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CROSS-TALK OF THE CORTICOTROPIN RELEASING HORMONE RECEPTOR SUBTYPE 1 WITH THE DOPAMINE SYSTEM:

Functional Evidence on Emotional Responses to Stress and Alcohol Dependence

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“ I love science,

and it pains me to think

that so many are terrified of the subject

or feel that choosing science means

you cannot also choose compassion,

or the arts, or be awed by nature.

Science is not meant to cure us of mystery,

but to reinvent and reinvigorate it. “

Robert M. Sapolski

ABSTRACT

The concept of "stress" is often associated with unpleasant feelings, even though it is the major trigger of the evolutionary processes. The refined system responsible for the regulation of stress in animals as well as humans controls the release of Corticotropin Releasing Hormone (CRH) in the brain. In the presence of threats, physiological reactions are activated as fast as deactivated once the stressful stimulus has vanished. However, prolonged stimulation of the CRH system leads to the development of anxiety-associated disorders. Increasing evidence indicates that the dopamine (DA) system plays a role in the modulation of emotional responses. The vulnerability to stress is not only a crucial characteristic of anxiety-related diseases, but it is also a major component underlying different stages in the development of addiction. Indeed, alcohol dependence is a chronic-relapsing disease which induces long-term neuroadaptations that recruit a negative emotional state leading to excessive alcohol ingestion motivated by relief of negative emotionality.

Thus, this PhD thesis aims to establish the cell-specific involvement of the CRH receptor subtype 1 (CRHR1) in the regulation of emotional responses to stress under healthy conditions and in alcohol dependence.

Specifically, Study 1 demonstrates that the amygdala-specific increase in D1 binding sites upon pharmacological activation of CRHR1 is accompanied by an augmented anxiety-like behavior, which is abolished by pretreatment with a D1 antagonist. Further experiments using mice lacking CRHR1 expression in D1-containing neurons revealed that a D1-CRHR1 interaction is due to co-localized receptors, and bioluminescence resonance energy transfer (BRET) assay suggests that D1 and CRHR1 forms heterocomplexes. Together these results demonstrate that amygdala CRHR1 acts via D1 to mediate anxiety-like responses. In alcohol dependence, a maladaptive interaction between the two receptors seems to underlie the increased vulnerability to stress, a hallmark of the post-dependent (PD) phenotype that subsequently triggers stress-induced relapse to alcohol drinking (Study 2). Study 3 reveals that CRHR1 is differentially involved in stress-induced alcohol drinking whether it is expressed in DAT- or D1- neurons. These findings are supported by the anatomical separation within the VTA of D1-containing neurons, expressed in the dorsal portion, and the dopaminergic neuronal population distributed in the ventral part, and both co-localized with CRHR1. The cell-specific impact of CRHR1 in stress-induced alcohol consumption is further investigated in Study 4. Virus-induced αCaMKII-dependent over-expression of the receptor in the central nucleus of the amygdala (CeA) resulted in an increased alcohol-seeking behavior during stress-, but not cue- induced reinstatement in transgenic rats. Finally, an opposite functional impact of D1-CRHR1 and dopaminergic CRHR1 has been established for cue-induced reinstatement of cocaine seeking behavior using two double transgenic mouse lines lacking CRHR1 in D1- and DAT-expressing neurons, demonstrating that the modulation of relapse-like behavior for cocaine is related to the neuron-specific expression of CRHR1 (Study 5).

In summary, the results presented in this thesis demonstrate that an interaction of D1 and CRHR1 receptors within the amygdala functions as a novel mechanism involved in the regulation of the emotional responses to stress under healthy condition. However, during alcohol dependence, the maladaptive functionality of the D1-CRHR1 interaction results in a hyper-responsivity to stress, leading to an increase in alcohol consumption as a relief to the negative affective state. Furthermore, our findings reveal the functional impact of dopaminergic and dopaminoceptive CRHR1 on stress-induced alcohol-seeking and cocaine-mediated behavior. In addition, this thesis gives support for the functional relevance of receptor-receptor interaction in stress and addiction-related processes, and thus the promise of the development of pharmacological compounds acting on these receptor-heterocomplexes as medication therapies.

ZUSAMMENFASSUNG

Der Begriff „Stress“ wird oft mit unangenehmen Gefühlen in Verbindung gebracht, obwohl es sich um den wichtigsten Auslöser der Evolution handelt. Das ausgeklügelte System, welches für die Regulierung von Stress in Tieren und Menschen verantwortlich ist, kontrolliert die Ausschüttung des Corticotropin-releasing Hormone (CRH) im Gehirn. Bei Gefahr werden physiologische Reaktionen aktiviert, und ebenso schnell auch wieder deaktiviert, sobald der Stress-verursachende Reiz verschwunden ist. Jedoch führt die anhaltende Anregung des CHR Systems zur Entwicklung von Angststörungen. Zunehmende Indizien deuten darauf hin, dass das Dopamin (DA)-System eine Rolle bei der Regulierung der emotionalen Reaktionen spielt. Die Anfälligkeit für Stress ist nicht nur eine wesentliche Eigenschaft der Angststörungen, sie ist auch ein wichtiger Bestandteil verschiedener Stadien in der Entwicklung von Sucht. Tatsächlich ist Alkoholabhängigkeit eine chronische, rezidivierende Krankheit, welche langfristige Veränderungen im Gehirn verursacht. Diese Veränderungen sorgen für ein negatives Gefühlserleben, was wiederum zu erhöhtem Alkoholkonsum sorgt, welcher diesem negativen Befinden entgegenwirken soll.

In dieser Doktorarbeit soll die zellspezifische Beteiligung des CRH Rezeptor Subtyp 1 (CRHR1) an der Regulierung der emotionalen Reaktionen auf Stress, sowohl unter normalen Bedingungen als auch in Alkoholabhängigkeit, ermittelt werden.

In Studie 1 wird gezeigt, dass die Amygdala-spezifische Zunahme an D1 Rezeptorbindungen durch pharmakologische Aktivierung des CRHR1 von verstärktem Angstverhalten begleitet wird, welches durch Vorbehandlung mit einem D1 Antagonisten verhindert wird. Weitere Experimente an Mäusen, die in D1-beinhaltenden Neuronen keinen CRHR1 exprimieren, zeigten, dass die Interaktion zwischen D1 und CRHR1 auf der Kolokalisierung der Rezeptoren basiert. Der Biolumineszenz-Resonanzenergietransfer (BRET)-Test deutet außerdem darauf hin, dass D1 und CRHR1 Heterokomplexe bilden. Zusammengenommen zeigen diese Ergebnisse, dass Amygdala-lokalisierte CRHR1 das Angstverhalten mithilfe von D1 vermitteln. In der Alkoholabhängigkeit scheint die verstärkte Stressanfälligkeit einer maladaptiven Interaktion der beiden Rezeptoren zu unterliegen, was charakteristisch für den „post-dependent“ (PD) Phänotyp ist. Dadurch lösen D1-CRHR1 vermutlich den stressinduzierten Rückfall in den Alkoholkonsum aus (Studie 2). Studie 3 deckt auf, dass CRHR1 unterschiedliche Aufgaben beim stressinduzierten Alkoholkonsum übernimmt, je nachdem ob es in Neuronen mit Dopamintransporter (DAT) oder D1 exprimiert wird. Diese Ergebnisse werden durch die anatomische Trennung von D1 Neuronen im dorsalen Teil des ventralen tegmentalen Areals (VTA) und dopaminergen Neuronenpopulationen im ventralen Teil der VTA unterstützt, welche beide jeweils mit CRHR1 kolokalisiert sind. Die zellspezifischen Effekte von CRHR1 im stressinduzierten Alkoholkonsum werden in Studie 4 weiter untersucht. Durch eine Virus-induzierte, αCAMKII abhängige Überexpression des Rezeptors im zentralen Kern der Amygdala (CeA) kommt es zu einem erhöhten Alkoholsuchverhalten bei stressinduziertem, aber nicht reizinduziertem Rückfall in transgenen Ratten. Letztlich wird eine gegensätzliche Funktion von CRHR1 auf D1-Neuronen und CRHR1 auf dopaminergen Neuronen gezeigt. Hierzu werden zwei doppelt transgene Mauslinien verwendet, welche kein CRHR1 in entweder D1- oder DAT-Neuronen exprimieren. Der reizinduzierte Rückfall in Kokainsuchverhalten zeigt, dass

dopaminerge und dopaminozeptive CRHR1 die Regulierung von Rückfall-ähnlichem Verhalten modulieren (Studie 5).

Zusammenfassend zeigen die Ergebnisse dieser Studien eine Interaktion von D1 und CRHR1 Rezeptoren innerhalb der Amygdala als einen neuen Mechanismus, durch den emotionale Stressreaktionen unter normalen Bedingungen reguliert werden. In Alkoholabhängigkeit führt eine fehlerhafte Interaktion von D1 und CRHR1 zu einer verstärkten Stressreaktion, welches wiederum zu erhöhtem Alkoholkonsum führt, durch den negative affektive Zustände gelindert werden sollen. Des Weiteren zeigen unsere Ergebnisse die funktionellen Auswirkungen von dopaminergen und dopaminozeptiven CRHR1 auf stressinduziertes Alkoholsuchverhalten und durch Kokain ausgelöstes Verhalten. Zusätzlich unterstützt diese Doktorarbeit die funktionelle Bedeutung der Rezeptor-Rezeptor-Interaktionen bei Stress und suchtbedingten Prozessen, und unterstreicht damit die Wichtigkeit der Entwicklung von pharmakologischen Präparaten mit Auswirkungen auf diese Rezeptor-Heterokomplexe.

TABLE OF CONTENTS

ABBREVIATIONS

AC	Adenylyl cyclase
ACTH	Adrenocorticotropic hormone
AcbC	Nucleus accumbens core region
AcbSh	Nucleus accumbens shell region
BL	Basal nucleus of the amygdala
BLA	Basolateral nucleus of the amygdala
BMA	Basomedial nucleus of the amygdala
BNST	Bed nucleus of the stria terminalis
BRET	Bioluminescence Resonance Energy Transfer
αCaMKII	Calcium/calmodulin-dependent protein kinase type II alpha chain
CeA	Central nucleus of the amygdala
CeL	Central nucleus of the amygdala-lateral portion
CeM	Central nucleus of the amygdala-medial portion
Cg	Cingulate cortex
CIE	Chronic intermittent exposure to alcohol vapor
CPM	Counts per minutes
CPu	Caudate putamen
CRH	Corticotropin releasing hormone
CRHR1	Corticotropin releasing hormone receptor subtype 1
CRHR2	Corticotropin releasing hormone receptor subtype 2
DA	Dopamine
DAG	Diacylglicerol
DAT	Dopamine transporter
D1	Dopamine receptor 1
D2	Dopamine receptor 2
GABA	Gamma-Aminobutyric acid
Glu	Glutamate
GPCR	G-protein coupled receptor
HPA	Hypothalamic-pituitary-adrenal
I.c.v	Intra-cerebroventricular injection
Idp	Dorsal paracapsular intercalated cell
IL	Infralimbic cortex
Ilp	Lateral paracapsular intercalated cell
Imp	Medial paracapsular intercalated cell
IN	Main nucleus of the intercalated cells
Ip	Intercalated paracapsular
Ip	Intra-peritoneal injection
IP_3	Inositol 1,4,5-Triphosphate
I.r.	Immuno-reactivity
ITC	Intercalated cell cluster
Ivp	Ventral paracapsular intercalated cell
LA	Lateral nucleus of the amygdala
LC	Locus coeruleus
M1	Motor cortex 1
MeA	Medial nucleus of the amygdala
PBP	Parabrachial nucleus
PFC	Prefrontal cortex
PIP_2	phosphatidyl inositol diphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
POMC	Propiomelanocortin
PrL	Prelimbic cortex

PVN	Paraventricular nucleus
RN	Raphe nucleus
SN	Substantia nigra
SNC	Substantia nigra pars compacta
SNR	Substantia nigra pars reticulata
Ucn	Urocortin
URO	Urotensin
VTA	Ventral tegmental area

PUBLICATIONS

1. R.E. Bernardi*, **L. Broccoli***, R. Spanagel, A.C. Hansson. *Sex differences in dopamine binding and modafinil conditioned place preference in mice*, Drug Alcohol Depend. 2015 Oct 1;155:37-44.
   *** authors contributed equally**

2. S. Uhrig, N. Hirth, **L. Broccoli**, M. von Wilmsdorff, M. Zink, J. Steiner, B. Malchow, P. Falkai, R. Spanagel, A.C. Hansson, A. Schmitt. *Reduced oxytocin receptor gene expression and binding sites in schizophrenia brain regions: a post-mortem study*. (Schizophrenia Research, invited publication)

3. N. Hirth, M.W. Meinhardt, H.R. Noori, H. Salgado, O. Torres-Ramirez, S. Uhrig, **L. Broccoli**, V. Vengeliene, M. Roßmanith, S. Perreau-Lenz, G. Köhr, W.H. Sommer, R. Spanagel, A.C. Hansson. *Convergent evidence from alcohol dependent humans and rats for a hyperdopaminergic state in protracted abstinence* (Under revision in PNAS)

4. **L. Broccoli** et al., *Dopamine D1 and Corticotropin Releasing Hormone Receptor subtype 1 interaction in emotional responses and alcohol dependence* (In preparation, Studies 1 and 2 in this thesis)

5. **L. Broccoli** et al., *Differential role of dopaminergic- and dopaminoceptive CRHR1 on stress-induced drinking behavior* (In preparation, Study 3 in this thesis)

6. **L. Broccoli** et al., *Dissociable role of CRHR1 on DAT and D1 neurons in cocaine cue-induced reinstatement* (In preparation, Study 6 in this thesis)

7. **L. Broccoli** et al., *αCaMKII-neuron specific CRHR1 modulates stress-induced alcohol seeking behavior* (In preparation, Study 4 in this thesis)

8. **L. Broccoli et al.,** *Dopamine D1 receptor activity is involved in the increased anxiety levels observed in STZ-induced diabetic rats* (In preparation)

PART I: INTRODUCTION

"Stress" is an intriguing word with numerous different meanings. Although it has a commonly-used negative connotation, which associates stress with unpleasant feelings, it is the major trigger of the evolutionary process. Indeed, all animals react to threatening stimuli through many physiological responses that help them to overcome stressful events. The system responsible for the regulation of stress in both animals and humans controls the release of Corticotropin Releasing Hormone (CRH) in the brain. Indeed, the CRH system responds perfectly to acute physical stressors: in the presence of immediate, real threats, "fight-or-flight" physiological reactions are activated and deactivated quickly upon the onset and termination, respectively, of a stressful stimulus. However, constantly worrying about non-life-threatening stressors, a peculiar human behavior, can have devastating consequences for the health: a prolonged activation of the CRH system causes impairment of the immune-system, depression, anxiety and other mental health disorders. Thus, as Dr. Sapolski stated during a lecture at the Stanford University: "*We've evolved to be smart enough to make ourselves sick*". The vulnerability to stress is not only a crucial characteristic of anxiety-related diseases, but is also a major component underlying different stages in the development of addiction (e.g. withdrawal, drug-seeking and relapse). For this reason this thesis aims to provide further insight into the molecular mechanisms regulating the responses to stressful stimuli under healthy conditions and dependence to drugs of abuse (e.g. alcohol and cocaine).

1.1 The Corticotropin Releasing Hormone (CRH) system

1.1.1 Brief history of the discovery of the CRH system

The CRH was first isolated by Vale and colleagues in the 1981 from the ovine hypothalamus (Vale et al., 1981) (Figure 1). However, only since the creation of antibodies directed to this molecule (Rivier et al., 1982) has it been possible to map the distribution of CRH in the rat brain (Bloom et al., 1982; Swanson et al., 1983). Pharmacological experiments performed on different animal models demonstrated the pivotal role of CRH in orchestrating functional responses to stress and maintaining homeostasis (Fisher et al., 1982; Sutton et al., 1982; Britton et al., 1986; Tazi et al., 1987). These findings were confirmed by clinical studies demonstrating elevated CRH levels in the brains of subjects with mental health disorders (Gold et al., 1984; Gold et al., 1986). Together, these results launched a new era focusing on drug targets in the pharmaceutical treatment of affective disorders and other stress-related diseases (Nemeroff et al., 1984; Nemeroff, 1998). Simultaneously,

the CRH receptors (CRHR) were discovered and characterized, giving further insight into the distribution, pharmacology and signaling pathway of the CRH system (De Souza and Van Loon, 1984; De Souza et al., 1985; Chen et al., 1986; Perrin et al., 1986). With the development of better molecular techniques, it was possible to identify more ligands belonging to the CRH family, such as urocortin (Ucn) I, Ucn II, Ucn III (Vaughan et al., 1995; Lewis et al., 2001; Reyes et al., 2001) and a second receptor, the CRHR2 (Chen et al., 2005). To date, a great deal of effort has been spent to better characterize the impact of the CRH system on the regulation of the responses to stressful stimuli under different physiological conditions (Lightman, 2008; Kovacs, 2013).

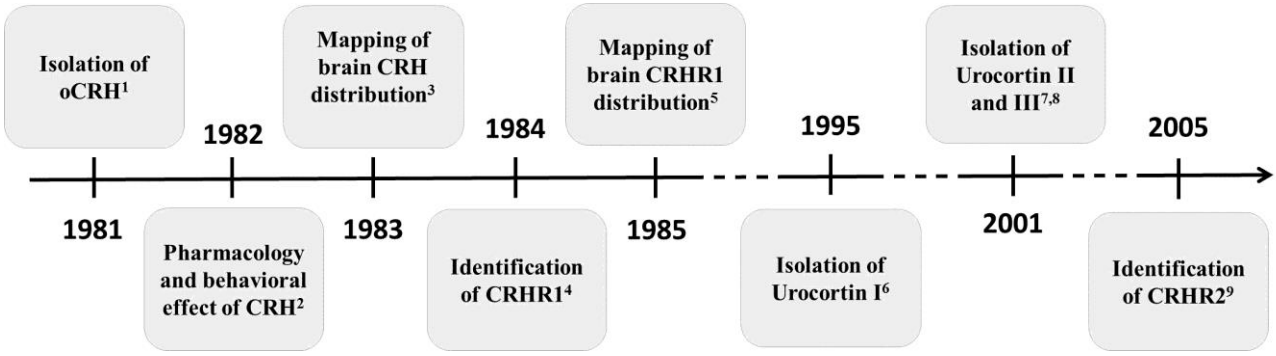

Figure 1: Timeline of the discovery of the Corticotropin-Releasing Hormone (CRH) system. Since the isolation of the ovine CRH, occurred at the beginning of the 80s (Vale et al., 1981), the quick identification of the CRH system and its involvement in stress responses launched a new era focused on the characterization and the treatment of stress-related disorders. 1. *Vale et al., 1981*; 2. *Sutton et al., 1982*; 3. *Bloom et al., 1983*; 4. *DeSouza et al., 1984*; 5. *DeSouza et al., 1985*; 6. *Vaughan et al., 1995*; 7. *Lewis et al., 2001*; 8. *Reyes et al., 2001*; 9. *Chen et al., 2005*.

1.1.2 The CRH and the other members of the family

CRH, also called corticotropin releasing factor (CRF), is a polypeptide composed of 41 amino acids (Vale et al., 1981), and is highly conserved through different mammalian species (Dautzenberg and Hauger, 2002). The C-terminal of the neuropeptide interacts with the extracellular binding pocket of the CRH receptor, while the N-terminal contacts other sites on the receptor to initiate intra-cellular signaling (Hoare et al., 2004). The protein structure is constituted by an alpha-helix (in residue 6-36) with an amphipathic portion (between the residue 6 and 20) (Dathe et al., 1996). It is obtained by cleavage of the C-terminal of a 196 amino-acid precursor. Human and rat CRH (h/rCRH) shared the same protein structure, but they differ from the ovine CRH (oCRH, Figure 2). The CRH family consists of other related molecules that are expressed in the mammalian brain and share a certain level of homology (Figure 2) (Dathe et al., 1996). The first one to be identified was Ucn I, a 40

amino acid protein (Vaughan et al., 1995). Its name derives from the homologous urotensin (URO), a neuropeptide discovered in fish (Alderman and Bernier, 2007). To date, two different homologous of Ucn I have been isolated from human, rat, mouse and sheep, called Ucn II and Ucn III. CRH and Ucn genes contain two exons, with the second one encoding the entire precursor protein (Zhao et al., 1998).

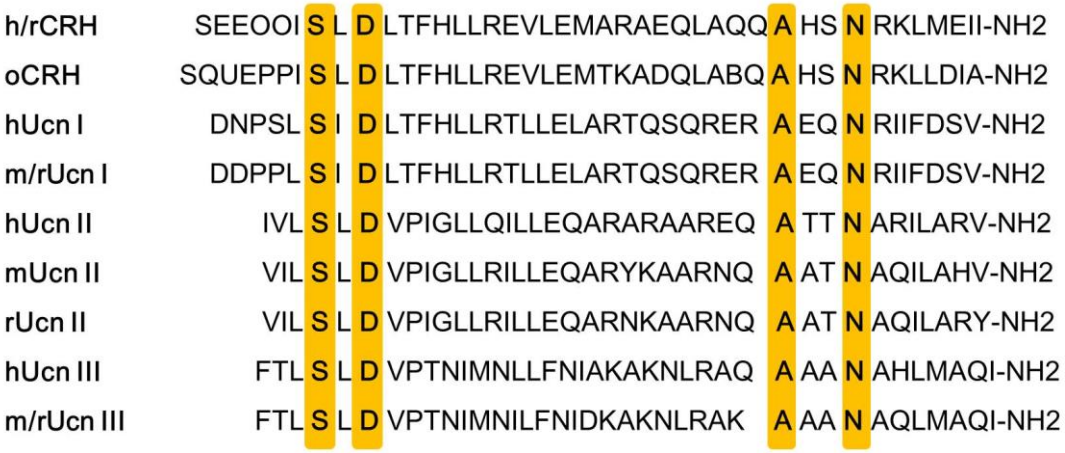

Figure 2: Alignment of the CRH and the other members of the family. The protein sequences of CRH and Ucn polipeptides reveals a certain degree of homology within humans (h), rats (r), mice (m) and ovine (o). Homologous residues are highlighted in orange.

