

5.2.4.5 Conditional deletion of CRHR2 tremendously affects the physiology of the serotonergic system

It has been shown several times that activation of CRHR2 in serotonergic neurons changes their firing rate and excitability (Lowry et al., 2000;Pernar et al., 2004). Furthermore, stimulation of raphe CRHR2 increases c-fos expression in the DRN and serotonin release in several stress- and emotion-related brain regions, i.e. prefronal cortex, nucleus accumbens basolateral and central

amygdala (Amat et al., 2004;Forster et al., 2006;Hale et al., 2010;Lukkes et al., 2008;Staub et al., 2006;Tanahashi et al., 2012). Additionally, CRHR2 KO mice show decrease activation of the DRN (Issler et al., 2014). Interestingly changes in serotonergic neurotransmission have also been reported in mice lacking CRHR2 ligands i.e. double and triple urocortin knockout mice (Neufeld-Cohen et al., 2010b;Neufeld-Cohen et al., 2010a) or mice overexpressing UCN3 (Neufeld-Cohen et al., 2012). Based on the altered emotional phenotype of CRHR2$^{CKO-5HT}$ mice as well as the literature citied above, we suspected a disruption in serotonergic neurotransmission to occur in these conditional CRHR2 knockout mice. For this purpose several stress-related brain regions along with subregions of the DRN and MnR were microdissected and basal levels of serotonin and 5-hydroxyindoloacetic acid were analyzed. Within the raphe complex we found a significant increase of serotonin tissue concentration in the DRC and decreased 5HT/5HIAA ratio in the DRVL, DRC parts and MnR. Both increased concentration of 5HT and 5HIAA in the DRC and decreased 5HT/5HIAA ratio in other raphe subredions and the MnR might reflect their decreased activity. Although changes observed in the DRC seem to be a primary effect of selective CRHR2 deletion, alterations in other subregions are most likely secondary to impairments in raphe-raphe interactions. The DRC and MnR project to the ventral part of the hippocampus, which is strongly connected with expression and extinction of contextual fear and emotional regulation and where we also found decreased content of serotonin in CRHR2$^{CKO-5HT}$mice. Importantly, it has been shown that animals treated with anxiolytic drugs such as benzodiazepines do not show any characteristic increase in hippocampal serotonin-levels and display a decrease in anxiety-related behavior. Interestingly, decreased contextual fear expression, was also observed after selective pharmacological inhibition of CRHR2 in the MnR (Ohmura et al., 2010). Taken together, decreased anxiety-related as well as decreased contextual freezing behavior are most likely due to decreased activity of the DRC and MnR in CRHR2$^{CKO-5HT}$ mice. Importantly, we found significantly increased serotonin and 5HIAA content in the lateral septum of CRHR2$^{CKO-5HT}$ mice what might be due to decreased activity of serotonergic projections and increased storage of monoamines. It has been shown that repeated swimming prevents a decrease in serotonin release to the LS and corresponds to development of behavioral despair in the forced swim test (Price et al., 2002). Such state is characterized by decreased active stress coping behavior and increased floating activity. Thus increased septal 5HT might favor active stress coping behavior indeed observed in CRHR2$^{CKO-5HT}$ mice. It is also worth to note that no change in serotonin, 5HIAA or their ratio were found in the CeA. The CeA is one of the major sites of CRH expression, which is strongly activated in response to proximal danger or during cued fear memory recall (Phillips

and LeDoux, 1992). It is also strongly innervated by the DRC (Fox and Lowry, 2013;Waselus et al., 2011). As mentioned before we found increased 5HT and decreased 5HIAA/5HT in the DRC, what might result in reduced serotonergic stimulation of the CeA. Such effect could at least in part contribute to the observed decrease in anxiety-related behavior. In line with reduced anxiety-related behavior a decreased 5HIAA/5HT ratio was found in the BNST, another structure rich in CRH containing neurons (Wang et al., 2011), indicating its diminished activity. In contrast to the CeA, the BNST is thought to be activated by distant danger cues and effects of such activation are long-lasting and refer to produce states of anxiety (Davis et al., 1997b;Davis et al., 1997a;Davis et al., 2010;Walker and Davis, 1997;Walker et al., 2003). Thus reduced activity of the BNST would inhibit anxiety-response reactions observed in CRHR2$^{CKO-5HT}$ mice. Interestingly the BNST as well as the CeA have been shown to project to the DRN (Fox and Lowry, 2013;Ogawa et al., 2014;Weissbourd et al., 2014). For instance the CeA is sending axons to the DRVL which could in turn modulate activity of the serotonergic system by means of CRH release and its interaction with CRH receptors, most likely CRHR1.