Immunohistochemical and radioimmunoassay techniques helped to map the anatomical distribution of CRH in the central nervous system (CNS) (Owens and Nemeroff, 1991). Consistent with its functional role in the endocrine and physiologic regulation of the responses to stressful stimuli, CRH mRNA and protein are highly concentrated in the paraventricular nucleus (PVN) of the hypothalamus, bed nucleus of the stria terminalis (BNST), central amygdala (CeA), and locus coeruleus (LC) (Merchenthaler et al., 1982; Swanson et al., 1983; Morin et al., 1999). Furthermore, CRH-containing fibers are distributed within the lateral septum (LS), raphe nucleus (RN), and spinal cord (Morin et al., 1999; Korosi et al., 2007). CRH is also expressed peripherally in blood vessels, skin, lung, testes, ovaries and placenta (Di Blasio et al., 1997; Saeed et al., 1997; Baigent, 2001).

Functionally, this neuropeptide mediates endocrine, autonomic, behavioral and immune responses to stress (Dunn and Berridge, 1990; De Souza, 1995), as well as anxiety-like behavior, arousal, food intake and learning and memory (Smagin et al., 2001).

1.1.3 The CRH receptors

The biological effects of CRH neuropeptide are mediated by its specific receptors: the CRH receptor subtypes 1 (CRHR1) and 2 (CRHR2). The *Crhr1* gene, subdivided in 13 exons, encodes a 415 amino-acid protein (De Souza and Van Loon, 1984; Perrin et al., 1986; Ross et al., 1994; Grammatopoulos, 2012), while the *Crhr2* locus contains 15 exons encoding three different isoforms: alpha (411 amino acids), beta (438 amino acids), and gamma (397 amino acids) (Valdenaire et al., 1997; Catalano et al., 2003). Both receptors belong to the class B of G-protein coupled receptors (GPCRs), consisting of seven transmembrane domains, three extracellular loops that bind to CRH and three intracellular domains involved in the signal transduction. More than 80% of the structure of both CRHR1 and CRHR2 is conserved between species: the highest degree of homology between the receptors is found in the seven transmembrane domains and in the three intracellular loops, while the total similarity is reached in the coupling site (Dautzenberg et al., 1999). However, CRHR1 and CRHR2 differ from each other in their selectivity to bind the various CRH analogues. UcnI binds to CRHR2 with a higher affinity than CRHR1, while CRH has considerably higher affinity to (Vaughan et al., 1995; Donaldson et al., 1996; Dautzenberg et al., 2001; Smagin et al., 2001) (Table 1). Furthermore, UcnII and UcnIII seem to be specific ligands for CRHR2 (Lewis et al., 2001; Reyes et al., 2001).

Table 1: Main ligand of the CRH system and their affinity with CRHR1 and CRHR2.

Peptide	CRHR1 (Ki)	CRHR2 (Ki)
CRH	0.95 nM	13 nM
UcnI	0.16 nM	0.5 nM
URO	0.43 nM	3.4 nM

CRHR1 and CRHR2 are both expressed in numerous brain regions, such as the bed nucleus of the stria terminalis (BNST), amygdala, paraventricular nucleus (PVN), ventral tegmental area (VTA), substantia nigra (SN), hippocampus, periaqueductal gray (PAG), and olfactory bulb (OB). However, CRHR1 alone has been found in high concentrations in the intermediate lobe of pituitary (IPit), cerebral cortex and cerebellum (Chalmers et al., 1995; Sanchez et al., 1999)(Figure 3), while CRHR2 is strongly expressed in the lateral septum (LS), posterior lobe of the pituitary (PPit), and ventromedial hypothalamic nucleus (VMH, Figure 3) (Chalmers et al., 1995; Sanchez et al., 1999). CRH receptors are also expressed in the periphery. CRHR1 expression occurs in the testis, ovaries,

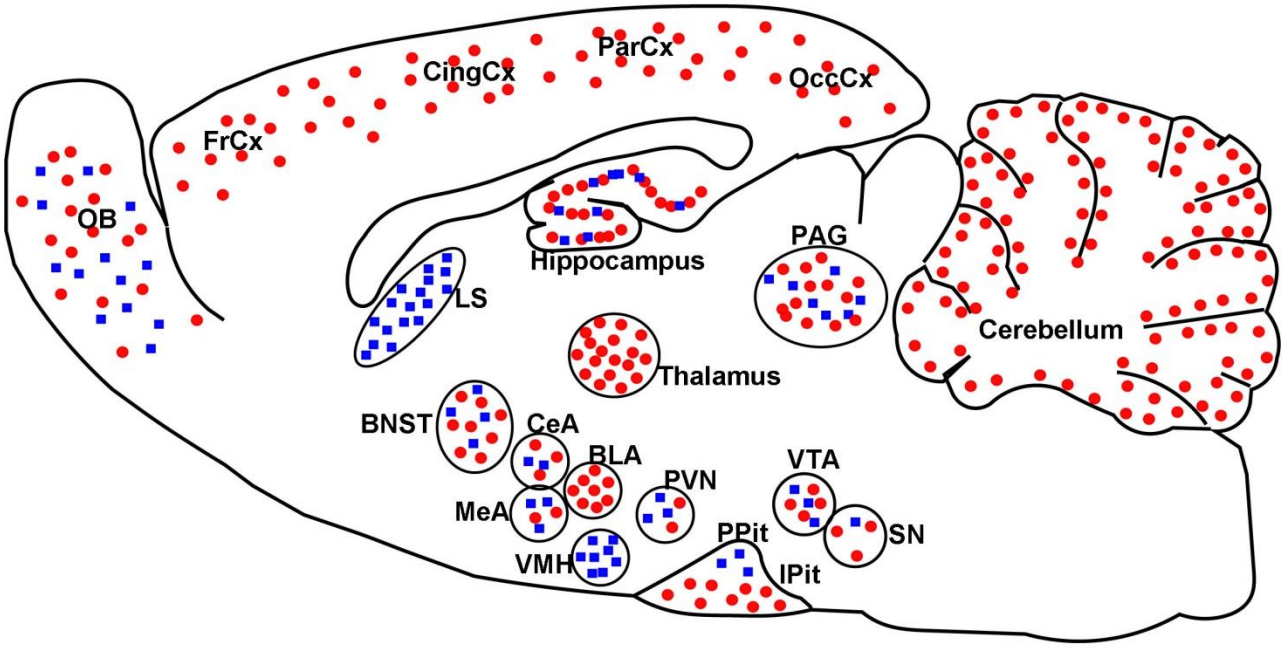

Figure 3: Map of CRHR1 and CRHR2 distribution in the rodent brain. Adapted from (Janssen and Kozicz, 2013). Red dots represent CRHR1, while blue squares represent CRHR2. Abbreviations: BLA, basolateral amygdala; BNST, bed nucleus stria terminalis; CeA, central amygdaloid nucleus; CingCx, cingulate cortex; FrCx, frontal cortex; IPit, intermediate lobe of the pituitary; LS, lateral septum; OB, olfactory bulb; OccCx, occipital cortex; PAG, periaqueductal gray area; ParCx, parietal cortex; PPit, posterior pituitary; PVN, paraventricular nucleus of the hypothalamus; SN, substantia nigra; VMH, ventromedial hypothalamic nucleus.

and adrenal glands (Palchaudhuri et al., 1998), while CRHR2 mRNA has been found in cardiac myocytes, gastrointestinal tract, lungs, ovaries, and skeletal muscles (Coste et al., 2000). Stimulation of either CRHR1 or CRHR2 by CRH and CRH-related peptides triggers activation of adenylyl cyclase and an increase in cAMP through the activation of the stimulatory GTP binding protein (Gs). However, several studies have demonstrated multiple G-protein activation by CRH receptors such as Gq and Gi (Grammatopoulos and Chrousos, 2002). Coupling of the α subunit of the Gs protein to the third intracellular loop of the receptor stimulates adenylyl cylclase activity which, in turn, produces an increase in cyclic AMP (cAMP). This second messenger activates Protein Kinase A (PKA), which phosphorylates downstream enzymes (ERK, MERK) and transcription factors (CREB, AP-1 and c-Fos).

1.1.4 The CRH system and stress

In response to acute stress challenges, the brain activates different neuronal circuits to adapt to the threatening situation. The endocrine responses to stress are driven by the release of CRH in the

hypothalamic-pituitary-adrenocrotical (HPA) axis (Plotsky and Vale, 1985). CRH, produced in the paraventricular nucleus (PVN), acts on pituitary corticotropes to stimulate propiomelanocortin (POMC) synthesis and, in turn, adrenocorticotrophic hormone (ACTH) is secreted into the blood. ACTH binds receptors in the adrenal gland cortex to activate the synthesis and release of glucocorticoids. Glucocorticoids mediate a variety of physiological and metabolic responses that are important for the survival of the organism, such as the increase of cardiovascular tone and blood pressure, the enhancement of immunity, and energy provision to the muscles (Seasholtz, 2000).

However, numerous studies have provided strong evidence supporting the involvement of CRH system in the responses to stressful stimuli through the activation of extra-hypothalamic pathways. CRH-driven fear responses were abolished by disruption of the central nucleus of the amygdala (CeA), but not the PVN (Liang et al., 1992), and peripheral blockade of ACTH and glucocorticoids production failed to affect the behavioral response to stress (Pich et al., 1993). Furthermore, CRH-induced locomotion activity was abolished in adrenalectomized animals (Korte et al., 1992). The role of CRH in the amygdala has been confirmed by preclinical studies demonstrating an augmented release of CRH, as well as upregulated expression of CRH mRNA, in the amygdala (Kalin et al., 1994; Merlo Pich et al., 1995; Merali et al., 1998; Muller et al., 2003)

The role of CRH in the regulation of anxiety-related behaviors is well established and several lines of evidence point to the participation of CRHR1 in these effects. Firstly, CRHR1 binds CRH with high affinity (see Table 1). Secondly, pharmacological treatment with CRHR1 antagonists (e.g. antalarmin, D-phe-CRH) reduces anxiety-related behavior when systematically or region specifically injected (Valdez et al., 2002; Zorrilla et al., 2002; Finn et al., 2007; Vicentini et al., 2014; Ettenberg et al., 2015). These findings were confirmed by the creation of CRHRl-deficient mice, as these knockout animals displayed decreased anxiety-related behavior (Muller et al., 2003; Refojo et al., 2011). Although numerous studies have demonstrated the involvement of a dysregulation of the HPA axis in the development of anxiety-related diseases (Steckler and Holsboer, 1999; Holsboer, 2000) the focus of this PhD thesis is on the role of the extra-hypothalamic CRH system.

All relevant functions in the “fight or flight” responses and in anxiety-related disorders are primarily driven by CRHR1. Indeed, CRHR2 appears to act opposite to CRHR1, mediating anxiolytic responses: CRHR2-deficient mice shows sensitivity to stress (Bale et al., 2000) and stress-induced drug consumption is prevented by CRHR2 activation (Valdez et al., 2004). Nevertheless, a role for CRHR2 in the response to stressful stimuli cannot be excluded, as studies aiming to disentangle its involvement in stress responses have shown contradictory results, pointing

to a context- and brain region- dependent function. For example, two lines of CRHR2-deficient mice showed an increase in anxiety-related behavior (Bale et al., 2000; Kishimoto et al., 2000) while, in contrast, Coste and colleagues did not find any changes in a third knock-out line (Coste et al., 2000).

1.2 The dopaminergic system

1.2.1 Brief history of the discovery of the dopaminergic system

Dopamine (DA) was first synthesized in 1910. However, its importance emerged only in the mid-1950s, when Arvid Carlsson (1957) identified dopamine as a fundamental neurotransmitter (Carlsson et al., 1957), a finding that resulted in his being awarded the Nobel Prize for Medicine in 2000, together with Paul Greengard and Eric Kandel, who characterized the physiological and functional effects of DA (Kebabian and Greengard, 1971) and its impact on short-term memory (Kandel and Spencer, 1968), respectively (Figure 4). With the development of fluorescent histochemical methods in the 1960s, Fuxe and Dahlstrom provided the first evidence of DA-containing pathways that project from the substantia nigra to the striatum, and the VTA to the limbic areas of the rat brain (Dahlstrom et al., 1965; Anden et al., 1966). During subsequent years, Kebabian and coworkers demonstrated the presence of receptors for DA by showing the effect of dopamine on the stimulation of adenylyl cyclase activity (Kebabian et al., 1972) and in 1979 published an important paper classifying the dopamine receptors into D1 and D2 based on their pharmacology (Kebabian and Calne, 1979). Later, the development of advanced experimental tools and the discovery of novel pharmacological molecules allowed further investigating of the impact of the dopamine system on behavioral responses, as well as in pathological diseases such as Parkinson’s, Alzheimer’s, anxiety-related disorders, and drug-addiction.

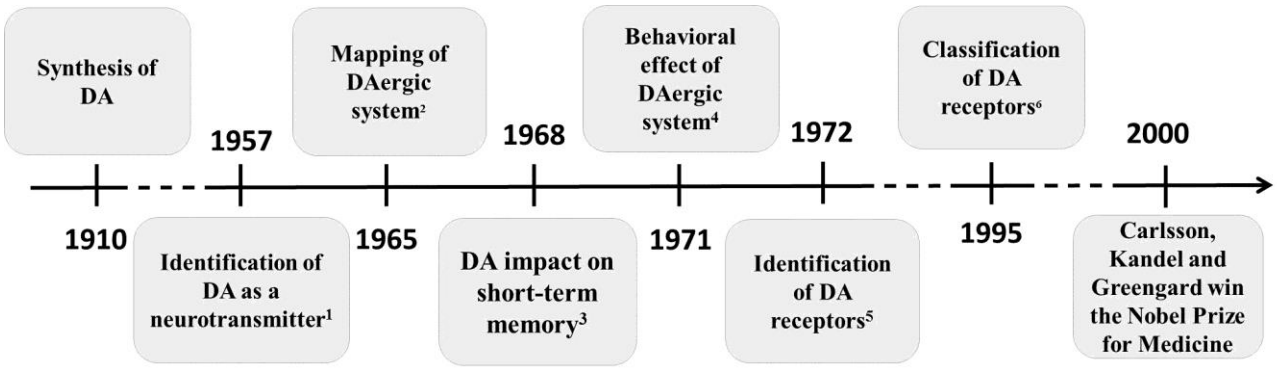

Figure 4: Timeline of the discovery of the Dopaminergic system. Even though dopamine (DA) was identified since the early 20th century, it took until the end of 1950´s to consider it as a neurotransmitter and to characterize the dopaminergic (DAergic) system. 1. *Carlsson et al., 1957*; 2. *Dahlstrom et al., 1965*; 3. *Kandel and Spencer, 1968*; 4. *Kebabian and Greengard, 1971*; 5. *Kebabian et al., 1972*; 6. *Kebabian and Calne, 1995*.

1.2.2 The dopaminergic pathways

The dopamine system is highly involved in the regulation of movement and reward (Salgado-Pineda et al., 2005), as well as in the modulation of cognition and emotional responses to fear (Guarraci et al., 1999). Dysregulation of the dopamine system induces a variety of disorders, ranging from Parkinson's disease to drug addiction (Ford and Williams, 2008; Noori et al., 2012). Within the brain there are three main dopaminergic pathways: 1) the nigrostriatal pathway, originating from the substantia nigra (pars compacta, SNC), and the 2) mesocortical and 3) mesolimbic pathways, highly interconnected pathways originating from the dopaminergic cell bodies of the VTA (Figure 5). Dopaminergic axons of the mesocortical pathway are highly involved in rewarding processes, and thus project mainly into the nucleus accumbens (Acb) and prefrontal cortex (PFC). The mesolimbic pathway consists of VTA dopaminergic projections innervating the amygdala and BNST, regions involved in cognitive and emotional processes. This PhD thesis will be mainly focused on the mesolimbic pathway, since it is highly involved in the responses to stressful stimuli (Noori et al., 2012).

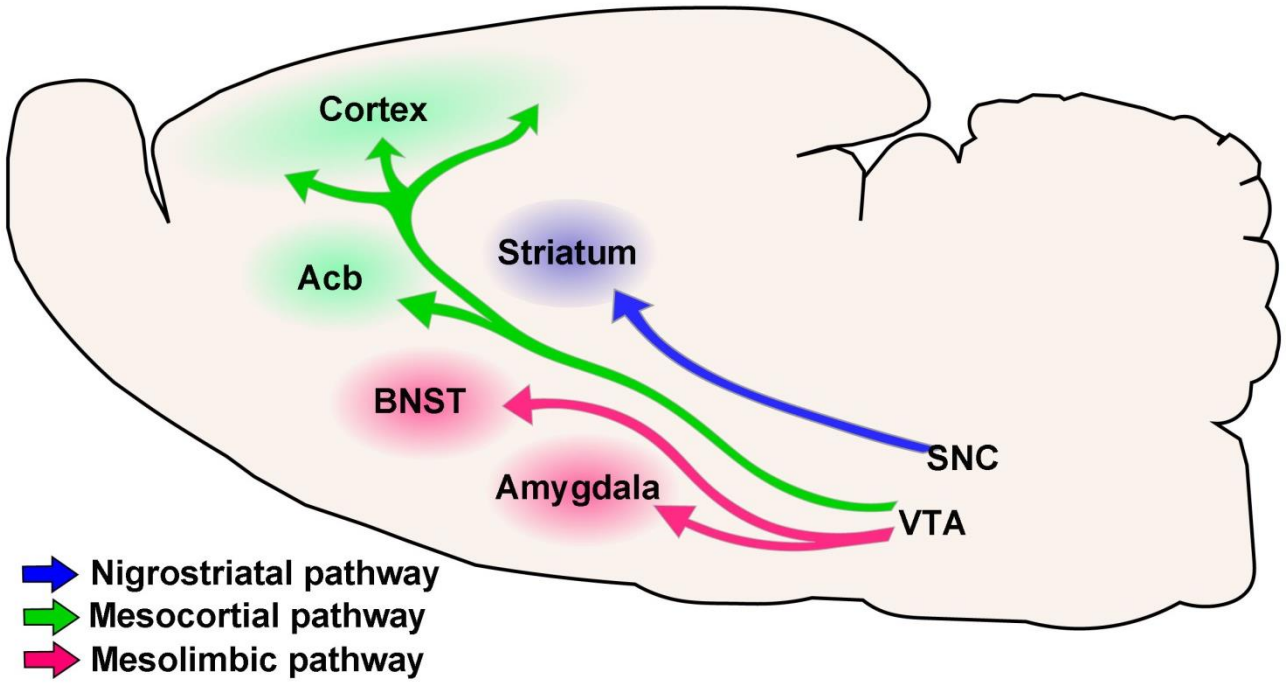

Figure 5: Dopaminergic pathways in the rodent brain. Dopaminergic cell bodies within the substantia nigra compacta (SNC) project via the nigrostriatal pathway to the striatum. Dopaminergic neurons within the ventral tegmental area (VTA) innervate the nucleus accumbens (Acb) and the cortical regions via the mesocortical pathway as well as the amygdala and the bed nucleus of the stria terminalis (BNST) via the mesolimbic one.

1.2.3 The dopamine receptors

Dopamine receptors have been classified into two groups in relation to their biochemical, pharmacological and physiological characteristics. The D1-like family includes D1 and D5 subtypes, while D2, D3 and D4 belong to the D2-like family (Kebabian and Calne, 1979). All receptors share a similar sequence within the trans-membrane domain, although homology is higher within the same receptor family. Although it is easy to discriminate between the two receptor families pharmacologically, it is more challenging to discriminate between members of the same family. Indeed, D1 receptors have a higher selectivity for the antagonist SCH23390 than for the selective D2/D3 antagonists raclopride and sulpiride (Andersen, 1988). Similarly, quinpirole behaves as a selective D2/D3 agonist (Levant et al., 1993) and SKF38393 as a D1-receptor agonist (Arnt et al., 1992).

D1 receptors are the most expressed dopamine receptor in the brain. Since they are strongly distributed in the areas receiving dopamine innervations, D1 receptors have been found in the striatum, Acb, olfactory tubercle, and hypothalamus. Interestingly, a high density of D1 has been

detected in the amygdala (Scibilia et al., 1992), with a greater expression within the intercalated cells masses (ITCs) (Dawson et al., 1986; Scibilia et al., 1992; Fuxe et al., 2003). D2 receptors are mainly distributed within the striatum and in the core part of the nucleus accumbens (AcbC) (Salgado-Pineda et al., 2005). Even though their expression level is low, they are also detected in the cerebral cortex, hippocampus, amygdala, cerebellum and midbrain (Bouthenet et al., 1987; Scibilia et al., 1992; Khan et al., 1998; Pinto and Sesack, 2008). Analyses of the primary structure of the dopamine receptors, together with biochemical and pharmacological assays, have revealed that all receptors belong to the GPCR family (Kebabian et al., 1972; Kebabian, 1978). Similar to the CRHR1, the D1 receptor stimulates adenylyl cyclase (AC) via the activation of the Gsα subunit and thus robustly stimulates cAMP production. Furthermore, D1 receptors appear to modulate intracellular calcium levels through a variety of mechanisms. One of these mechanisms is via the stimulation of phosphatidylinositol (PI) hydrolysis by phospholipase C (PLC), which results in the production of inositol 1,4,5-trisphosphate and mobilization of intracellular calcium stores. In contrast, D2-like receptors are mainly associated with the Gi subunit, which blocks the activation of AC and the PKA-dependent signaling pathway.

1.2.4 The role of the dopaminergic system in the response to stress

The involvement of the dopaminergic system in stress responses has received increasing attention in recent years due to the importance of dopaminergic transmission for neuropsychiatric disorders such as schizophrenia, depression and drug addiction (Fibiger, 1995; Weiss et al., 2001; Oda et al., 2015). Furthermore, it is well established that stressful stimuli activates the dopaminergic system (Coco et al., 1992). For example, mild foot shock induces metabolic activation of dopaminergic innervations in the PFC (Thierry et al., 1976), and in the accumbal regions (Deutch and Roth, 1990). However, the critical brain region emerging from the literature regarding the interaction between stress and the dopaminergic system is the amygdala. Pharmacological studies have demonstrated that an injection of the D1 antagonist SCH23390 into both the BLA and CeA diminished anxiety-like behavior in rodents (de la Mora et al., 2005; Bananej et al., 2012), as does sulpiride, a D2-like receptor antagonist (Bananej et al., 2012; Perez de la Mora et al., 2012).

1.3 Interaction between the CRH and dopamine system

The dopamine and the CRH systems have long been thought to modulate rewarding and stress-related processes, respectively, and have been considered independent. However, recent studies have demonstrated that these two systems work in concert to modulate physiological responses to stressful stimuli in different brain regions, and have suggested that dysregulation of this interaction may underlie some aspect of drug dependence (e.g., withdrawal and relapse) and the development of stress-related disorders and drug dependence.

Stress enhances activity of the mesolimbic dopamine system and increases dopamine release in the VTA through the actions of CRH (Bagosi et al., 2006; Wanat et al., 2008; Walsh et al., 2014). Furthermore, a knockout of CRHR1 in the mesolimbic dopaminergic system reduces DA release in the PFC and increases anxiety-related behavior. Other studies have provided further evidence supporting an interaction between the CRH and dopaminergic systems in drug addiction. Chronic cocaine treatment results in a co-activation of CRH and dopamine receptors, which modulate the BLA-mPFC connection through an increase in excitatory postsynaptic current (EPSC) (Orozco-Cabal et al., 2008). Furthermore, CRH pre-treatment significantly attenuates the cocaine-induced increase of dopamine release in the Acb and VTA, suggesting an interaction also in these regions. During cocaine withdrawal, a state associated with low levels of dopamine, CRHR1-dependent long term potentiation (LTP) in the CeA is increased (Pollandt et al., 2006; Fu et al., 2007), while the consequent impairment of D1-dependent LTP within the BNST can be mimicked by chronic treatment with CRH (Francesconi et al., 2009).

Although these studies demonstrate an interaction between the CRH and the dopaminergic system in the brain, and dopaminergic projections have been shown to directly innervate CRH-containing neurons in the CeA (Eliava et al., 2003) and BNST (Meloni et al., 2006), few studies have investigated the role of DA-CRH interaction in these regions.

1.4 Receptor-receptor interaction: A new concept in psychopharmacology

The first indication that GPCRs might interact in cell membranes occurred in the early 1980s (Agnati et al., 1982; Fuxe et al., 1983). However, in recent years, receptor-receptor interaction models have been extensively investigated due to the evolution of powerful techniques used to identify receptor heteromers in vitro (e.g., fluorescence and bioluminescence resonance energy transfer methods and atomic force microscopy), as well as the need to identify new targets for the development of novel pharmaceutical treatments for neurological disorders. Heterocomplex

formation of membrane receptors was first demonstrated for tyrosine kinase- (TH) containing neurons (Ullrich and Schlessinger, 1990), but in 1993 the term 'heteromerization' was introduced to describe a specific direct interaction between different types of GPCRs (Zoli et al., 1993). Since then, the concept of receptor-receptor heteromerization has extended the therapeutic potential of drugs targeting GPCRs. Initially, the functional relevance of receptor heteromerization was attributed exclusively to the effect of the distinct signaling pathways activated by the receptor crosstalk. However, recent studies have demonstrated that at the plasma membrane, receptor-receptor interactions can positively or negatively mediate ligand binding, resulting in specific pharmacological profiles (Jordan and Devi, 1999; Albizu et al., 2006; Franco et al., 2008). Furthermore, heteromerization stimulates new signaling pathways, leading to new G protein coupling (Mellado et al., 2006) or to the recruitment of internalization processes via β-arrestin activation (Terrillon and Bouvier, 2004). The first experimental evidence demonstrating the formation of dimeric complexes within the dopamine receptors was provided by the discovery of dopamine receptors homodimers (Ng et al., 1996). Subsequently, numerous studies have revealed D1-D3 (Marcellino et al., 2008), D2-D3 (Scarselli et al., 2001), and D1-D2 (Rashid et al., 2007) heteromerization, as well as their functional properties and roles in neuropsychiatric disorders (Table 2).

Table 2: List of hetercomplexes and their implications in neurological disorders

Heterocomplex	Functional relevance	Clinical relevance	Reference
D1-D2	Signaling, Internalization	Schizophrenia, Parkinson´s disease	*(Rashid et al., 2007)* *(So et al., 2005)*
D1-D3	Pharmacology, Behavior	Parkinson´s disease	*(Fiorentini et al., 2008)* *(Marcellino et al., 2008)*
D2-D3	Pharmacology, Signaling	Schizophrenia, Parkinson´s disease	*(Maggio et al., 2003)* *(Scarselli et al., 2001)*
D1-A1	Pharmacology, Signaling, Internalization	Schizophrenia, Parkinson´s disease, Addiction	*(Gines et al., 2000)* *(Cao et al., 2007)* *(Yabuuchi et al., 2006)*
D2-A2a	Pharmacology, Signaling, Internalization	Schizophrenia, Parkinson´s disease, Addiction	*(Canals et al., 2003)* *(Ferre et al., 1999)* *(Fuxe et al., 2005)*
D2-mGluR5	Pharmacology	Schizophrenia	*(Ferre et al., 1999)* *(Popoli et al., 2001)*
CRHR1-V1b	Signaling	Mood disorder, Depression	*(Young et al., 2007)*

Numerous studies have demonstrated that dopamine receptors also form complexes with receptors from different systems, interacting with adenosine A1 and A2 receptors (Ferre et al., 1999; Gines et al., 2000) and glutamatergic receptors, which create heterodimers with mGluR5 (Ferre et al., 1999). To our knowledge, there are no studies demonstrating the receptor-receptor heteromerization between dopamine and CRH receptors. However, CRHR1 is also able to form heteromers, as shown by a study revealing an interaction between CRHR1 and vasopressin receptor subtype 1b (V1b) (Young et al., 2007).

In summary, the concept of receptor-receptor heteromerization considerably expands the potential of pharmacological targets for the development of novel medication for anxiety-related disorders and drug dependence.

1.5 The amygdala

1.5.1 Neuroanatomy

The amygdala is an heterogeneous brain region highly involved in the detection and the orchestration of the adequate responses to stressful stimuli (Davis and Shi, 2000; LeDoux, 2000; Pare et al., 2004; Perez de la Mora et al., 2008; Ehrlich et al., 2009; Amir et al., 2011). Its name derives from the Greek "ἀμυγδαλή, *amygdalē*" which means "almond", due to the shape of its main portion, the basolateral nucleus (BLA). It is located on both hemisphere in the rostro-medial portion of the temporal lobes and is constituted by the BLA, the central (CeA), the basomedial (BMA) and the medial (MeA) nucleus of the amygdala.

Here we focus on the BLA and the CeA complexes. The basolateral amygdala, constituted by the basal (BA) and lateral (LA) subnuclei, consists mainly of glutamatergic neurons and inhibitory GABA-ergic interneurons. While the neurons within the CeA, which is divided in lateral (CeL) and medial (CeM) subnuclei, are primarily GABAergic. Situated in the space between the BLA and the CeA are the called intercalated cells (ITCs) of the amygdala (Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993; Pare and Smith, 1993; Marcellino et al., 2012). These clusters of GABAergic interneurons surrounding the BLA act as a filter for the information flow within the amygdala (McDonald, 1985; Royer et al., 1999). As shown in Figure 6 there are different intercalated paracapsular (Ip) clusters of cells which are labelled based on their distribution in the dorsal (Idp), lateral (Ilp), medial (Imp) and ventral (Ivp) portion of the BLA nucleus. The main

nucleus (IN) of the ITCs extends from the latero-ventral part of the BLA through the bottom of the CeA, forming a hug-shape in the dorsal portion. The small GABAergic ITCs are medium spiny neurons and showed to be rich in D1 (Fuxe et al., 2003; Jacobsen et al., 2006) and CRHR1 (Justice et al., 2008) receptors.

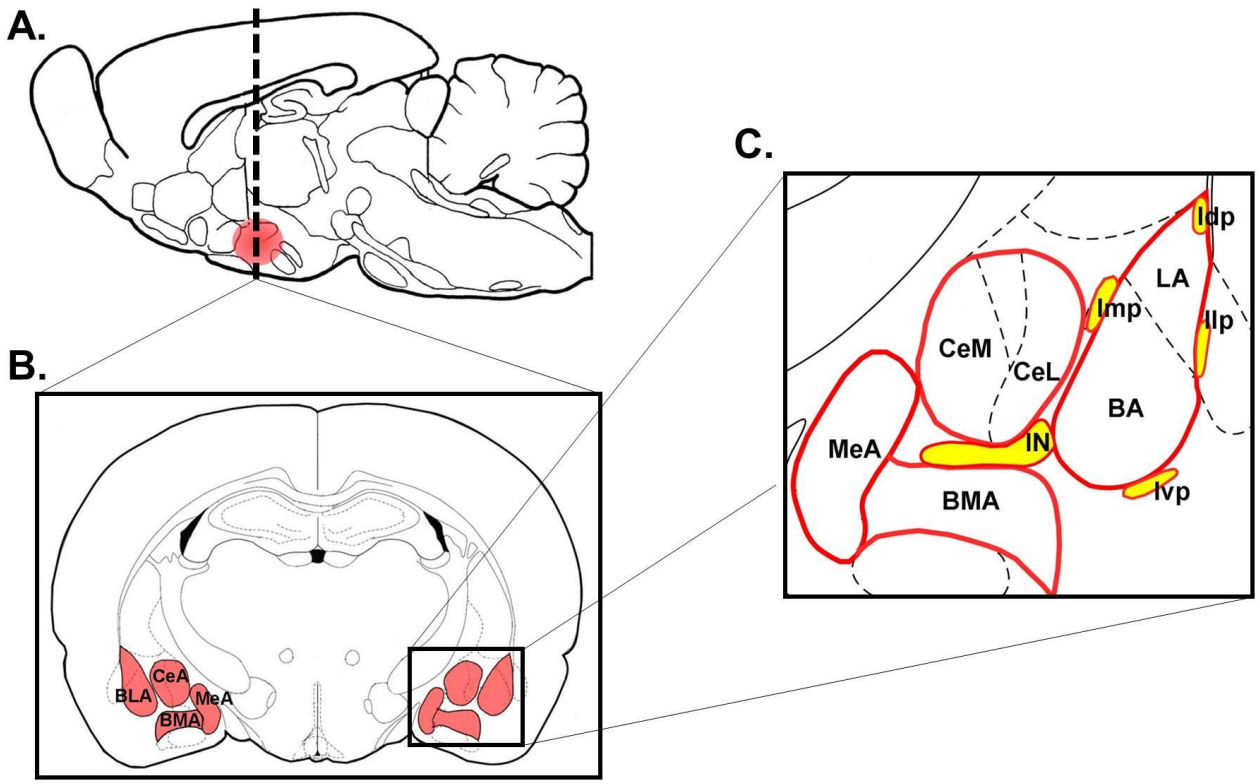

Figure 6: Schematic representation of the different nuclei and intercalated paracapsular cell clusters in the rodent amygdala. The amygdala is located in the rostro-medial portion of the temporal lobes as showed in the sagittal section of the brain (panel A) The amygdala is constituted by the basolateral (BLA), central (CeA), BMA (basomedial) and medial (MeA) nuclei (panel B: coronal section of the brain). The BLA is divided into latera (LA) and basal (BA) subnuclei, while the CeA is constituted by the lateral (CeL) and the medial (CeM) portion (panel C: amygdala magnification). The intercalated paracapsular (Ip) cell clusters surround the BLA and according to their position are called medial (Imp), dorsal (Idp), lateral (Ilp), ventral (Ivp) and main nucleus (IN) (Panel C).

1.5.2 Inter- and intra-connections of the Amygdala

The distribution of information within the amygdala is extremely complex. There are many parallel networks between the nuclear divisions, which can be travelled in both directions or unilaterally, converging in the same area (for review (Janak and Tye, 2015)).

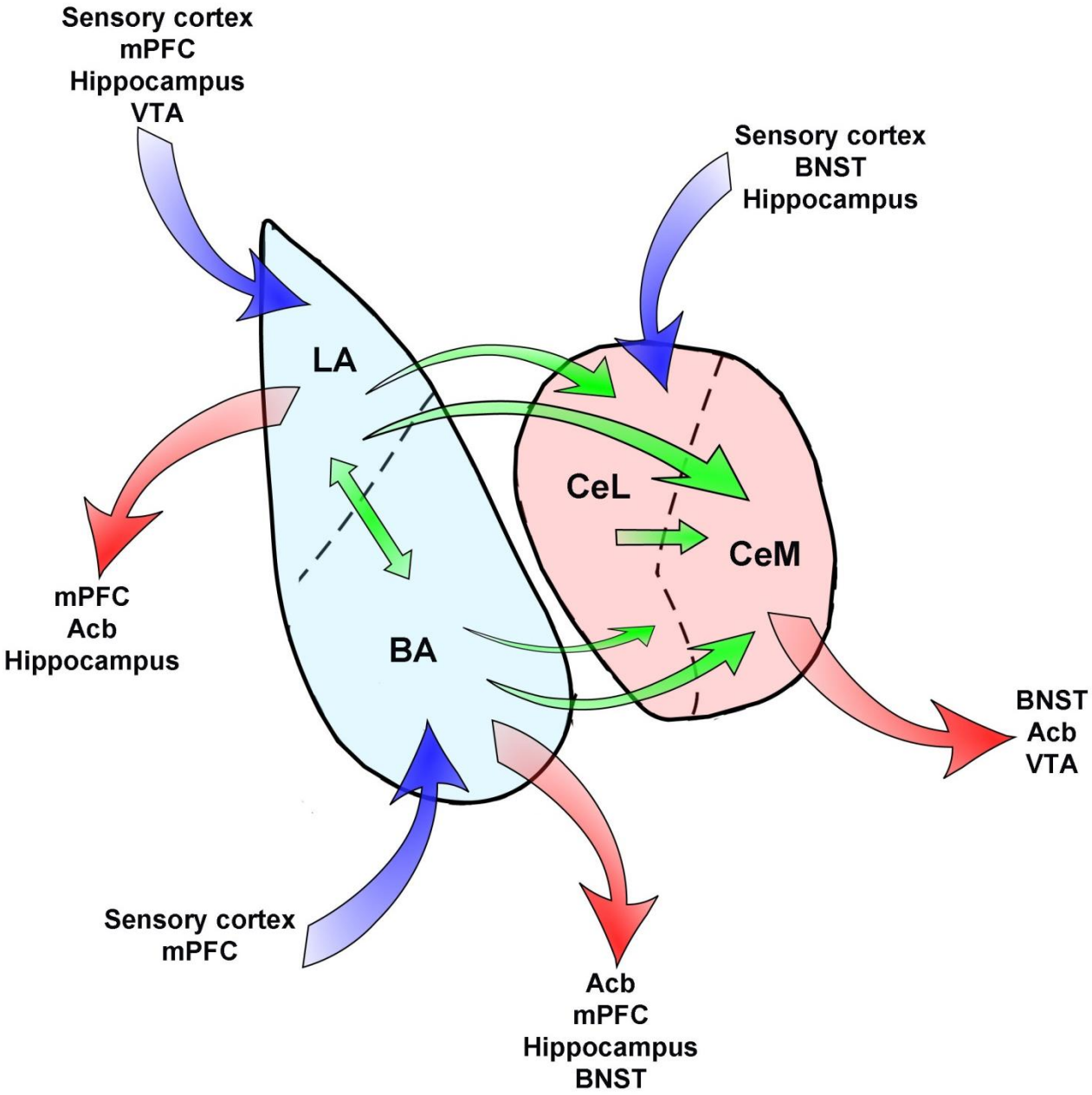

Figure 7: Schematic representation of the main afferent, efferent and intra-amygdala connections. The BLA receives and integrates the inputs coming from the cortical regions. The information flow innervates the CeM directly or via the CeL, which modulates physiological responses by efferent projections to downstream regions. Afferent and efferent projections are represented with blue and red arrows respectively. Green arrows represent intra-amygdala connections.

A highly simplified view of information flow through the amygdala is the following. The amygdala receives information about the external environment from the sensory thalamus and sensory cortices, which project to the LA (Quirk et al., 2003; Berretta, 2005). LA projects to the BLA and to the neighbouring subnuclei of the CeA (CeL and CeM) (Ciocchi et al., 2010; Haubensak et al., 2010). The CeM is the main output region of the amygdala, projecting to downstream regions such

as the BNST, Acb, and VTA, and receiving direct inputs from the BLA and the ITCs and indirect inputs from the CeL (Ciocchi et al., 2010; Haubensak et al., 2010), which acts as a modulator of CeM activity (Figure 7). The BLA is reciprocally connected with cortical regions, in particular the medial prefrontal cortex (mPFC) and hippocampus (McDonald, 1998). It also projects unilaterally to the CeA, as well as to the BNST and striatum, in particular the Acb (Heimer and Alheid, 1991; LeDoux, 2000). The trafficking of nerve impulses between these two nuclei appears, however, to be controlled by inhibitory interfaces consisting of ITCs (Royer et al., 1999, 2000; de la Mora et al., 2010), which are in a strategic position to influence the flow of information from the basolateral complex to the CeA (Collins and Pare, 1999). It has been shown that ITCs receive innervation from the BLA (Millhouse, 1986; McDonald and Augustine, 1993; Pare and Smith, 1993; Busti et al., 2011), mPFC, in particular infralimbic regions (McDonald, 1998; Pinto and Sesack, 2000), and thalamus (Royer et al., 1999; Vertes, 2002). The different ITC clusters are functionally connected to each other (Busti et al., 2011), as well as with the central, medial, and basolateral nuclei of the amygdala, extended amygdala, and extra-amygdaloid regions (Pare and Smith, 1993; Royer et al., 2000). Furthermore, electrophysiological experiments have shown that based on the nature, intensity, and origin of the glutamatergic input that innervates the ITCs clusters, different area of the CeA are inhibited, thus demonstrating the role of ITCs in the modulation of CeA activity (Royer et al., 1999).

1.5.3 Functional role of the amygdala

Numerous studies have demonstrated the pivotal role of the amygdala in anxiety in both humans and rodents. Amygdalar volume is positively correlated with anxiety in humans (LeDoux, 2000; Qin et al., 2014), and patients diagnosed with anxiety disorder display a higher stimulation of this brain region during anticipatory anxiety relative to the healthy controls (Boehme et al., 2014). In rodents, immediate early gene assays in rodents have demonstrated amygdala activation following exposure to anxiogenic contexts (Silveira et al., 1993; Butler et al., 2012), and pharmacological inactivation of the amygdala induces anxiolytic effects in the elevated plus maze (Moreira et al., 2007). Furthermore, lesion and pharmacological inactivation studies have demonstrated the involvement of the amygdala nuclei in responses to fear conditioning (Davis and Shi, 1999; LeDoux, 2000) and fear learning (Duvarci and Pare, 2014). Thus, both human and rodent studies implicate the amygdala in the regulation of anxiety-related behaviors.

1.6 The Ventral Tegmental Area

1.6.1 Neuroanatomy

The ventral tegmental area (VTA) is located close to the midline in the ventral portion of the midbrain, and is highly involved in reward and addiction (Nestler and Carlezon, 2006; Kauer and Malenka, 2007; Yetnikoff et al., 2014). This is not surprising, as the majority of VTA neurons (approximately 60–65%) are dopaminergic, while approximately 35% are GABAergic, while a small proportion are glutamatergic (Nair-Roberts et al., 2008; Sesack and Grace, 2010). Although recent studies have identified neuronal subclasses based on their projection targets and physiological characteristics (Lammel et al., 2011; Lammel et al., 2012; Lammel et al., 2014b; Lammel et al., 2014a), dopaminergic neurons of the VTA remain highly anatomically and physiologically heterogeneous. Furthermore, VTA modulation of the dopaminergic system and its dysregulation are highly involved in stress-induced drug-seeking behavior and anxiety-related diseases (Koob and Volkow, 2010; Wise and Morales, 2010). This is due to the presence of CRH-containing axon terminals originating in the limbic system and hypothalamus (Rodaros et al., 2007). Wanat and colleagues demonstrated that CRH increases the action potential firing rate of dopaminergic neurons within the VTA through the activation of CRHR1 (Wanat et al., 2008). In addition, CRHR2 stimulation via glutamatergic transmission modulates dopaminergic VTA neuronal populations (Fiorillo and Williams, 1998; Ungless et al., 2003).

1.6.2 Inter- and intra-interconnections of the ventral tegmental area

Dopaminergic neurons of the VTA project to the PFC and Abc, as well as to the hypothalamus, amygdala, lateral habenula, pallidum, and BNST (Kauer and Malenka, 2007; Sesack and Grace, 2010), brain regions comprising the mesocorticolimbic pathway. However, the same VTA dopaminergic neurons projecting to the accumbens shell receive excitatory glutamatergic and cholinergic inputs from the laterodorsal tegmental nucleus (LDT) (Lammel et al., 2012) and inhibitory GABAergic input from the rostromedial tegmental nucleus (RMT) (Goncalves et al., 2012; Lammel et al., 2012). In contrast, the PFC-projecting neurons receive glutamatergic input from the lateral habenula, which also modulates LDT and RMT, thus regulating dopamine release in accumbal regions. Furthermore, a minority of dopaminergic neurons found in the medial VTA also receive an inhibitory GABAergic input from the BNST. In addition to these defined subcircuitries of the VTA, several other regions strongly innervate the VTA. The Acb sends GABAergic projections to the VTA (Nauta et al., 1978; Kalivas et al., 1993), which may be involved in the disinhibition of the dopaminergic neurons (Kalivas et al., 1990; Klitenick et al.,

1992; Xi and Stein, 1998) via the activation of GABA-A receptors (Bocklisch et al., 2013). Furthermore, a population of dopaminergic neurons also receives excitatory, CRH-mediated inputs from the BNST and CeA (Rodaros et al., 2007; Jennings et al., 2013). Considerably less is known about the connectivity and diversity of non-dopaminergic neurons of the VTA, but GABAergic neurons within the VTA play a significant role in modulating dopaminergic cell activity and thus driving behavior (Tan et al., 2012; van Zessen et al., 2012).