Taken together, we found changes in tissue content of 5HT, 5HIAA or their ratio support the behavioral phenotype observed in CRHR2$^{CKO-5HT}$ mice. These data also confirm that the deletion of CRHR2 in serotonergic neurons deeply affects the physiology of the serotonergic system on functional level. Thus pharmacological, raphe targeted, inhibition of CRHR2 might represent an attractive strategy for developing further medication aiming to treat anxiety and mood spectrum disorders.

5.3 Activation of CRHR2 in the DRN decreases active stress coping behavior

In order to study effects of activation of CRHR2 in the DRN we injected a selective CRHR2 agonist, i.e. mUCN2, into the ventral part of the dorsal raphe nucleus. It has been shown that the DRV subnucleus contains the highest amount of double positive CRHR2/TPH2 neurons (Day et al., 2004;Lukkes et al., 2008). We found that mice injected with mUCN2 display increased passive stress coping behavior in the FST. It has been shown that activation of CRHR2 on serotonergic neurons of the DRN increases their activity in vitro and most likely serotonin release in vivo (Kirby et al., 2000;Lowry et al., 2000). Indeed, the direct infusion of mUCN2 into the DRN increases expression of c-Fos protein in serotonergic neurons of the DRN and stimulates release of serotonin in the basolateral amygdala (Amat et al., 2004). Moreover, microinjection of CRH into the DRN was shown to promote freezing behavior and enhance immediate 5HT release in the CeA and delayed release in the mPFC (Forster et al., 2006). Interestingly, increased septal serotonergic neurotransmission was also negatively correlated with active stress coping behavior in the FST (Kirby and Lucki, 1997). These results are also in positive relation to our previous data obtained from CRHR2$^{CKO-5HT}$ mice, devoid of CRHR2 exclusively in serotonergic neurons, which show increased active stress coping behavior in the FST. Summing up this experiment we showed that the acute activation of raphe CRHR2 promotes a state of behavioral despair.

5.4 UCN2 in the LC as a possible agonist of CRHR2 in the DRN

Previous studies reported that the locus coeruleus projects to the raphe nucleus (Kim et al., 2004;Samuels and Szabadi, 2008). By injecting a virus carrying a cre-activate GFP-synaptophysin fusion protein into Nat-Cre mice we were able to confirm those previous results (Chen et al., 2006;Reyes et al., 2001). We found noradrenergic projections of LC neurons in all subregions of the DRN. Taking into account that the LC was shown to be an endogenous site of UCN2 expression, we assume that released at the axon terminals UCN2 could indeed activate CRHR2 in the DRN in vivo. Based on these results we aimed at solving a possible role of UCN2 in the LC on CRHR2 function in the DRN and its relation to emotional behavior.

5.4.1 Generation and characterization of UCN2-COE^{NE} mice

To study effects of UCN2 expression in the LC and its possible interactions with CRHR2 in the RNs we generated UCN2-COE mice. In UCN2-COE^{NE} mice UCN2 is overexpressed in the noradrenergic neurons of the LC only. We confirmed increased mUCN2 gene expression in the LC of

UCN2-COE[NE] mice by radioactive in situ hybridization with a mUCN2 specific riboprobe. In addition we showed restricted expression of the transgene by detecting activity of the β-galactosidase reporter expressed along with mUCN2 gene in the LC.

Under non-stress conditions we did not find significant changes in anxiety-related parameters in the open field, elevated plus maze and dark-light box tests. Nevertheless, we observed decreased general locomotion in UCN2-COE[NE] mice. Interestingly, in the forced swim test we found a significant decrease in active stress coping behavior in UCN2-COE[NE] mice, which was further exaggerated during re-exposure 24 hours later. All together, these results might indicate that UCN2-COE[NE] mice display signs of behavioral despair which can be enhanced by stress exposure.

Indeed animals exposed to chronic stress, as during chronic social defeat stress, often display decreased general locomotion similarly to UCN2-COE[NE] mice (Sterlemann et al., 2008). Decreased exploratory behavior is also believed to be a sign of behavioral despair (Cryan and Holmes, 2005). Importantly, decreased time spent swimming in the UCN2-COE[NE] model during the FST closely resembles outcomes from our former experiment where we stimulated CRHR2 in the DRV by acute injection of mUCN2 indicating the possibility of similar mechanisms mediating observed effects, i.e. changed 5HT neurotransmission. As it was discussed before, a decreased time spent swimming is correlated with increased release of serotonin to target structures and might be achieved by direct stimulation of raphe CRHR2 (Amat et al., 2004). Additionally, impaired active stress coping behavior in UCN2-COE[NE] mice is obvious and as expected in opposite to mice deficient in UCN2, which display increased active stress coping behavior (Chen et al., 2006). Nevertheless, taking into account the fact that UCN2-COE[NE] mice show under basal conditions decreased general locomotion, which might be related to decreased expression of tyrosine hydroxylase or altered physiology of the noradrenergic system. It is debatable whether observed effects are indeed caused by primary activation of CRHR2. The molecular characterization of the LC of UCN2-COE[NE] mice could shed light to this problematic circumstance, but does not provide explicit explanations. Changes in expression of TH or activity of the noradrenergic system might either be the primary source of observed effects, i.e. decreased locomotion and active stress coping behavior in the FST, or secondary to changed serotonergic neurotransmission in the DRN, i.e. enhanced stimulation of raphe CRHR2 by overexpressed UCN2, which in turn decreases the LC activity. Indeed the DRN and LC are reciprocally connected what means that both are able to communicate among each other and in response to changes in their neurophysiology initiate adaptive mechanisms (Fink and Gothert, 2007;Kim et al.,