1.6.3 Functional role of the ventral tegmental area

The VTA has long been implicated in rewarding and reinforcing processes, as release of dopamine from the VTA into the Acb is induced not only by natural stimuli required for the survival, such as food and sex (Kelley and Berridge, 2002), but also by drugs of abuse (Di Chiara and Imperato, 1988). However, aversive and stressful events are also able to augment dopaminergic function within the mesolimbic system (Tidey and Miczek, 1996; Anstrom and Woodward, 2005; Berton et al., 2006; Brischoux et al., 2009; Ungless et al., 2010). For example, dopamine release in the Acb and PFC increases during social-defeat stress (Tidey and Miczek, 1996), and VTA dopaminergic neurons are activated by foot shock and during acute restraint stress (Anstrom and Woodward, 2005; Brischoux et al., 2009). Furthermore, stress-induced reinstatement of drug-seeking is blocked by intra-VTA injection of CRH antagonist (Wise and Morales, 2010). Thus, it is highly important to improve our knowledge regarding the mechanisms of this brain region critically involved in both the reinforcement associated with drugs of abuse and negative affective state associated with dependence.

1.7 Alcohol addiction

Alcoholism is a major public health problem, and alcohol use constitutes 4% of the global burden of disease (Ezzati et al., 2002). Alcoholism is an etiologically and clinically heterogeneous disorder characterized by chronic relapsing episodes, loss of control over alcohol intake, and dysregulation of stress-related brain emotional systems (Zorrilla et al., 2014).

The beginning of the long process leading to alcohol dependence is characterized by a rewarding experience. This represents a positive reinforcing force that drives the motivational emotion during subsequent drug intake. However, during chronic use of alcohol, stress circuits are progressively recruited, which may contribute to a chronic negative affective state that leads to a negatively

reinforced use of alcohol (Figure 8). This phase is considered crucial for the transition to compulsive alcohol use, and subsequent alcohol addiction (Koob and Le Moal, 2008b; Koob and Volkow, 2010). With chronic, repeated periods of intoxication and withdrawal, the processes regulating the negative affective states predominates over the rewarding pathways. As a result, more alcohol is needed to maintain a normal, stable state (Zorrilla et al., 2014). Furthermore, extreme vulnerability to stressful stimuli underlie protracted abstinence and alcohol is consumed in order to relieve the negative affective state (negative reinforcement) (Heilig and Koob, 2007; Koob and Volkow, 2010; Koob and Zorrilla, 2010; Zorrilla et al., 2013).

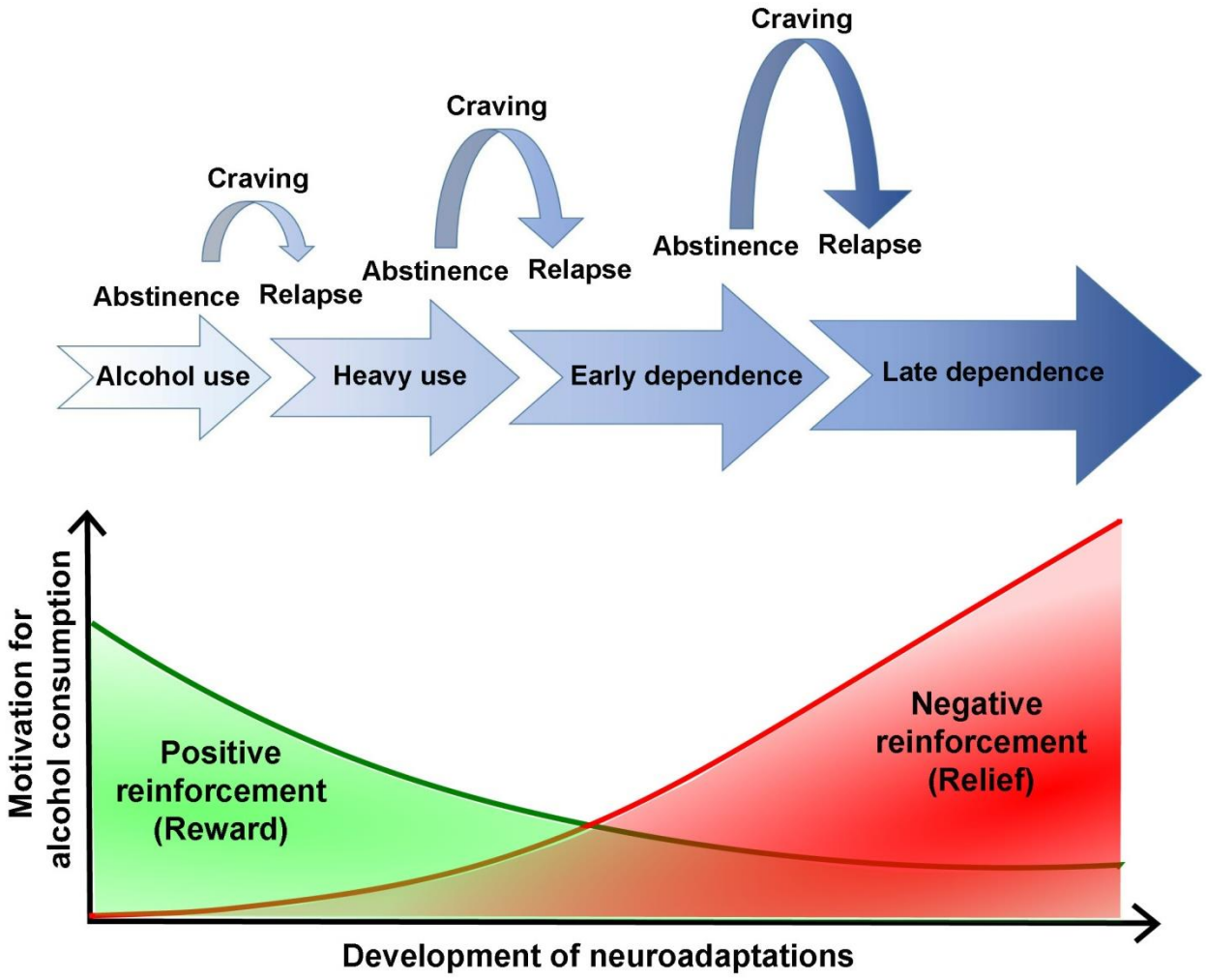

Figure 8: Development of alcohol dependence over time. (A) Initial alcohol use that is linked to positive reinforcing effects of alcohol is followed by the loss of control and compulsive alcohol intake. (B) This progression is accompanied by the shift of positive to negative reinforcement where alcohol is consumed to achieve relief from negative emotional states. The state of late dependence is characterized by long-lasting neuroadaptations that also persist into protracted abstinence. Adapted from Heilig and Koob, 2007 and Meinhardt and Sommer, 2015.

The initial positive reinforcing effects of the drug are regulated in part by reward circuitry comprised of dopaminergic inputs from the VTA that activate accumbal regions, the PFC, and the limbic system (Noori et al., 2012). However, chronic consumption of the drug, leading to negative emotional symptoms, induces a recruitment of the CRH system in limbic areas such as amygdala. Numerous studies have demonstrated that animals with experimentally-induced alcohol dependence display increased anxiety-like behavior during acute and protracted abstinence, as well as long-lasting up-regulation of the CRH system in amygdala nuclei (Rimondini et al., 2002; Valdez et al., 2003; Sommer et al., 2008). Furthermore, systemic and CeA-directed infusion of CRH antagonists attenuate stress-induce alcohol consumption (Rassnick et al., 1993; Funk et al., 2007).

In recent years, several approaches have been investigated to help alcoholic patients to control not only alcohol drinking but also alcohol craving and relapse (Volpicelli et al., 1992; Gehlert et al., 2007). Medications tested for their potential therapeutic effect on alcohol abuse (i.e. naltrexone, acamprosate, disulfiram, pexacerfont) are currently used (Volpicelli et al., 1992; Ades and Lejoyeux, 1993; Monti et al., 1993; Hughes and Cook, 1997; Kwako et al., 2015). However, despite some promising results, none of these medications is sufficiently effective. Thus, understanding the neurobiology of alcoholism will strongly contribute to the development of effective new pharmacotherapies for alcoholism.

1.8 Modelling alcohol dependence in rodents

The use of an animal model of alcoholism is of crucial importance to addiction research. Several models have been created with varying degrees of face, predictive and construct validity, which aspire to reproduce the symptomatic, pharmacological and molecular characteristics, respectively, of alcohol addiction in humans. The animal models applied in this thesis to study alcohol dependence and drug-seeking behavior, consist of the post-dependent animal model and the reinstatement model.

1.8.1 Post-dependent animal model

Alcohol dependence is induced by repeated periods of intoxication and withdrawal from the drug, resulting in an increased withdrawal severity. Chronic intermittent exposure to alcohol vapor (CIE) is a well-established and reliable method (Rimondini et al., 2002; Sommer et al., 2008). As a consequence of the high blood alcohol level in the brain, kept constantly between 150–250 mg/dL

during the intoxication phase (Gilpin et al., 2009), and the subsequent withdrawal periods, the post-dependent (PD) state is achieved. The post-dependent state consists of excessive, voluntary and compulsive behavior toward the drug, increased tolerance to the drug, and high vulnerability to stress (Figure 9) (Sommer et al., 2008; Meinhardt and Sommer, 2015). Furthermore, post-dependent animals show long-lasting neuroadaptations that remain even after long periods of abstinence, involving the glutamatergic system (Hermann et al., 2012) and the CRH system in the amygdala (Sommer et al., 2008; Vendruscolo et al., 2012). Although alcohol administration in this model is passive, thus differing from the voluntary mode of intake in human alcoholics, the symptoms in post-dependent animals correspond well to the human situation and fulfill several criteria of the DSM-V, such as withdrawal signs, tolerance, loss of control, and an increase in voluntary alcohol consumption.

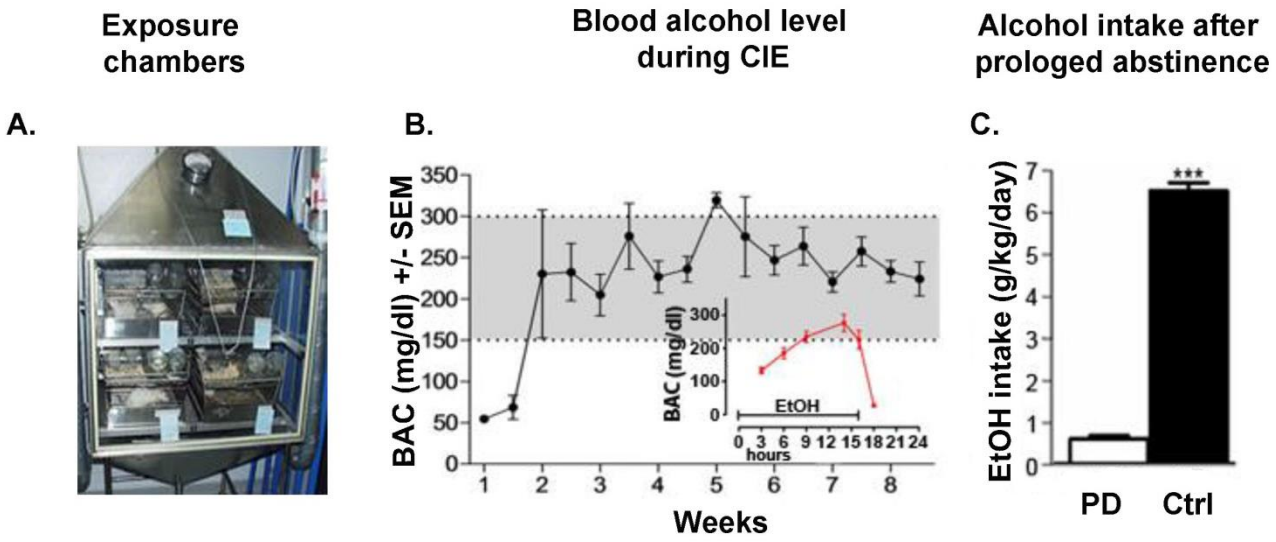

Figure 9: The post-dependent animal model. (A) Rats are housed in their home-cages in the exposure chambers and are exposed to chronic intermittent cycles of alcohol vapor (CIE) or air (controls) for 7 weeks. (B) Ideally, blood alcohol concentrations (BAC) rise daily to levels of 150-250 mg/dl. The inlet shows the time course of blood alcohol levels over one day of alcohol exposure. During the 16 hours of alcohol vapor exposure levels increase continuously. The remaining hours, animals are exposed to air and blood alcohol concentrations decrease rapidly. This procedure results in the post-dependent phenotype. (C) Following three weeks of abstinence, the post-dependent rats consume high amounts of alcohol in a two-bottle fee choice paradigm. Adapted from Meinhardt and Sommer, 2015.

1.8.2 Reinstatement model of drug seeking

Relapse to drug consumption is one of the major events responsible for the development of drug dependence, and the main impediment the treatment of the addiction (Hunt et al., 1971; Koob and Volkow, 2010). Thus, preclinical researchers have developed the reinstatement model, a robust and widely applied approach to investigate the behavioral, environmental, and neural mechanisms underlying drug-seeking and relapse in drug addiction (Davis and Smith, 1976; de Wit and Stewart, 1981; Shaham et al., 2003; Spanagel, 2003). Using an operant conditioning paradigm, animals are first trained to self-administer the drug, usually by associating an operant response, such as lever pressing, and a drug-associated stimulus (which can be an olfactory, visual, tactile or acoustic cue), with the delivery of the drug and its putative rewarding properties,. Following stable responding for the drug for variable periods of time, and an extinction phase in which responding is decreased via omission of the cue and drug delivery, lever pressing can be induced by drug priming injections (de Wit and Stewart, 1981), the presentation of drug cues (Meil and See, 1996; Crombag and Shaham, 2002), or external stressors (Shaham and Stewart, 1995; Shaham et al., 2003; Spanagel, 2003). Thus, the reinstatement model corresponds to behavior in humans, in which relapse to drug intake during abstinence can be induced by exposure to the drug itself, environments previously associated with drug-taking, and stressors. Thus, the reinstatement paradigm is a robust and reliable model to study drug-seeking and in preclinical research.

A large body of work has been published regarding the neuronal networks involved in the responses to different types of reinstatement. This thesis will present results obtained by the use of the reinstatement approaches. In Study 4, a visual cue (blinking light) and a stressor (yohimbine) were used to trigger alcohol-seeking behavior in rats, while in Study 5 cocaine-mediated seeking-behavior was tested via cue-induced reinstatement.

Because it receives dopaminergic afferent from the VTA and projects to both the PFC and Acb (Figure 10) (Di Ciano and Everitt, 2001; Stefanik and Kalivas, 2013), the BLA critically mediates conditioned cue-induced drug-seeking behaviors (Kalivas and McFarland, 2003; Kalivas and Volkow, 2005; See, 2005). In fact, many pharmacological studies have demonstrated a crucial role for the D1 receptor in the BLA in cue-induced reinstatement. For example, systemic treatment with D1 antagonists have been shown to attenuate reinstatement induced by cues previously paired with a drug (Ciccocioppo et al., 2001; Alleweireldt et al., 2002). Furthermore, intra-BLA injections of a D1, but not D2-like, receptor antagonist blocked cue-induced reinstatement of cocaine seeking (See et al., 2001). In addition, dopamine release and Fos immunoreactivity, which are increased in the

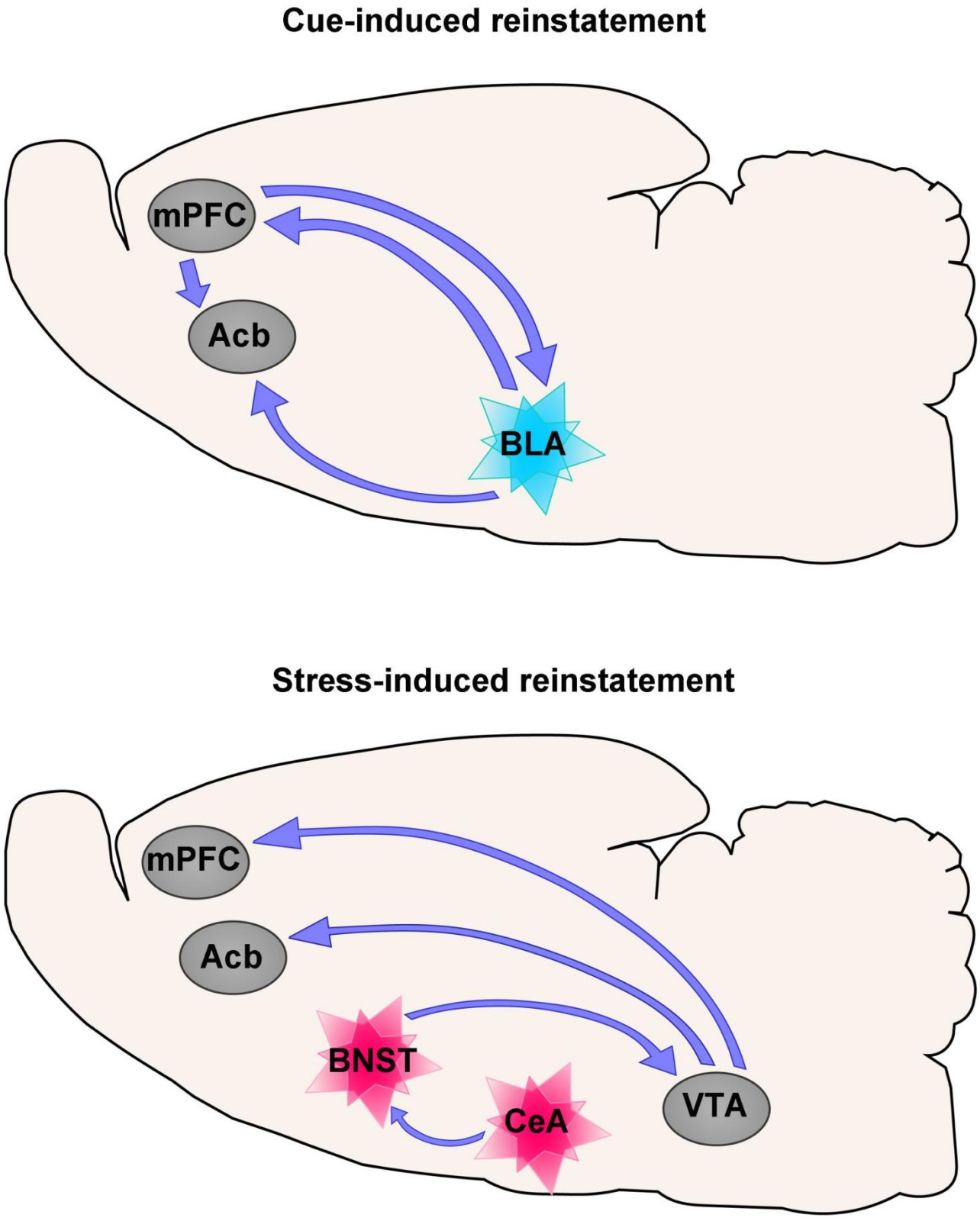

Figure 10: Schematic representation of mechanisms mediating cue- and stress-induced reinstatement. Cue induced reinstatement: BLAstimulation due to the mPFC, activates its projection to the Acb and PFC, both regions which drives the rewarding and motivational behaviors. Stress-induced reinstatement: The extended amygdala, constituted by the CeA and the BNST, mediates stress-induced drug-seeking behavior by projecting to the VTA which innervates the Acb and mPFC.

BLA upon presentation of a drug-associated cue, are blocked by D1 antagonists (Ciccocioppo et al., 2001). In addition, during stress-induced reinstatement, the activation of the CRH and the noradrenergic systems stimulate activity of the CeA and the BNST, which together orchestrates responses to stressful stimuli via both direct (Acb and PFC) and indirect (VTA) actions on the dopaminergic system (Erb et al., 2001; Shaham et al., 2003) (Figure 10). Selective CRHR1 and non-selective CRH receptor antagonists attenuate foot shock-induced reinstatement in heroin-, cocaine-, and alcohol-trained rats (Shaham et al., 1997; Erb et al., 1998; Le et al., 2000). Furthermore, CRHR1 is required for D1-mediated signaling in the BNST (Kash et al., 2008), which is also involved in drug-seeking behaviors as part of the extended amygdala (Pollandt et al., 2006; Koob and Le Moal, 2008a; Krishnan et al., 2010).

1.9 Genetically modified animals

The development of genetically modified animals has been a crucial step in identifying the role of specific neuronal mechanisms in the brain.

There are different ways to obtain genetically manipulated animals. To create a transgenic animal, exogenous DNA is incorporated into the genome of the mouse, achieved by retroviral infection or pronuclear injections methods. The animals created are generally gain-of-function mutants because the transgene is designed to express a new protein or to modify the activity of the normal one. However, the most precise method for gene manipulation is homologous recombination in embryonic stem cells, used mainly to induce a complete or partial loss of function in the gene, creating a knock-out mutant animal (Capecchi, 1989; Sauer, 1998). A DNA construct corresponding to the locus of interest is transfected into pluripotent embryotic stem cells and afterward injected into a pseudo-pregnant female, creating chimeric founders. Chimeric founders are crossed with wild-type animals and the heterozygous first generation offspring are used to establish a new mouse line.

In conventional mouse lines obtained with gene targeting methods, the mutation is globally presented in the animal. However, to increase the understanding of individual gene function in the development and pathogenesis of disease and to investigate the role of a protein within specific cell-population, a powerful tool has been developed: Cre site-specific DNA recombinase of bacteriophage P1 (Sternberg et al., 1986; Sauer, 1998). Cre is a 38 kDa prokaryotic enzyme which is capable to recognize a 34-bp sequence called loxP (locus of C-over of P1). The loxP site consists

in two inverted repeats of 13-bp, flanking a sequence of 8-bp. Cre-mediated recombination between two loxP cassettes, determines the excision of the DNA between them (Hoess et al., 1990) (Figure 11). In order to obtain a conditional knock-out mouse line for a specific gene, a mouse carrying loxP cassettes flanking the target gene is crossed with a Cre-transgenic conspecific. The double-transgenic offspring obtained will then express both Cre and loxP; thus the sequence targeted is excised, resulting in a loss of the functional protein. This type of mutation is called "conditional gene modification."

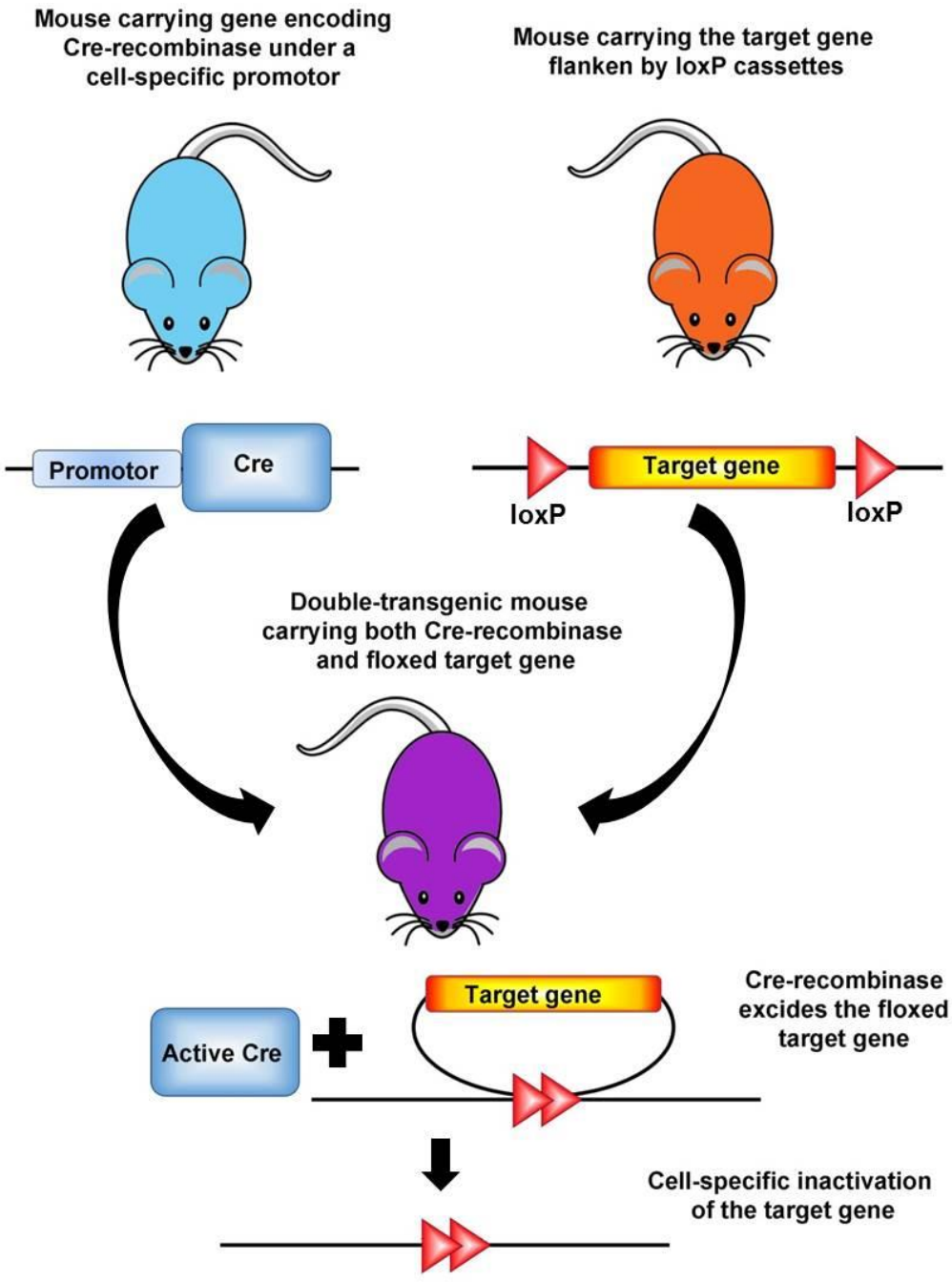

Figure 11: Strategy to obtain a conditional gene modification

It often occurs that the mutations induced alter the correct development of the embryos and thus, it is difficult to obtain healthy off-springs. In order to overtake this limitation and to temporally control the activation of Cre-expression, molecular engineering developed a method based on an inducible-Cre expression. Here we briefly explain how tamoxifen-dependent Cre recombinases (CreER) works and the focus will be on the CreERT2 recombinase, which is currently the most successful CreER version (Branda and Dymecki, 2004). Tamoxifen is a specific antagonist of the estrogen receptor and thus, tamoxifen-inducible CreERT2 systems are based on its effect on the internalization of these receptors into the cellular nucleus. Usually the receptors binds to heat shock protein 90 (hsp90) and they are free in the cytoplasm. However, when tamoxifen displays hsp90, this factor activates a nuclear localization cascade and the DNA-binding domain for the estrogen receptor into the nucleus becomes accessible. This event leads to the translocation of the receptor into the nucleus. Thus, in CreERT2-mice treated with tamoxifen, the recombinase, fused together with the estrogen receptor, is introduced into the nucleus where it can reach the loxP cassettes (Garcia and Mills, 2002).

HYPOTHESIS

Recent studies have demonstrated that the CRH and dopaminergic systems work in concert to modulate physiological responses to stressful stimuli in a cell-type specific manner. Here we suggest that a dysregulation of this interaction underlie some aspect of drug dependence (e.g. withdrawal and relapse) and the development of stress-related disorders.

OVERALL AIM

To demonstrate a cell-type specific function of CRHR1 in mediating the responses to stress and addiction-like processes

Specific Aim 1: To establish an interaction of CRHR1 and D1 receptor on anxiety related behavior under physiological condition and in alcohol dependence

Specific Aim 2: To determine the functional impact of cell-type specific CRHR1 on alcohol-and cocaine related behavior

LIST OF STUDIES

Study 1: Cross-talk of CRHR1 and D1 on anxiety-related behavior (Aim 1)

Study 2: Impact of CRHR1-D1 interaction on alcohol dependence (Aim 1)

Study 3: Differential role of dopaminergic and dopaminoceptive CRHR1 on stress-induced drinking behavior (Aim 2)

Study 4: αCaMKII-dependent CRHR1 modulates stress-induced alcohol seeking behavior in the central nucleus of the amygdala (Aim 2)

Study 5: Opposite effects of CRHR1 on DAT and D1 neurons in cocaine cue-induced reinstatement (Aim 2)

PART II: MATERIALS AND METHODS

2.1 Animals

Male **Wistar rats** (Charles Rivers, Germany), weighing 220 – 250g at the beginning of the experiment, were used to establish D1-CRHR1 interaction by pharmacological treatments (Study 1) and to characterize the dopaminergic system in the post-dependent model of alcohol addiction (Study 2).

Male **αCaMKII^{CreERT2} rats** (Sprague-Dawley genetic background), were used to determine the alcohol seeking behavior upon injection of CRHR1-overexpression virus in the CeA (Study 4). Cre-recombinase is expressed under the promotor of αCaMKII gene (Schonig et al., 2012). The transgene was induced by tamoxifen treatment. At the beginning of the experimental procedures all rats weighted between 190g and 290g and were housed 4 per cage. Animals were provided by Dr. Kai Schönig and Dr. Dusan Bartsch from the department of Molecular Biology of the Central Institute of Mental Health of Mannheim (Germany).

Male **D1^{Cre}-CRHR1$^{f/f}$ mice** (C57Bl/6 genetic background) were tested to address their response to acute stressful stimuli under healthy conditions (Study 1), during alcohol dependence (Study 2) and after repeated stress (Study 3). This mouse line was further characterized on the behavioral level under baseline conditions (Study 1). D1^{Cre}-Crhr1$^{f/f}$ mouse line was bred in order to obtain a cell-type specific knockout of CRHR1 in D1-containing neurons: Cre-recombinase, constitutively expressed under *Drd1* promotor, binds the loxP cassettes flanking the exon 8 and 12 of the *Crhr1* gene, generating a non-functional CRHR1 protein. The generation of the CRHR1-floxed (CRHR1$^{f/f}$) line was previously described by Müller (Muller et al., 2003), while the D1^{Cre} driving line was showed from Lemberger (Lemberger et al., 2007).

Male **DAT^{CreERT2}-CRHR1 $^{f/f}$ mice** (C57Bl/6 genetic background) were used to assess stress-induced alcohol drinking (Study 3) and cocaine-mediated seeking behavior (Study 5). Cell-specific knockout is induced by i.p. administration of tamoxifen (1 mg) twice a day for 5 consecutive days (Erdmann et al., 2007). Thus, Cre-recombinase, expressed under the promotor for the dopamine transporter (DAT), is free to bind the loxP cassette, which flanks the exon 2 of *Crhr1* gene, preventing the formation of a functional receptor (Refojo et al., 2011).

Male **D1^{CreERT2}-CRHR1$^{f/f}$ mice** (C57Bl/6 genetic background) were applied in Study 5 in order to determine cocaine seeking behavior. As previously described for D1^{CreERT2}-CRHR1$^{f/f}$ line, tamoxifen treatment (Erdmann et al., 2007) was necessary to induce Cre activity, which is under the control of the D1 promotor, and consequently causing exon 2 excision in *Crhr1* gene.

All neuron-specific CRHR1 knockout mouse lines were generated and provided by Dr. Jan Deussing (Max Plank Institute of Munich, Germany). At the beginning of the experiments, all mice were 10-12 weeks old and they were single housed for the entire experimental period. The respective CRHR1$^{f/f}$ littermates were used as control group in all the experiments.

Double immunohistochemistry analysis was performed on brain tissue from ***Crhr1*-GFP** reporter line and **C57BL/6 mice**. *Crhr1*-GFP transgenic line was created to map the brain distribution of CRHR1 by immunohistochemistry, due to the lack of specific CRHR1 antibodies (Justice et al., 2008). The reporter green fluorescent protein (GFP) was inserted under the promotor and enhancer of the *Crhr1* gene by using a BAC-based approach. *Crhr1*-GFP transgenic line was generated by Dr. Nicholas J. Justice from the Institute of Molecular Medicine and Department of Integrative Biology and Pharmacology, University of Texas Health Sciences Center, Houston, Texas (USA). Breeding of the animals was performed at the Central Institute of Mental Health of Mannheim (Germany). Mice were group-housed.

Male ***Gad67*-GFP** transgenic line expresses GFP under the promotor of glutamic acid decarboxylase **(**GAD67), an enzyme involved in GABA synthesis (Tamamaki et al., 2003). For this reason these mice were applied for whole cell patch-clamp recordings in the intercalated cells of the amygdala by Dr. Kai Jüngling and Dr. Hans-Christian Pape from the Institute of Physiology I (Neurophysiology), Westfälische Wilhelms-University Münster, Münster, Germany.

Food and water were available *ad libitum.* Holding rooms for all animals were kept under controlled conditions of light (12h light-dark cycle from 07:00 to 19:00), temperature (20-22°C) and humidity (65%). Animal manipulations were conducted at the Central Institute of Mental Health in Mannheim (Germany). Experimental procedures were carried out according to the NIH ethical guidelines for the care and use of laboratory animals, and were approved by the local animal care committee (Regierungspräsidium Karlsruhe, Germany).

2.2 Genotype assessment

Standard polymerase chain reaction (PCR) was used to determine the genotype in CamKII^{CreERT2} rats, D1^{Cre}-CRHR1$^{f/f}$, D1^{CreERT2}-CRHR1$^{f/f}$, DAT^{CreERT2}-CRHR1$^{f/f}$ and *Crhr1*-GFP mouse line. Primers used for each line are listed in Table 3. DNA was isolated from tail tissue of CamKII^{CreERT2} rats and *Crhr1*-GFP reporter line, while micro-punches of caudate putamen (CPu) and cortical brain tissue were analyzed in D1^{Cre}-CRHR1$^{f/f}$ and inducible lines (D1^{CreERT2}-CRHR1$^{f/f}$, DAT^{CreERT2}-CRHR1$^{f/f}$), respectively using the DNEasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

Table 3: List of primers used for PCR in rat and mice tissue for genotyping

Target gene	Forward	Reverse
αCaMKII^{CreERT2} rats *(Schonig et al., 2002; Schonig et al., 2012)*		
CreERT2	5′-TCGCTGCATTAC CGGTCG ATGC-3′	5′-CCATGAGTGAAC GAACCT GGTCG-3′
D1^{Cre}-CRHR1$^{f/f}$ mice *(Müller et al., 2003)*		
Cre	5´-GATCGCTGCCA GGATATACG-3´	5´-AATCGCCATC TTCCAGCAG-3´
Thy1-F1 (Control)	5´-TCTGAGTGGCAA AGGACCTTAGG-3´	5´-CCACTGGTG AGGTTGAGG-3´
Crhr1 (exon 8-12)	5´-TCACCTAAGTCC AGCTGAGGA-3`	5´-GAGCGGATCT CAAACTCTCC-3`
D1^{CreERT2}-CRHR1$^{f/f}$ mice and DAT^{CreERT2}-CRHR1$^{f/f}$ mice *(Refojo et al., 2011; Kuhne et al., 2012)*		
CreERT2	5´-GGCTGGTGTGTC CATCCCTGAA-3´	5´-GGTCAAATCCA CAAAGCCTGGCA-3´
Crhr1 (exon 2)	5´-AGTCAATCTG CATGTCCTCAT T-3´	5´-CACCCATGGTT AGTCCCAGT-3´
***Crhr1*-GFP mice** *(Justice et al., 2008)*		
Crhr1-GFP	5´-AGGATTGGGA AGACAATAGC-3´	5´-TCTCTAATAA AAACCCCACTGG-3´

Figure 12 shows PCR products for constitutive D1^{Cre}-CRHR1$^{f/f}$ mice and inducible D1^{CreERT2}-CRHR1$^{f/f}$ mice, respectively. Cre (574bp) and Cre^{ERT2} (405bp) PCRs confirm the genotype of the animals (left panels), while Crhr1 exon-specific products (right panels) demonstrate the *Crhr1*exon-specific deletion. After recombination, the exons are deleted, which results in a loss of functional CRHR1 protein. PCRs products for the knockout mice display two bands: the lower band (~500bp) corresponds to the remaining portion of *Crhr1* gene after Cre recombination, while the upper band, observable also in control littermates, corresponds to the entire floxed *Crhr1* gene (2377 bp). The presence of intact *Crhr1* gene in knockout mice is due to the cell-specificity of Cre recombination.

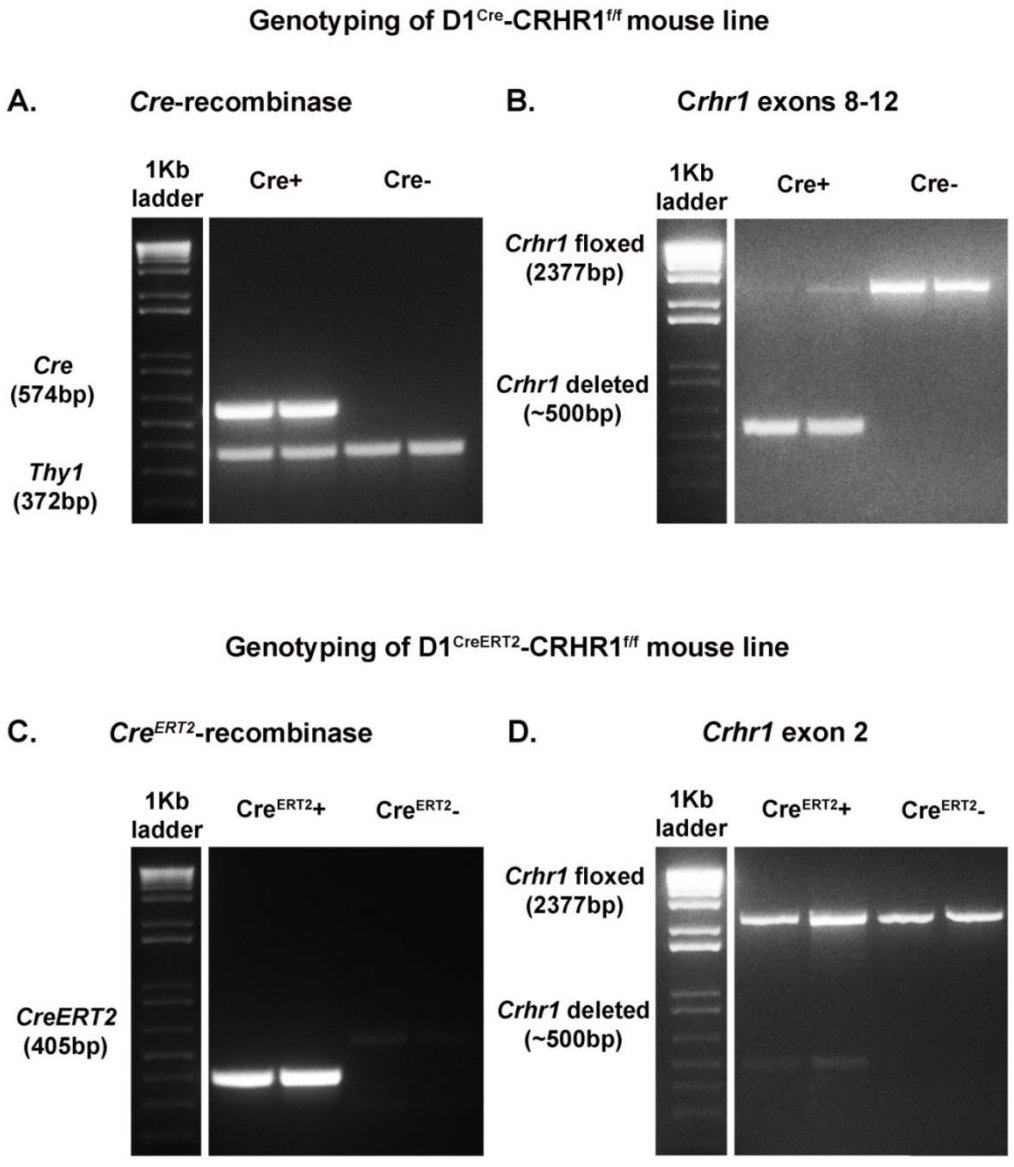

Figure 12: Genotyping of mice lacking Crhr1 in D1-containing neurons. PCRs products of Cre-recombinase (A.) and exons 8-12 of *Crhr1* gene (B.) in constitutive D1^{Cre}-CRHR1$^{f/f}$ mice (Cre+) and their control littermates (Cre-). Similar analysis was performed for D1^{CreERT2}-CRHR1$^{f/f}$ (Cre^{ERT2}+) mouse line and their control littermates (Cre^{ERT2}-) for inducible recombinase (C.) and exon2-specific PCR product of *Crhr1* gene (D.)

2.3 Experimental designs

2.3.1 Study 1: Cross-talk of CRHR1 and D1 on anxiety-related behavior

Firstly, co-localization between D1 and CRHR1 was assessed in different brain regions, with a focus in the amygdala nuclei and intercalated cells masses by double immunohistochemistry assays performed on brain sections of *Crhr1*-GFP mice.

Secondly, pharmacological experiments (Figure 13A) were performed on male Wistar rats and constitutive $D1^{Cre}$-$CRHR1^{f/f}$ mice to establish a functional interaction between D1 and CRHR1 under healthy conditions. Guide cannulas were first implanted and after one week of recovery, animals were microinjected (intracerebroventrally (i.c.v.) or bilaterally in the amygdala). Intra-amygdala infused rats were functionally tested for anxiety 30 min after the treatment. All animals were sacrificed 1 hour after the infusion and D1 receptor autoradiography was performed on brain sections.

In order to characterize the receptor-receptor interaction in the amygdala under the functional aspect, electrophysiological studies were performed on intercalated cells cluster of *Gad67*-GFP mice. To further investigate the molecular mechanism underlying D1-CRHR1 cross-talk, bioluminescence resonance energy transfer (BRET) assay was carried out in HEK293 cells.

2.3.2 Study 2: Impact of CRHR1-D1 interaction on alcohol dependence

D1 mRNA and protein level was assessed on brain of post-dependent rats by *in situ* hybridization and receptor autoradiography, respectively. Furthermore, post-dependent phenotype of $D1^{Cre}$-$CRHR1^{f/f}$ mice and their littermates was observed to identify the role of D1 and CRHR1 interaction in alcohol dependence (Figure 13B). Mice were gradually habituated to consume 12% ethanol (EtOH; v/v) solution with a two-bottle free-choice paradigm. Once the drinking baseline was reached, animals were exposed to repeated cycles of alcohol vapor intoxication for 4 weeks. Withdrawal signs were checked during the subsequent 12 hours following the last cycle of intoxication. After 3 days of withdrawal, mice had free access to both water and alcohol solution in order to observe a post-dependent drinking behavior. A new baseline was obtained after almost 4 weeks and the CRH system was further challenged with 3 consecutive days of forced swim stress. Alcohol intake was monitored for the next days. Body weight was recorded once a week.

Figure 13: Experimental designs showed by timeline for each study

2.3.3 Study 3: Differential role of dopaminergic and dopaminoceptive CRHR1 on stress-induced drinking behavior

The anatomical organization of the dopaminergic system and its relationship with the CRH system was assessed by immunohistochemistry. Double-immunostainings were performed on C57Bl/6 mice and *Crhr1*-GFP reporter line. Furthermore, to identify the impact of CRHR1 expression on pre- or post- synaptic dopaminergic neurons during stress-induced drinking, the behavior of inducible $DAT^{CreERT2}$-$CRHR1^{f/f}$ and constitutive $D1^{Cre}$-$CRHR1^{f/f}$ mouse lines was observed. As previously described for Study 2, mice were habituated to drink 12% EtOH solution until the achievement of a stable alcohol consumption. One week of vapor exposure (Molander et al., 2012), followed by 3 days of abstinence was applied as a stressful paradigm and the alcohol consumption was constantly monitored during the subsequent days (Figure 13C). Control group for each genotype were not stressed by repeated alcohol vapor exposure, however they were alcohol deprived and daily treated with pyrazole as well as their littermates.

2.3.4 Study 4: αCaMKII-dependent CRHR1 modulates stress-induced alcohol seeking behavior in the central nucleus of the amygdala

The aim of this study consists in defining the role of CRHR1 in αCaMKII-expressing neurons of the central nucleus of the amygdala (CeA) in alcohol seeking behavior. Thus, firstly co-localization between CRHR1 and αCaMKII within the amygdala was assessed by immune-histochemistry in brain sections of *Crhr1*-GFP mice. Then, CRHR1 over-expression was induced in CeA of $αCaMKII^{CreERT2}$ rats and their operant drinking behavior was observed. As the timeline in Figure 13D shows, rats were trained to press first for saccharine- and then for alcohol. After 5 weeks, animals received intra-CeA injections of CRHR1-overexpression virus and were treated with tamoxifen to induce the expression of the recombinase. Before the retraining, locomotor activity of all the animals was established. Cue-, stress and a combination of stress and cue- induced reinstatements were performed after subsequent extinction trainings. Animals were euthanized immediately after the last reinstatement test and the brains were isolated to detect the virus injection sites by *in-situ* hybridization.