2004;Matsumoto et al., 1995;Samuels and Szabadi, 2008;Weissbourd et al., 2014). To further solve these questions we currently continue characterizing UCN2-COE^{NE} mice in terms of activity and possible compensatory effects in noradrenergic, serotonergic and CRH/UCN systems.

5.4.1.1 Stress increased anxiety-related behavior of Ucn2-COE^{NE} mice

Lack of changes in anxiety-related behavior of UCN2-COE^{NE} mice under basal condition raised our concerns that simple overexpression of a neuropeptide might not guarantee its enhanced release in non-stress conditions. Both, available literature and our own observations seem to confirm that hypothesis. For instance, it was described that immobilization prior to septal UCN2 administration promotes increased anxiety-related behavior (Henry et al., 2006). Second, mice lacking both, UCN1 and UCN2, display significant decrease in anxiety-related behavior after stress exposure indicating importance of urocortins in recovery processes (Neufeld-Cohen et al., 2010a). Therefore, an independent group of animals was exposed to immobilization stress preceding behavioral testing in the next set of experiments. Indeed, stressed UCN2-COE^{NE} mice showed potent increase in anxiety-related behavior when they had been immobilized before. Interestingly, when the same animals were let undisturbed before testing, no change in anxiety-related behavior was detected. In addition, also during this round of testing we found a robust decrease in active stress coping behavior of UCN2-COE^{NE} mice in the FST and extended these results by the tail suspension test. To further investigate additional features of anhedonia, which do not depend on locomotor activity of animals, we performed sucrose preference test. The sucrose preference test has been shown to be a sensitive indicator of an anhedonic state in animals. Rodents naturally prefer sweet treats and liquids, but this preference might be diminished during periods of stress due to decreased activity of the reward system. Such rodent endophenotype is thought to resemble features of depression in humans (Cryan and Holmes, 2005;Krishnan and Nestler, 2011). Over the period of testing the amount of sucrose consumed by UCN2-COE^{NE} mice was tending to be decreased. This outcome suggests that observed phenotypic changes in UCN2-COE^{NE} mice are true signs of a behavioral despair state and not secondary, unspecific effects due to a decrease in general locomotion.

Stress is known to promote anxiety-related behaviors in animals (Reul et al., 2000). The proper stress recovery is an important process aiming to establish new physiological and psychological balance when the stressor is over. Inadequate stress recovery increases the risk of mental disorders including, depression and anxiety disorders (McEwen, 2002). In our model UCN2-COE^{NE} mice show evident signs of behavioral despair which are significantly exaggerated by stress

exposure. In these animals a prolonged or intensified psychological stress response increases anxiety-related behavior and promotes depression-related phenotypes. We assume that mechanisms standing behind these effects are mediated by release of excess UCN2 from the noradrenergic projections of the LC terminating in the DRN what leads to overactivation of CRHR2. We previously showed dichotomy in behavioral/emotional effects mediated by CRHR2 using genetic CRHR2^{CKO} mouse models. In line with most studies, general activation of CRHR2 is linked to anxiolysis and proper stress recovery, as it has been shown for constitutive CRHR2 KO mice (Bale et al., 2000;Issler et al., 2014;Kishimoto et al., 2000). We were able to show that these anxiolytic effects are mediated by stimulation of CRHR2 in GABAergic, most likely long projecting neurons, since mice devoid of CRHR2 in those neurons display increased anxiety-related behavior. Nevertheless, the function of CRHR2 in serotonergic neurons of the raphe nucleus is in obvious contrast to its previously reported role in GABAergic or Camk2a positive neurons. We found that selective deletion of CRHR2 in serotonergic neurons significantly decreases anxiety-related behavior and improves stress coping behavior. Similar effects were observed by pharmacological inhibition of CRHR2 in DRN and MnR (Hammack et al., 2003b;Ohmura et al., 2010). Long lasting overactivation of CRHR2 in DRN, as most likely occurring in UCN2-COE^{NE} mice or during periods of chronic stress in humans, might produce secondary effects like changed dynamics of the serotonergic system, expression of serotonergic receptors in DRN and target structures as well as impairments in proper function of other neurotransmitter systems, i.e. noradrenergic and dopaminergic circuits. Taken together, all these alterations finally increase the risk of depression or anxiety disorders, what is resembled in behavioral phenotypes of UCN2-COE^{NE} mice. Although some questions still require answers, our results underscore the important interaction between the neuropeptidergic stress system and classic monoaminergic neurotransmission. Such interplay between CRHR2, UCN2 and 5HT opens new venues to improve available antidepressant and anxiolytic treatments in the future, what is of particular importance for drug-resistant patients.

6 Conclusions

The presented study aimed to elucidate roles of CRHR2 and its endogenous ligand UCN2 in anxiety and mood disorders. Using a conditional approach we showed that the deletion of CRHR2 from the entire central nervous system results in mildly increased anxiety-related behavior and decreased active stress coping behavior. A similar phenotype was found in animals devoid of CRHR2 exclusively in GABAergic neurons. We showed that long-lasting disruption of CRHR2 signalling in GABAergic neurocircuitries hypersensitizes the HPA axis reactivity to chronic social defeat stress but excess of circulating corticosterone does not contribute to increased anxiety-related behavior. Interestingly, deletion of CRHR2 in Camk2a expressing neurons during adulthood, revealed its essential role in modulation of anxiety-related behavior but not of stress coping behaviour or the HPA axis function. These data support the original notion that activation of CRHR2 is primarily serving anxiolytic effects and is crucial for proper stress coping.

In contrast to these, conditional CRHR2 mouse lines (CRHR2^{CNS}, CRHR2$^{CKO\text{-}GABA}$ and CRHR2$^{CKO\text{-}Camk2a}$) deletion of CRHR2 in serotonergic neurons of raphe nuclei significantly decreased anxiety-related behavior and contextual fear expression as well as increased active stress coping behavior. Furthermore, CRHR2$^{CKO\text{-}5HT}$ mice showed increased social preference, which was sustained in chronically stressed animals. Behavioral changes present in CRHR2$^{CKO\text{-}5HT}$ mice are particularly explained by underlying changes in general physiology of the serotonergic system. In addition, we found that selective removal of serotonergic CRHR2 disrupted the sleep architecture.

In order to complement this study we showed that the acute activation of CRHR2 in the DRV decreases active stress coping behavior, an effect opposite to the phenotype observed in CRHR2$^{CKO\text{-}5HT}$ mice. Moreover, we confirmed that the native site of UCN2 expression, i.e. locus coeruleus, projects to the DRN. By generation of UCN2-COE^{NE} mice, we corroborated behavioral despair of mutant animals.

Taken together our data provide evidence that CRHR2 plays diverse roles in modulation of mood anxiety-related behaviors and responses to stress. In particular opposite functions of CRHR2 are segregated between GABAergic and serotonergic neurotransmitter systems.

	CRHR2^{CKO-CNS}	CRHR2^{CKO-GABA}	CRHR2^{CKO-Camk2a}	CRHR2^{CKO-5HT}	UCN2-COE^{NE}
Anxiety-related behavior	↑/↔	↑/↔	↑↑	↓↓↓	↑↑ after stress
Active stress coping behavior	↓↓↓	↓	↔	↑↑↑	↓↓↓
The HPA axis	n.a.	↑ after stress	↔	↔	n.a.

Table 2. Behavioral phenotypes of conditional CRHR2 knockout and UCN2 overexpressing mouse lines

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Acknowledgements

Curriculum Vitae

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Scientific conferences and publications

- Society for Neuroscience annual meeting 2014 (SfN), **Poster presentation**

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1) **Activation of CRFR2 in serotonergic neurons induces a behavioral despair state and anxiety-like responses in mice (working title).**

Kolarz A, Donner N, Kumar D, Dedic N, Issler O, Flachskamm C, Schieven M, Wotjak C, Kimura M, Wurst W, Lowry CA, Chen A, Deussing JM.

Manuscript in preparation

2) **Behavioral phenotyping of Nestin-Cre mice: implications for genetic mouse models of psychiatric disorders.**

Giusti SA, Vercelli CA, Vogl AM, **Kolarz A**, Pino NS, Deussing JM, Refojo D.

J Psychiatr Res. 2014 Aug;55:87-95.

3) **The amphetamine-chlordiazepoxide mixture, a pharmacological screen for mood stabilizers, does not enhance amphetamine-induced disruption of prepulse inhibition.**

Douma TN, **Kolarz A**, Postma Y, Olivier B, Groenink L.

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4) **Tail suspension test does not detect antidepressant-like properties of atypical antipsychotics.**

Wesołowska A, Partyka A, Jastrzębska-Więsek M**, Kolarz A**, Mierzejewski P, Bieńkowski P, Kołaczkowski M.

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