2.3.5 Study 5: Opposite effects of CRHR1 on DAT and D1 neurons in cocaine cue-induced reinstatement

The role of CRHR1 in D1- or DAT- containing neurons in cocaine-related behavior was investigated using inducible $D1^{CreERT2}$-$CRHR1^{f/f}$ and $DAT^{CreERT2}$-$CRHR1^{f/f}$ mouse lines,

respectively. Firstly, home cage-locomotion after tamoxifen treatment was measured in both lines. Subsequently, locomotor sensitization to acute and chronic cocaine treatment (Figure 13E) was assessed. Cocaine self-administration experiment was performed as showed in Figure 13E: before the implantation of the intra-jugular catheter, mice were trained to press the levers giving food-reward. Once they recover from the surgery, mice self-administered cocaine for 8 consecutive days (2h/day). The extinction phase, which lasted in average for 2 weeks, was followed by cue-induced reinstatement.

2.4 Pharmacological experiments

2.4.1 Intracranial surgeries and microinjections

For intracranical implantation of guide cannulae (Plastic One, Roanoke, VA) animals were anesthetized with isoflurane (CP-Pharma Handelsgesellschaft mbH, Burgdorf, DE) and placed in a stereotaxic frame. The exact position of the cannula was calculated by Bregma identification (rat brain atlas (Paxinos and Watson, 1998); mouse brain atlas (Paxinos and Franklin, 2004)). The implantation was secured with stainless screws (Bondeye Optical Ltd, Birmingham, UK) and dental cement (Paladur, Heraeus Kulzer GmbH, Hanau, DE). Animals were single housed for 7 days in order to recover completely from the surgery. All cannulae were kept open with 28- or 33-gauge dummies (PlasticsOne, Roanoke, VA).

2.4.1.1 I.c.v. injections in Wistar rats (Study 1) Wistar rats (N=6-7 rats/group) received i.c.v. injections of cerebro-spinal fluid (CSF, 2µl), CRH (2µg/2µl inj volume) (Spina et al., 2002) and Stressin I (4µg/2µl inj volume) (Rivier, 2008). All Ki values are listed in Table 4. The coordinates for the micro-infusions were: Bregma posterior -0.80 mm, lateral ±1.40 mm and ventral -3.2 mm, according to rat brain atlas (Paxinos and Watson, 1998).

2.4.1.2 Intra-amygdala injections in Wistar rats (Study 1) Wistar rats were treated with bilateral infusion in the amygdala of CSF (0.5µl/ hemisphere) or 0.01µg/side of stressin I or with the combination of stressin I with 120ng/side (de la Mora et al., 2005) of SCH23390, a D1-specific antagonist (D1: Ki=0.2 nM; D2 and D3: Ki=0.3nM) (Bourne, 2001). The compounds were injected with a total volume of 0.5µl per each side (Bananej et al., 2012; Zarrindast et al., 2012). Coordinates of the injections were: Bregma posterior -1.80 mm, lateral ±4.2 mm and ventral -7.9 mm (Paxinos and Watson, 1998).

2.4.1.3 I.c.v. injections in D1^{Cre}-CRHR1$^{f/f}$ line (Study 1) D1^{Cre}-CRHR1$^{f/f}$ and control littermates received i.c.v. injections (n=5-7/group) of CSF (2µl inj volume) or Stressin I (0.5µg/2µl inj volume) (Bruchas et al., 2009), according to the following coordinates: Bregma posterior -0.10 mm, lateral +/-0.85 mm and ventral -2.0 mm (mouse brain atlas Paxinos and Franklin, 2004).

Table 4: Affinity values (Ki) of the compounds with CRHR1 and CRHR2

Compound	CRHR1 (Ki)	CRHR2 (Ki)
CRH	0.95 nM *(Donaldson et al., 1996)*	13 nM *(Donaldson et al., 1996)*
Stressin I	1.5 nM *(Rivier et al., 2007)*	224 nM *(Rivier et al., 2002)*

All the compounds were injected with a speed rate of 250nl/min, using a micro-infusion pump (PHD22/2000, Harvard Apparatus, Massachusetts, USA) and Hamilton syringe (25µl) with a microinjector (Plastics One, Roanoke, VA) protruding 0.5 mm below the implanted guide cannula to reach the target area. The needle (28-gauge for rats and 33-gauge for mice infusions) was kept in position for one minute after the end of the injection to avoid back-flow. Animals showing a misplacement of the infusions were excluded from the analysis.

Animals were sacrified by decapitation 1 hour after the microinjection. Brains were quickly removed, frozen in -40°C isopentane (VWR International GmbH, Vien, A) and kept at -80°C until use.

2.4.2 Mapping of the injection sites

For study 2.4.1.2, extra-brain sections were collected and stained using cresylviolet to verify the correct placement of the intra-amygdaloid injections in Wistar rats. Slides were merged for 10 minutes in pure acetone (Sigma Aldric Chemie, Schnelldorf, DE), then dipped in distilled water with 10µl of acetic anhydride (Caesar&Loretz, Hilden, DE) for 2 minutes. Brain sections were then incubated in cresylviolet for 20 minutes and washed again with fresh distilled water, added with

acetic anhydride. Slides were dunked quickly in different solutions with increased concentrations of ethanol (70%, 80%, 90% and 100%), in isopropanol (Merck KGaA, Darmstadt, DE) and finally in xylol (Merck KGaA, Darmstadt, DE) for 5 minutes. Coverslipped brain sections were observed with the optical microscope.

2.5 Expression analysis: mRNA and protein quantification in brain tissues

2.5.1 Quantitative saturated receptor autoradiography

D1 antagonist [^{3}H]-SCH23390 (specific activity = 80.5 Ci/mmol; Kd= 0.7 nM, B_{max}= 347.0 (Schulz et al., 1985), Perkin-Elmer, Massachusetts, USA) was used as radio-labeled ligand for D1 receptors. SKF38393 hydrobromide (Tocris Bioscience, Bristol, UK), a D1-like selective partial agonist, was the cold competitor to identify non-specific binding (Sommer et al., 2014; Bernardi et al., 2015). First, slides were dipped in the pre-incubation buffer containing 50mM Tris (pH 7.4), 5mM MgCl2, 1mM EDTA at room temperature for 15 minutes. This step was repeated a second time with fresh buffer. Then, the incubation buffer (50mM Tris (pH 7.4), 5mM MgCl2, 1mM EDTA, 100mM NaCl, 1mM DTT, 0.1% bovine serum albumin-BSA) was applied on each slide and kept at 30°C for 2 hours. This solution contained also [^{3}H]-SCH23390: 10nM for rat brain sections; 1nM for mouse and 3nM for post-mortem human brain added with 10µM mianserin (Tocris, Bristol, UK). Non-specific binding was estimated by incubating adjacent sections in a buffer containing a mix of [^{3}H]-SCH23390 and SKF38393 hydrobromide (1µM for brain rat sections; 10µM for brain mouse sections) while for human tissues the cold competitor was flupenthixol (10µM, Tocris, Bristol. UK). Slides were washed twice for 2 minutes in fresh 50mM Tris-HCl buffer (pH 7.4). Finally the sections were washed for 2 minutes in ice cold distilled water before fan-drying.

2.5.2 *In situ* hybridization on rat brain tissues

In situ hybridization was performed as previously described (Hansson et al., 2006; Sommer et al., 2008; Bernardi et al., 2014).

2.5.2.1 Fixation Brain sections were first defrosted and then incubated in 4% PFA in PBS for 15 minutes, washed for 10 minutes in PBS, and twice in sterile water for 5 minutes. After treatment in 0.1M HCl solution for 10 minutes and two times with PBS for 5 minutes, brain slides were incubated in 0.1M triethanolamine (pH=8) and 0.25% acetic anhydride buffer for 20 minutes

in order to acetylate proteins. Subsequently, sections were washed twice in PBS for 5 minutes, once in sterile water for 1 minute and dehydrated in a graded series of ethanol (70%, 80%, 99%) leaving the section in each concentration for 2 minutes. After air drying, sections were stored at -80°C in sealed boxes with silica gel to avoid moisture.

2.5.2.2 RNA probe synthesis Rat specific riboprobes were generated from rat cDNA templates showed in the table below. Antisense and sense RNA probes were obtained by incubating 200 ng DNA with transcription buffer (Ambion® Applied Biosystems, Darmstadt, Germany). The solution contained also 12.5 nmol of ATP, CTP, GTP, 50pmol UTP and 125pmol [^{35}S]-UTP (1250 Ci/mmol, Perkin Elmer, Rodgau, Germany), 1 U RNase inhibitor and 1 U RNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany) at 37°C for 90 minutes. Afterwards, the DNA was digested with RNase-free DNase at 37°C for 20 minutes and the transcripts were purified using spin columns (illustra^{TM} Microspin^{TM} S-200 HR Colums, GE Healthcare, Munich, Germany). The counts per minute (cpm) were assessed with the Liquid Scintillation Analyzer (1600 TR).

Table 5: Gene-specific RNA riboprobes used for *in situ* hybridizations on rat brain sections

mRNA	RefSeq	Position (bp) from- to	
D1 *(study 2)*	NM_012546.2	60	1400
Cre^{ERT2} *(study 4)*	Plasmid	307	672
αCaMKII *(study 4)*	NM_009792.3	229	439
Crhr1 *(study 4)*	NM_030999	85	492

2.5.2.3 In situ hybridization The pre-hybridization step consists in incubating the slides with pre-hybridization buffer (50 % deionized formamide, 50 mM Tris-HCl pH7.6, 25 mM EDTA pH8.0, 20 mM NaCl, 0.25 mg/ml yeast tRNA, 2.5 x Denhardt's solution (Invitrogen, Darmstadt, Germany) at 37°C for 2-4 hours. Then, the sections were hybridized with 100 µl hybridization

buffer (50% deionized formamide, 20 mM Tris-HCl pH 7.6, 10x Denhardt's solution, 5 mg/ml yeast tRNA, 1 mg/ml polyadenylic acid, 10 mM EDTA pH 8.0, 150 mM DTT, 330 mM NaCl, 10% dextransulphate) added with 1 x 106 cpm of either the labeled antisense RNA or sense RNA. The sections were immediately covered with siliconized coverslips and incubated at 55°C overnight in a humidified chamber. Coverslips were removed with 3 consecutive washing steps using 1x standard saline citrate (SSC) solution at 42°C for 40 minutes. Afterwords, the sections were washed in 0.5x SSC/50% formamide for 1 hour at 42°C. Following two additional washing steps in 1x SSC for 30 min at 42°C the sections were treated with 1 µg/ml RNaseA in RNase buffer (0.5 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 7.5) for 1 hour at 37°C. After two washing steps in 1x SSC for 30 minutes at 55°C followed by a brief washing in 1xSSC at RT, the sections were dehydrated in graded ethanol and air-dried.

BAS-TR 2025 imaging plates were exposed to the dry slides for 1 week and then scanned by the phosphorimager (Fuji phosphorimager Typhoon FLA 700, GE Healthcare Life Sciences, Pittsburgh, USA). Densitometric analysis was performed using the MCID Image Analysis Software (Imaging Research Inc., UK). Mean density values were measured as minimal detectable change (MDC) units per mm^2, compared against standard curves generated using [^{14}C]-Microscales (Amersham, GE Healthcare Life Sciences, Pittsburgh, USA). Data are expressed as nCi/g (means ± SEM).

2.5.3 Densiometric measurements

Brain sections were dried under a stream of cold air and were exposed to Fuji Imaging Plate BAS-TR2025 (GE Healthcare Life Science, Pittsburgh, USA). After 1 week, Fuji plates were scanned with the phosphorimager (Typhoon FLA 700, GE Healthcare, Germany). The digital images obtained, were used to measure the signal density by MCID Image Analysis Software (Imaging Research Inc., UK). Signal density was measured as minimal detectable change (MDC) units per mm^2. For receptor autoradiography assays, the measurements were converted into fmol of receptor per mg protein tissue by plotting values in a standard curve obtained by a [^{3}H]-microscales (Amersham, GE Healthcare Life Sciences, Pittsburg, USA). Binding in femtomoles per milligram (fmol/mg) was calculated based on the specific activity of the radioligand. For *in-situ* hybridization assays the values were compared against standard curves generated using [^{14}C]-Microscales (Amersham, GE Healthcare Life Sciences, Pittsburgh, USA) and the data were expressed in nCi/g (means ± SEM).

2.6 Fluorescent double-labelling immunohistochemistry

Crhr1-GFP (N=3) and C57Bl/6 (N=4) mice were deeply anesthetized with isoflurane and intracardially perfused with 0.9% NaCl solution with 10000 IE/1 heparine and then with fixative solution (phosphate buffer, containing 4% paraformaldehyde and 14% saturated picric acid). Brains were collected, post-fixed for 1 hour, dehydrated in 1xPBS solution added with 10% sucrose for 3 days and finally frozen at -80°C. Fluorescent double-labeling immunohistochemistry were performed as described by Hansson and co-workers (Hansson et al., 1998; Hansson et al., 2003).

Table 6: Table. Primary antibodies applied for double-immunostainings

Double-immunostaining	Target	Host	Dilution	Source
NeuN-TH *(study 3)*	NeuN	Mouse	1:200	Millipore
	TH	Sheep	1:500	Millipore
D1-NeuN *(study 3)*	D1	Rat	1:400	Sigma
	NeuN	Mouse	1:200	Millipore
D1-TH *(study 3)*	D1	Rat	1:400	Sigma
	TH	Sheep	1:500	Millipore
***Crhr1*GFP-NeuN** *(study 3)*	*Crhr1*-GFP	Rabbit	1:300	Invitrogen
	NeuN	Mouse	1:700	Millipore
***Crhr1*GFP-TH** *(study 3)*	*Crhr1*-GFP	Rabbit	1:500	Invitrogen
	TH	Sheep	1:500	Millipore
***Crhr1*GFP-D1** *(study 3)*	*Crhr1*-GFP	Rabbit	1:500	Invitrogen
	D1	Rat	1:800	Sigma
***Crhr1*GFP-CaMKII** *(study 4)*	*Crhr1*-GFP	Rabbit	1:500	Invitrogen
	CaMKII	Mouse	1:500	Pierce Biotechnology

12µm thick coronal sections were cut with the cryostate, mounted on gelatin-coated slides and stored in -20°C until the use. Sections were brought to room temperature, rehydrated, rinsed in 0.01M PBS buffer, and incubated with a mixture of two primary antibodies (see Table 6) at 4°C overnight. The sections were rinsed in 0.01M PBS/ 0.3% Triton buffer, and a mixture of the two secondary antibodies (conjugated with Alexa 488 or Alexa 594) were added and incubated for 1–2 h at room temperature. Sections were rinsed briefly, mounted in a mounting medium for fluorescent signals (Dako, Carpinteria, CA), and coverslipped. Slides were investigated using a Leica TCS SP2 imaging system mounted on a DM IRE2 microscope (Leica Microsystems, Wetzlar, Germany) using an X63 oil lens and either an argon ion laser (458–514 nm) or a green neon laser (543 nm). For the quantification, Z-stacks of 0.49µm were acquired on both hemispheres from region of 4-3 mice. The number of stained cells was counted using the cell counter analysis macro of Image J software. The percentage of co-localization was calculated in Means ± SEM for all the animals.

2.7 Crhr1 over-expression virus production and injection

2.7.1 AAV vector generation and administration.

Production of pseudo-typed rAAV1/2 mosaic vectors and determination of genomic titers was performed by the group of Dr. Matthias Klugmann at the University of New South Wales, Australia (Klugmann et al., 2005) and applied in study 4. After a stable self-administration baseline was reached, rats were bilaterally injected with either the *CRHR1* overexpression virus either the control virus. Animals were anaesthetized with isofluran and placed in a Kopf stereotaxic instrument. A WPI microinjection pump with a 33 gauge blunt needle was used to deliver 500nl of virus (5 x 10 vector genomes) into the CeA (coordinates: AP: -2.3 mm, ML: ±4.2 mm, DV: -7.8 mm according to (Paxinos and Watson, 1998)) at a speed rate of 100nl/min. The needle was left in place for 5 minutes after the end of the injection to avoid any backflow.

2.7.2 Induction of the overexpression by tamoxifen injection

After a recovery period of 4 days, tamoxifen was injected i.p. for 5 consecutive days, with each one injection on day 1, 3 and 5 and two injections on day 2 and 4 (Schönig et al., 2012). Rats were allowed to recover for 6 weeks during which the CRHR1 overexpression in αCaMKII-containing neurons of the CeA occurred.

2.8 Electrophysiological experiments

Preliminary electrophysiological experiments in study 1 were performed by Dr. Kai Jüngling and Dr. Hans-Christian Pape from the Institute of Physiology I (Neurophysiology), Westfälische Wilhelms-University Münster, Münster, Germany.

Naïve and post-dependent *Gad67*-GFP mice will be anesthetized and killed by decapitation. Amygdala coronal sections (300 µm thickness) from *Gad67*-GFP mice were cut with the vibratome and whole-cell patch-clamp recordings were performed on ITCs as described previously (Jungling et al., 2008). Briefly, GFP-expressing ITCs were identified by fluorescent microscopy and the recording was carried out either in the current-clamp or voltage-clamp mode to analyze dopamine-dependent effects on the membrane potential or stressin I-induced currents, respectively. All recordings were done in CSF at 32°C. Dopamine (10 µM) was applied by fast bath perfusion (2.5 – 3 ml/min) together with 0.5µM tetrodotoxin (TTX), GluR and GABA-R antagonists to avoid unspecific spontaneous action-potential generation. To investigate CRHR1-dependent effects on D1-signaling, a subset of slices were pretreated with the CRHR1-specific agonist stressin I (250 nM) 45 minutes prior to recordings. Dopamine-induced currents and/or changes of the membrane potential were analyzed and compared with the stressin I treatment effect.

2.9 Bioluminescence Resonance Energy Transfer (BRET) Assay

BRET assay were performed by Dr. Dasiel O. Borroto-Escuela and Dr. Kjell Fuxe from the department of Neuroscience, Karolinska Institutet, Stockholm, Sweden.

Human dopamine D1 and CRHR1 coding sequences without their stop codons were amplified from 3xHA-D1R-pcDNA and CRHR1-pcMV6 vectors and then sub-cloned into humanized pGFP2-N1 vectors (PerkinElmer, Waltham, MA, USA) and humanized pRluc-N3 vector (Packard Bioscience, Barcelona, Spain) respectively. For transfection, HEK293T cells were plated in 6-well dishes at a concentration of 1 × 106 cells/well or in 75cm2 flasks and cultured overnight before transfection. Cells were transiently transfected using TransFectin (Bio-Rad, Sweden). Forty-eight hours after transfection, HEK293T27 cells transiently transfected with constant (1µg) or increasing amounts (0.12-5µg) of plasmids encoding for D1-Rluc and CRHR1-GFP2 respectively, were rapidly washed twice in PBS, detached, and resuspended in the same buffer. Cell suspensions (20µg protein) were distributed in duplicate into the 96-well microplate black plates with a transparent bottom (Corning

3651) (Corning, Stockholm, Sweden) for fluorescence measurement or white plates with a white bottom (Corning 3600) for BRET determination. For BRET2 measurement, coelenterazine-400a also known as DeepBlue™C substrate (VWR, Sweden) was added at a final concentration of 5µM. Readings were performed 1 minute after using the POLARstar Optima plate-reader (BMG Labtechnologies, Offenburg, Germany) that allows the sequential integration of the signals detected with two filter settings: 410 nm (with 80 nm bandwidth) and 515 nm (with 30 nm bandwidth). The BRET2 ratio is defined as described in (Borroto-Escuela et al., 2010; Borroto-Escuela et al., 2013).

2.10 Behavioral experiments

2.10.1 Home cage locomotion

Locomotor activity was measured using a passive infrared sensor incorporated in the Mouse-E-motion Universal Data Logger (Infra-E-Motion GmbH, Hamburg, Germany). Warmth radiation emitted by the animal was recorded by the sensor. Since plastic cages were impermeable for infrared radiation, external inputs from outside or neighbor cages were not detected. All movements were recorded every 4 minutes for 3 consecutive days and were expressed as activity/4h. The devices were used to monitor the locomotion on $D1^{Cre}$-$CRHR1^{f/f}$, $DAT^{CreERT2}$-$CRHR1^{f/f}$ and $DAT^{CreERT2}$-$CRHR1^{f/f}$ mouse lines as well as on post-dependent rats and their controls.

2.10.2 Open field

Open field experiments were performed to observe the effect on locomotion in $\alpha CaMKII^{CreERT2}$ rats injected with either CRHR1-overexpression or control virus (Study 4). Rats were placed in the center of the arena (51 cm x51 cm x 50 cm) and left free to explore it for 15 minutes with a light intensity of 10.5 lux. The observation program used for the analysis in rat was Viewer^2 (Biobserve GmbH, Bonn, Germany). Boxes were cleaned with 50% ethanol solution and dried after each session.

For study 1, locomotion activity of $D1^{Cre}$-$CRHR1^{f/f}$ (n=29) and control $CRHR1^{f/f}$ (n=22) mice was assessed for 15 minutes in a TruScan activity arena (Coulbourn Instruments, USA). Each arena (27 x 27 x 39 cm) was surrounded by photocells which detect mouse movement. Light intensity was set up at 20lux. Total locomotion activity was measured as distance travelled (cm) and the time spent in the center, expressed in seconds (s) was used as index for anxiety-like behavior.

2.10.3 Elevated-Plus-Maze (EPM)

The elevated plus-maze (EPM) test was applied to measure anxiety-like responses 30 minutes after the intra-amygdaloid injections in Wistar rats and for the characterization of the $D1^{Cre}$-$CRHR1^{f/f}$ mouse line (Study 1). The experimental setting consisted of two open arms (50 cm x 10 cm for rats, 38 x 7 cm for mice) crossed at right angle with two enclosed arms of the same size. All arms had an open roof and the entire apparatus was elevated 50 cm above the floor. At the beginning of the experiment, each animal was placed in the center of the maze, facing a closed arm, and left free to explore in all four directions for 5 minutes. The light intensity in the apparatus was set at 30 lux. The percentage of time spent in the open arms was used as a measure of anxiety-like behavior, while the number of total arm entries was considered as an indicator of general locomotor activity. All arms were cleaned with 50% ethanol solution and dried after each trial.

2.10.4 Dark-light Box

The dark-light box paradigm was used to characterized the baseline behavior of $D1^{Cre}$-$CRHR1^{f/f}$ mice (N=14) and their littermate (N=10) (Study 1). It consists in a dark, protected compartment connected with a short tunnel to a bright (40 lux) arena. After 30 seconds of habituation in the dark compartment, the animal was allowed to move freely in both arenas for 5 minutes. The time spent in the light compartment and the number of entries between the two areas was considered as a measurement of anxiety. The apparatus was cleaned with 50% ethanol solution and dried after each round.

2.10.5 Fear Conditioning

To further characterized the baseline behavior, $D1^{Cre}$-$CRHR1^{f/f}$ mice (N=15) and their littermate (N=11) were tested for fear conditioning procedure (Study 1). The conditioning apparatus consisted in two different chambers inserted in a sound- and light-protected isolation cubicle (Habitest H10-24TA, Coulbourn Instruments, USA). Context A (17 cm × 18 cm × 32 cm) had two transparent walls and stainless steel grid floors (H10-11M-TC, Coulbourn Instruments, USA) from which 0.6 mA scrambled footshock was delivered from a precision animal shocker (H13-15, Coulbourn Instruments, USA). Context B had four transparent walls, stainless steel grid floors, and was 30cm × 25cm × 25cm in size (Med Associates, USA). The sound stimulus was a 5000-Hz, 80- to 85-dB tone and delivered via speakers into the chamber. The movements of the tested animal were recorded with a digital video camera mounted at the ceiling of the cubicle and analyzed for the percentage of freezing using FreezeView software (Actimetrics Software) (Waltereit et al., 2008).

During the acquisition phase, mice were placed in context A. After the first 3 minutes of adaptation, conditional fear was induced by presenting five pairings of 30 seconds acoustic stimulus which terminated with a single foot shock. Stimulus-free periods had a random length. On the next day context-conditioning was assessed by placing the mice in context A without deliver any tone or foot shock. Freezing was analyzed for 6 minutes. Mice were then moved back into their home cages for at least 5 hours before testing them for the cue-conditioning. Auditory cue conditioning was assessed by placing the mice in context B. After 3 minutes of acclimatization, the sound stimulus was presented for 6 minutes. Freezing behavior was recorded during the whole experimental session.

2.11 Alcohol drinking behavior in mice

2.11.1 Long-term voluntary alcohol consumption in mice (two-bottle free-choice paradigm)

Voluntary alcohol intake was measure in constitutive $D1^{Cre}$-$CRHR1^{f/f}$ (Studies 2 and 3), inducible $DAT^{CreERT2}$-$CRHR1^{f/f}$ (Study 3) and their control littermates by a two-bottle free-choice paradigm, according to standardized procedures (Spanagel and Holter, 1999). During the initial phase of the experiment, mice were habituated to alcohol taste by substituting one of the two tap water bottles with an ethanol solution. Alcohol concentration was increased every 3 days (2%, 4%, 8%, v/v) up to 12% ethanol for the rest of the experiment. The position of the bottles was switched randomly to avoid preference to any particular side of the cage. All bottles were weighted and fresh prepared every 3 days, while mice were weighted once a week. The amount of alcohol consumption was expressed as the absolute amount of solution (taking into account ethanol density (0.8 g/ml)) consumed each day with respect to the weekly weight of the animal (g/kg/day). Baseline drinking was defined as level of alcohol intake maintained stable for three consecutive measurements.

2.11.2 Chronic Intermittent Ethanol Exposure in mice

Once a stable baseline was achieved, mice were divided into chronic ethanol exposed group and controls. The ethanol vapor exposed mice received a cycle of 16 hours of continuous exposure to ethanol vapor in inhalation chambers, followed by 8 hours periods of withdrawal. In order to investigate the post-dependent phenotype in constitutive $D1^{Cre}$-$CRHR1^{f/f}$ mice (Study 2), each cycle was repeated for 5 consecutive days per week, for 4 weeks (modified from Becker and Lopez,

2004). While in Study 3, inducible DAT^{CreERT2}-CRHR1$^{f/f}$ and D1^{CreERT2}-CRHR1$^{f/f}$ mice were chronically stressed through one week of ethanol vapor exposure.

The vapor chamber system was purchased from La Jolla Alcohol Research (La Jolla, California, USA). A peristaltic Q-pump (Knauer, Berlin, Germany) delivered 96% alcohol into a flask at a flow rate of 0.44ml/min. This round-bottom flask was placed inside the heater so that drops of alcohol on the bottom were vaporized. Airflow controlled by a pressure gauge was delivered to the flask and serves to carry the alcohol vapor to the individual chambers through the tubes connected to the 4 side-arms. The airflow was adjusted to 5.9 l/min to maintain the ethanol concentration of 10-15 mg/l air in the chamber. Each chamber was connected to a vacuum to guarantee a constant circulation of primed air. Before each cycle of vapor exposure mice were i.p. injected with ethanol (1.6 g/kg; 8% w/v; 96% EtOH) and blood alcohol concentration (BAC) was stabilized by administration of 1 mmol/kg of pyrazole (Sigma Aldric, Germany), an alcohol dehydrogenase inhibitor. Control mice were handled similarly, but received injections of saline and pyrazole (Becker and Lopez, 2004; Lopez and Becker, 2005; Griffin et al., 2009). The BAC of exposed mice was weekly assessed after the second and fourth cycle (Lopez and Becker, 2005). Blood samples (20 µl), obtained from the saphenous vein were centrifuged for 30 minutes at 800 rpm to obtain 5 µl of plasma and ethanol concentration was measured using an AM1 Analox system (Analox Instruments Ltd, London, UK). BAC range was maintained between 150 and 250 ml/dl according to Becker and Lopez study (2004).

2.11.3 Ethanol withdrawal severity

After the last intoxication cycle, ethanol withdrawal severity was assessed (Mutschler et al., 2010). All mice were scored for withdrawal symptoms immediately after the last vapor exposure cycle (time 0), and during the next 4, 8 and 12 hour. Neuro-vegetative withdrawal signs like tremor, piloerection, tail rigidity, vocalizations, teeth chattering and wet dog shake (WDS) were assessed by observing each mouse for maximum 5 minutes. Each symptom was scored as 0, 1 and 2, indicating low, middle and high severity respectively. The sum of the incidence of each sign represents the entire withdrawal score.

2.11.4 Forced Swim Stress

Repeated forced swim stress was performed on post-dependent mice and their controls in order to observe a stress-induced drinking behavior. Mice were placed in a glass cylinder (25 cm high, 14 cm wide), filled for two thirds with water, to avoid mice to touch the bottom with the tail. The water

temperature was maintained at 21°C. Each trial lasted for 5 minutes and mice were tested for 3 consecutive days. After each trial, mice were gently dried and moved back to their home cage with free access to water and alcohol. On the following three days the bottles were weighed daily to observe any variation in alcohol intake.

2.12 Alcohol drinking behavior in rats

2.12.1 Chronic Intermittent Ethanol Exposure in rats

Rats were weight-matched, assigned into the two experimental groups (N=8rats/group) and exposed to either ethanol vapor or normal air using a rodent alcohol inhalation system (Rimondini et al., 2002; Rimondini et al., 2003; Meinhardt et al., 2013). Three weeks after the last cycle of ethanol vapor intoxication, rats were sacrified and the brains were quickly collected, frozen in −40°C isopentane (VWR International GmbH, Vien, Austria) and kept at -80°C for molecular analysis.

2.12.2 Alcohol self-administration and alcohol seeking behavior

In Study 4, alcohol-seeking experiments were performed in operant chambers (MED Associates) enclosed in ventilated sound-attenuating cubicles. Each chamber is equipped with a retractable response lever on each side panel of the camber. Pressing of the left lever activated a syringe pump, which delivered 30 µl of fluid into a liquid receptacle next to the lever. A light stimulus (house light) is mounted above the response lever. An IBM-compatible computer controlled the delivery of fluids, presentation of stimuli, and data recording.

To train the rats to press the active lever while omitting the inactive lever, a saccharine-fading procedure was employed. During the first six training sessions, left lever presses were rewarded with the delivery of 30 µl of 0.2% saccharine on a fixed-ratio 1 (FR1) schedule, while right lever presses were recorded, but did not have any programmed consequences. The rats were water-deprived 16 hours before each of the first three training sessions, after this they had again *ad libitum* access to water. The six 0.2% saccharine sessions were followed by one day of 0.2% saccharine + 5% (v/v) ethanol, one day of 5% ethanol, one day of 0.2% saccharine + 8% ethanol, one day of 8% ethanol, one day of 0.2% saccharine + 10% ethanol, finally concluded with two sessions where only 10% ethanol was delivered.

Once the rats were trained to press the lever, the actual self-administration training commenced. Here, responses to the left lever was rewarded by delivery of 10% ethanol and the activation of the house light (3 seconds blink light), which also indicated a 3 seconds "time-out" period, during which additional lever presses of the active lever was recorded but did not lead to further delivery of 10% ethanol. In addition to the conditioned light stimulus, orange odor was presented during the entire session as an environmental stimulus, by adding 6 drops of orange extract to the bedding of the operant chambers. The bedding was changed and the trays cleaned at the end of each training session. After 10 self-administration sessions, a stable baseline was reached. The extinction phase took place after four additional training sessions following the behavioral tests. Here, response to the left lever did not result in the delivery of ethanol or the activation of the blink light, and the environmental stimulus was omitted. Extinction criterion was active lever responses <10% of the baseline of the last 3 training sessions. The conditions during cue reinstatement were identical to training sessions, except that the pressing of the left lever did not result in ethanol delivery. Both the conditioned and environmental stimuli were presented. For the stress-induced reinstatement, yohimbine was injected (1.25 g/kg, i.p.) 30 minutes before the start of the test session. The conditions in the operant chamber were identical to the extinction phase. During the combination of stress- and cue- induced reinstatement, rats were injected with yohimbine (1.25 g/kg, i.p.) 30 minutes before the start of the test sessions. As for the cue reinstatement, the conditioned and environmental stimuli were presented, and active lever pressing did not result in the delivery of ethanol.

2.13 Cocaine-mediated behavior in mice

2.13.1 Cocaine sensitization

The acute and sensitized locomotor response to cocaine was assessed in eight TruScan activity monitors (Coulbourn Instruments, USA) (Study 5). Each monitor consists of a clear acrylic plastic test cage (27 x 27 x 39 cm) placed inside a monitoring unit that records via computer ambulatory beam interruptions from infrared photocell emitter/detector pairs evenly spaced along each axis. Mice were habituated to the locomotor activity monitors for 60 min/day for three consecutive days. Following habituation, mice received 15 mg/kg cocaine i.p. or saline (10 ml/kg) on subsequent days for 5 days (5 injections). Cocaine injections on days 1 and 5 were followed by 60 minutes of

locomotor measurements in the activity monitors. Locomotor activity was measured as distance travelled (cm).

2.13.2 Cocaine self-administration

Cocaine self-administration was performed in 12 operant chambers (Med Associates, USA) housed in light- and sound-attenuating cubicles (Study 5). Each chamber (24.1 x 20.3 x 18.4 cm) was equipped with two levers (left and right), a food dispenser and a drug delivery system connected via infusion pump (PHM-100, Med-Associates, USA) located outside the cubicle. Operant chambers were controlled using Med-PC IV (Med Associates, USA) software. Mice first underwent lever training with 14mg sweetened food pellets (TestDiet, USA), as previously described (Bernardi and Spanagel, 2013). Following lever training, mice were implanted with an indwelling intravenous catheter (made in-house) into the jugular vein. Catheter patency was maintained with 0.15ml heparanized saline (100i.u./ml) containing Baytril (0.7mg/ml) administered daily throughout the experiment. After 3days of recovery, mice underwent to 2 hours to daily cocaine self-administration for 8 consecutive days. Cocaine (0.50 mg/kg/14 µl infusion) delivery was delivered together with pressing on the active lever under an FR1 schedule of reinforcement and paired with the 20 seconds presentation of a blinking light stimulus (Conditioned Stimulus, CS), which also served as a timeout period, during which lever presses were not reinforced. For all experiments, presses on the inactive lever were recorded but had no scheduled consequence. Following cocaine self-administration, mice were exposed to daily 2 hours extinction sessions, during which presses on the active lever were not reinforced, and the CS was omitted. Extinction concluded when each mouse reached a criterion level of responding on the active lever (less than 33% responding over two days relative to the mean active lever presses during the last two days of self-administration). Once extinction criteria were reached, cue reinstatement was tested during a single 2 hours session that began with a 5 seconds blinking of the light CS, and during which active lever presses resulted in the presentation of the 20 seconds blinking light CS, but no cocaine.

2.14 Statistical analysis

All experimental data are presented as Means ± SEM and the chosen criteria of significance for all the analysis was *p < 0.05, **p< 0.01, ***p<0,001. Statistical analysis was performed by the software Statistica 10 (StatSoft, Tulsa, USA) or SPSS22 (IBM, Chicago, USA). Graphical

representations of the data were provided by using the Prism 5 software (GraphPad, San Diego, USA).

Results obtained by receptor autoradiography and *in-situ* hybridization assays had homogeneous variance within respective regions and were therefore compared by region-wise one-way parametric analysis of variance (ANOVA), followed by Bonferroni correction. Two-way ANOVA analysis was applied to the D1 binding data gathered from pharmacological treatment of $D1^{Cre}$-$CRHR1^{f/f}$ mice, followed by Fisher's PLSD post-hoc test.

For electrophysiological experiments, statistical analysis during perfusion of drugs was performed for the last 10 min of every condition using two-way ANOVA followed by Bonferroni's post-hoc test and comparisons between control and dependent rats were performed using unpaired Student's t-test.

Data obtained from BRET assays were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison post-test. BRET isotherms were fitted using a nonlinear regression equation assuming a single binding site, which provided $BRET_{max}$ and $BRET_{50}$ values.

All drinking data (daily alcohol consumption and withdrawal scores) were analyzed by two-way analysis of variance (ANOVA) with repeated measures (factors were: genotype, or vapor exposure/stress). Whenever significant differences were found in the main effect, post-hoc Newman–Keuls tests were performed. Percentage of floating during FSS and stress-induced ethanol intake after repeated FSS were analyzed by two-way ANOVA (factors were: genotype, treatment) with Fisher´s post-hoc test.

Data obtained by alcohol self-administration and reinstatement in rats and cocaine-mediated behaviour in mice were analyzed using two-way ANOVA with repeated measures (with-in analysis). When allowed, Newman-Keuls post-hoc test was applied.

Results provided by locomotion and anxiety-like behaviors, such as Open Field, Elevate Plus Maze and Dark-Light Box tests, were analyzed by one-way ANOVA statistics. Time and genotype differences in fear conditioning and home cage-locomotion tests were assessed by repeated measures ANOVA followed by Newman-Keuls post-hoc test.

PART III: RESULTS

3.1 STUDY 1: CROSS-TALK OF CRHR1 AND D1 ON ANXIETY-RELATED BEHAVIOR

Refojo and colleagues (2011), demonstrated the cell-type specific (i.e. dopaminergic, glutamatergic, GABAergic and serotonergic) impact of CRHR1 in the regulation of anxiety. Furthermore, immuno-histochemical studies demonstrated a co-distribution of D1 and CRHR1 in the amygdala, mainly detectable in the intercalated cell (ITC) clusters (Jacobsen et al., 2006; Justice et al., 2008). Based on these findings we hypothesize that CRHR1, via the interaction with the dopamine receptor D1 may play a role in the regulation of emotional responses to stressful stimuli.

Here, we first applied immunohistochemistry to demonstrate a co-localization of the two receptors. We then performed pharmacological experiments using CRHR1 agonists (CRH and stressin I) and D1 antagonist (SCH23390) to show consequences of D1-CRHR1 interaction on D1 availability by receptor autoradiography and on anxiety-like behavior by Elevated Plus Maze (EPM), using naïve Wistar rats and knockout mice lacking CRHR1 receptors within D1 expressing neurons ($D1^{Cre}$-$Crhr1^{f/f}$). Furtheron, electrophysiological studies and bioluminescence resonance energy transfer (BRET) assays were performed to give insight into mechanistic aspects underling D1-CRHR1 interaction.

The $D1^{Cre}$-$Crhr1^{f/f}$ mouse line was generated and provided by Dr. Jan Deussing from the Max-Plank Institute of Munich, Germany. Preliminary results from electrophysiological experiments were carried out by Dr. Kai Jüngling and Dr. Hans-Christian Pape from the Institute of Physiology I (Neurophysiology), Westfälische Wilhelms-University Münster, Münster, Germany. BRET assays were performed by Dr. Dasiel O. Borroto-Escuela and Dr. Kjell Fuxe from the department of Neuroscience, Karolinska Institutet, Stockholm, Sweden.

3.1.1 Co-localization of D1 and CRHR1 using a *Crhr1*-GFP reporter mouse line

Since it has been difficult to obtain specific CRHR1 immunostaining, we used transgenic mice expressing GFP under the control of *Crhr1* promoter (Justice et al., 2008) to demonstrate that D1 receptor immunoreactivity (ir) is present in *Crhr1*-GFPir positive cells. The *Crhr1*-GFP mouse line was kindly provided by Dr. Nicholas J. Justice from the University of Texas Health Sciences Center, Houston, Texas, USA.

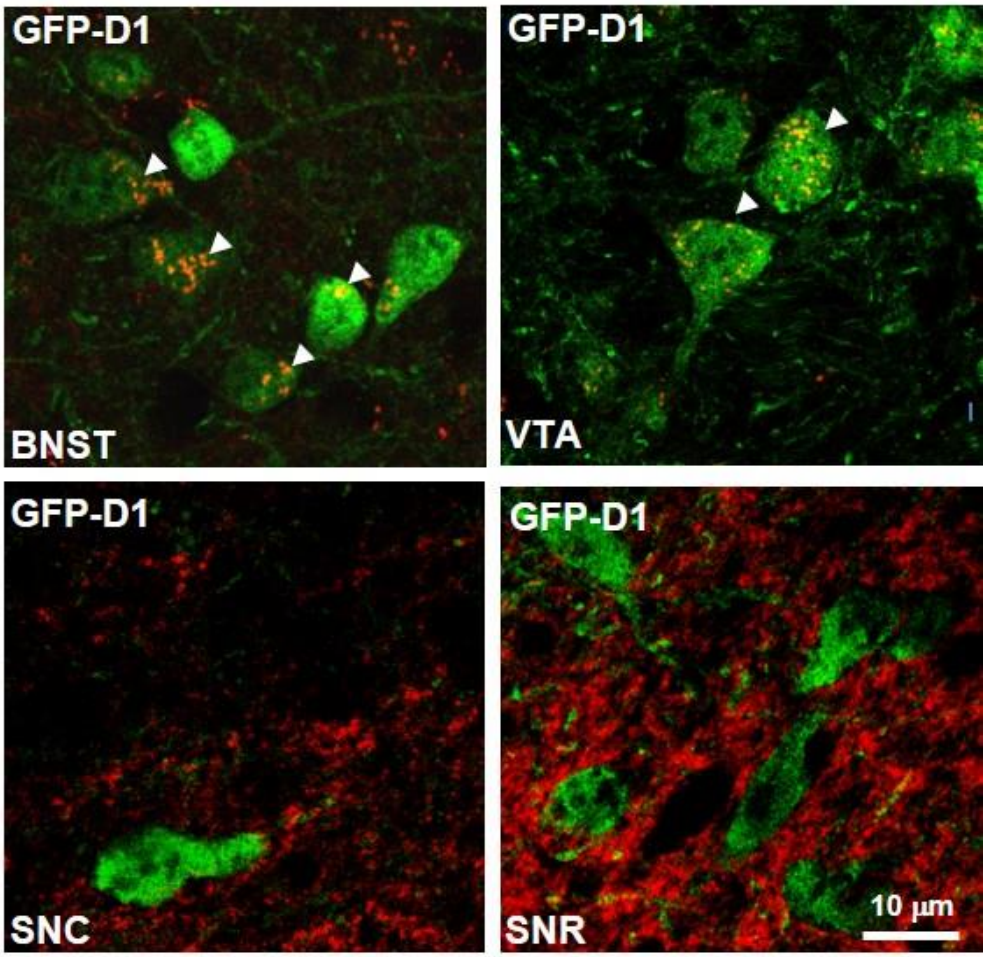

Figure 14: Co-localization of CRHR1 and D1 in different forebrain regions using the *Crhr1*-GFP reporter line. Confocal microscopic images show immunofluorescence staining for GFP-ir (green) and D1-ir (red) in merged channels. The substantial neuronal population of GFPir-stained cells overlaps with D1ir-expressing neurons in the bed nucleus of the stria terminalis (BNST) and the ventral tegmental area (VTA). No co-expression was found in the Substantia Nigra compacta (SNC) or reticulata (SNR)**.** White arrow points to co-localized receptors.

Coronal brain sections of 12 µm of thickness were taken from different Bregma levels including bed nucleus of the stria terminalis, (BNST, Bregma: +0.26 to -0.10 mm), amygdala (Bregma: -0.82 to -1.46 mm), ventral tegmental area (VTA), substantia nigra pars compacta (SNC) and reticulata (SNR) (Bregma: -2.92 to -3.28 mm) according to mouse brain atlas (Paxinos and Franklin, 2004). For co-localization studies, immunohistochemistry was performed using species-matched, fluorochrome-conjugated secondary antibodies (Alexa 488-green (GFP), Alexa 594-red (D1)) and a confocal laser-scanning microscope with filter sets for excitation/emission wavelengths at 495/515 nm (green) and 555/605 nm (red).

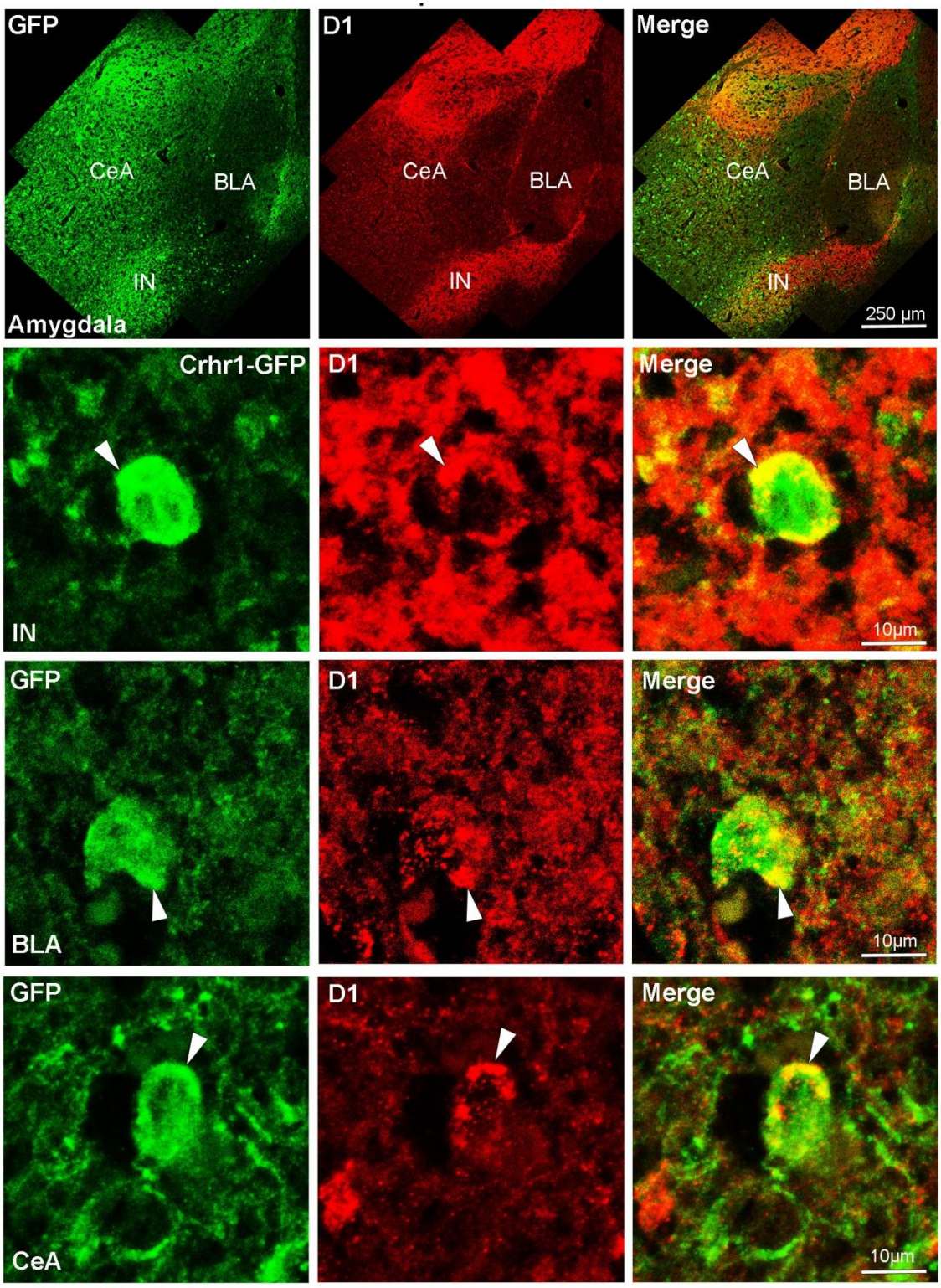

Figure 15: D1-CRHR1 co-localization in the amygdala brain region. A-C: Confocal images present an overview of the entire amygdala obtained by double-immunostaining for GFPir (green), D1ir (red) and co-localized receptors (yellow) in merged channels. Amygdala sections co-stained for GFP and D1 reveal substantial overlap in the IN (D-F), BLA (G-I) and CeA (J-L). White arrow points to co-localized receptors. BLA: basolateral nucleus of the amygdala; CeA: central nucleus of the amygdala; IN: main nucleus of the intercalated cells.

The *Crhr1*-GFPir (green color) is found in the nucleus and cytoplasm of the neurons, while D1ir (red color) is mainly directed to the neuronal dendrites (Figures 14 and 15). For this reason, the co-localization between GFPir- and D1ir is visualized in orange-to yellow color-labelled dendrites overlapping with the *Crhr1*-GRFir -green labelled cellular soma of merged images. Both BNST and VTA regions displayed co-localization of both receptors, while no co-localization was found in SNC and SNR (Figure 14).

As described in previous studies by Jacobsen et al. (2006), and Justice et al (2008), the ITC clusters of the amygdala were highly enriched by D1ir- and *Crhr1*-GFPir. A co-localization was found in all ITCs, i.e. main nucleus of the intercalated cells (IN), ventral, lateral, medial intercalated nucleus and amygdala subregions BLA and CeA (Figure 15).

3.1.2 Pharmacological experiments

3.1.2.1 CRHR1 activation increased D1 binding sites exclusively in the amygdala brain region

Wistar rats received intra-cerebroventricular (i.c.v.) injections of CRH (n=5) which is the natural ligand for both CRHR1 and CRHR2, stressin I (n=6), a CRHR1 specific agonist and vehicle cerebrospinal fluid (CSF, n=6). One hour after the micro-infusions, animals were sacrificed and D1 receptor autoradiography was performed on coronal brain sections. D1 binding sites are expressed as fmol/mg (mean ± SEM) and presented in Supplementary Table 1 for all analyzed regions. Statistical analysis was performed by one-way ANOVA followed by Fisher's PLSD post-hoc test, F- and p-values are summarized in Supplementary Table 2 for each region.

A 42% increase of D1 binding sites was observed in the ventral paracapsular island (Ivp) upon CRH receptor activation by CRH (main effect treatment: $F[2,14]= 14.5$, $p<.001$). Specific activation of CRHR1 by stressin I induced a stronger effect on increased D1 binding sites as compared to the vehicle-treated rats (53% increase, $p<.001$, Figure 16A). A similar regulation was found in the IN and in the dorsal paracapsular intercalated (Idp) cells of the amygdala, but only a trend or no effect on D1 was found in the medial (Imp) and lateral (Ilp) paracapsular clusters, repectively (see Supplementary material Tables 1 and 2). In the BLA, stressin I induced D1 binding sites were increased by 80% ($p<.001$; Figure 16B). A similar effect towards increased D1 was found in the other amygdala nuclei (i.e. CeA: 109%; $p<.001$; MeA: 60%; $p=.001$ and BMA: 125%; $p<.001$). No change on D1 binding sites upon CRH receptor activation was observed in other extra-amygdala brain regions such as BNST, CPu, Acb and VTA (Figure 16 C-D).

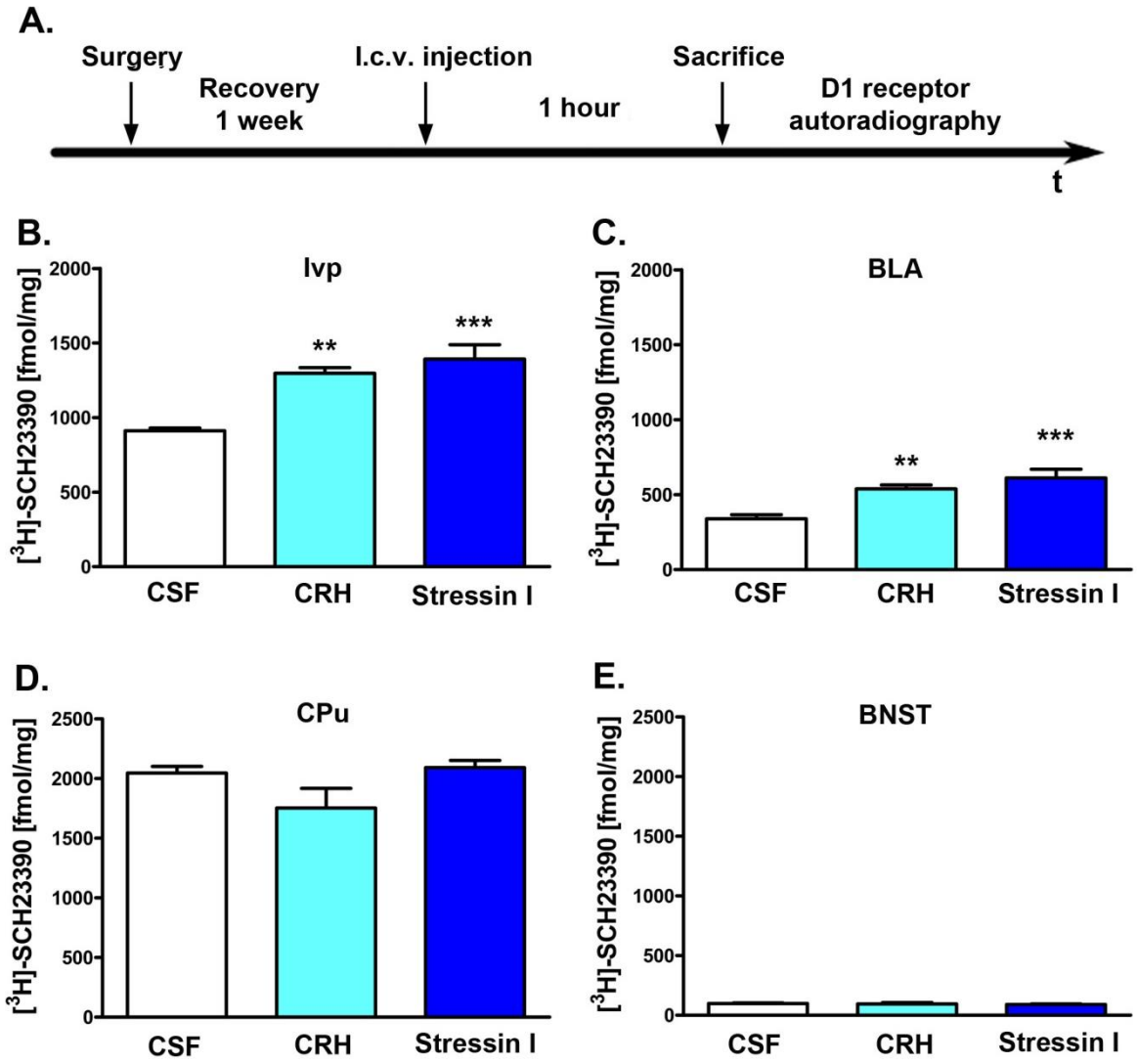

Figure 16: CRH receptor activation increases D1 receptor binding sites in the amygdala nuclei but not in extra-amygdala regions. A: Time line of the experiment: Wistar rats i.c.v. injected with 2µl of CSF (white bars) are compared to the animals treated with CRH (2µg/2µl inj volume, light blue bars; Spina et al., 2002) and CRHR1-specific agonist stressin I (4µg/2µl inj volume, dark-blue bars; Rivier et al., 2008). Data show D1 binding sites measured by saturated [^{3}H]-SCH23390 receptor autoradiography in two amygdala nuclei such as Ivp (B) and BLA (C) and in extra-amygdala regions CPu (D) and BNST (E). Data are expressed as mean ± SEM (n=6-7/group). Statistical analysis was performed by one-way ANOVA followed by Fisher's LSD post-hoc test, *p<0.05, **p<0.01, ***p<0.001. BLA: basolateral amygdala, BNST: bed nucleus of the stria terminalis, CPu: caudate putamen, Ivp: ventral paracapsular island.

3.1.2.2 CRHR1 is mediating anxiogenic behavioral responses via D1

Since the effect on D1 binding sites upon activation of CRH receptor was circumscribed to the amygdala nuclei, the anatomical specificity was confirmed by intra-amygdala injections of stressin I. Here we found an increase of amygdala D1 binding in a range of 20-24% in all amygdala nuclei,

which was prevented by co-treatment with D1 antagonist (SCH23390, Figure 17 B-C). This pharmacological interaction was accompanied by an anxiety-like response on the EPM test after intra-amygdala injection of stressin I injection (42% time spent in the open arms, F[2,11]=5.7, p=.019) and it was fully blocked by co-treatment with the D1 antagonist SCH23390 (Figure 17D). Locomotion activity, measured by the total number of entries in the arms, is similar within the treatment groups (F[2,11]=2.8, p>.05, Figure 17E, Supplementary material: Tables 3 and 4).

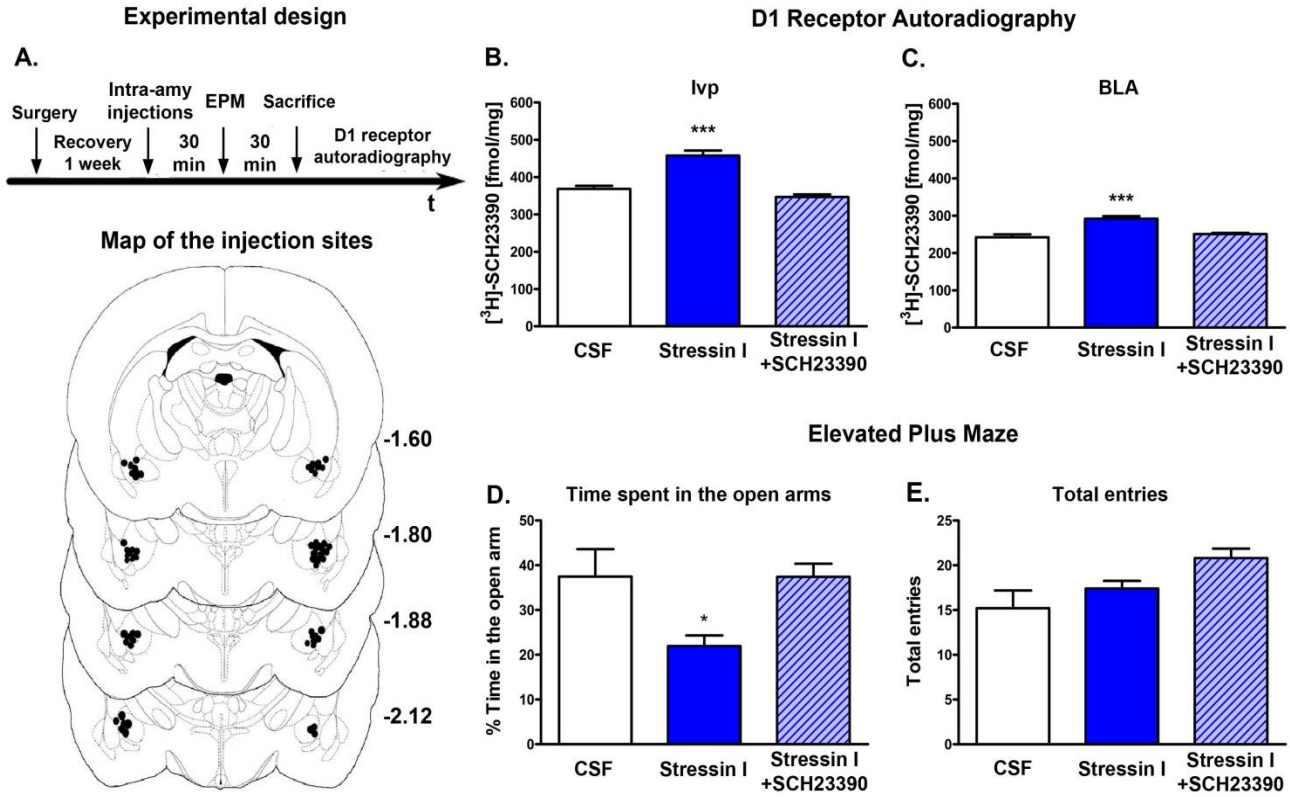

Figure 17: CRHR1 activation regulates anxiety-like behavior via activation of D1. A: Wistar rats were bilaterally injected in the amygdala with either CSF (0.5µl/ hemisphere), stressin I (0.01µg /hemisphere) or a combination of stressin I and SCH23390 (10 ng stressin I + 120ng SCH23390/ hemisphere; De La Mora et al., 2005). Anxiety-like behavior was assessed 30 min later on the Elevated-Plus-Maze (EPM) and rats were sacrified 1hour after injection. B: Images show quality of microinjections. C-F: The increase of D1 binding sites in the Ivp (C) and BLA (D) by CRHR1 agonist stressin I is completely abolished with co-treatment of D1 receptor antagonist SCH23390. E-F: EPM showed an increase level of anxiety-like behavioral response after stressin Ialone, while no effect was observed when D1 receptor is blocked by SCH23390 (E). F: No difference in the number of total entries. CSF (white bar): Cerebrospinal fluid, CRHR1-specific agonist stessin I (dark-blue bar), D1-specific antagonist SCH23390 (dashed line bar). Data are expressed as means ± SEM (n=5-7/group). Statistical analysis was performed by one-way ANOVA followed by Fisher's PLSD post-hoc test, *p<0.05, **p<0.01, ***p<0.001. BLA: basolateral amygdala; Ivp: ventral paracapsular island.

3.1.2.3 Increase of stressin I-induced D1-binding sites is dependent on co-localized receptors

To demonstrate weather the increased of D1 expression level upon activation of CRHR1 is due to co-localized receptors, we performed a similar experiment in mice lacking CRHR1 specifically in D1-expressing neurons as previously described in rats.

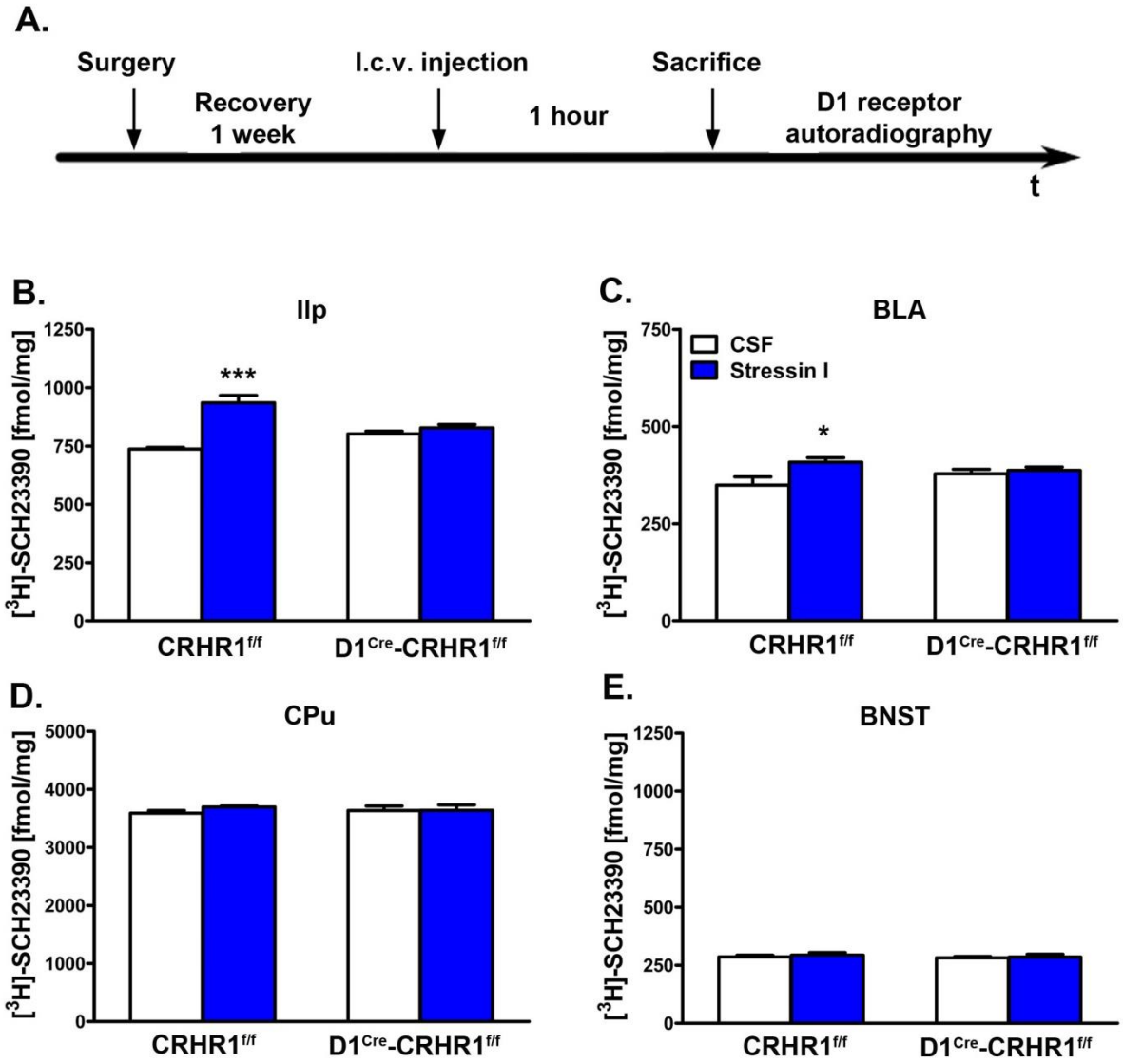

Figure 18: Increase of D1 availability upon activation of CRHR1 is due to co-localized receptors. Timeline (A.): D1 receptor autoradiography after i.c.v. injection of stressin I (0.5µg/2µl inj volume, dark-blue bar; Bruchas et al., 2009) or CSF (2 µl, white bar) in D1-CRHR1 knockout mice ($D1^{Cre}$-$CRHR1^{f/f}$) and their control littermates ($CRHR1^{f/f}$). Saturated [^{3}H]-SCH23390 receptor autoradiography show increased D1 binding sites in $CRHR1^{f/f}$ control mice, but not in $D1^{Cre}$-$CRHR1^{f/f}$ knockouts in amygdala Ivp (B) and BLA (C). No effect of stressin I is found in extra-amygdala regions (CPu (D) and BNST (E)). Data are expressed as fmol/mg (mean ± SEM, n=5-7/group. Statistical analysis was performed by two-way ANOVA followed by Fisher's LSD post-hoc test, *p<0.05, **p<0.01, ***p<0.001. BLA: basolateral amygdala, BNST: bed nucleus of the stria terminalis, CPu: caudate putamen, Ivp: ventral paracapsular island.

One hour after i.c.v. injection of stressin I (0.5µg/2µl inj volume; Bruchas et al., 2009) in CRHR1$^{f/f}$ mice, D1 binding sites increased in all amygdala regions (range: 13-38%) (Figure 18 B-C). The effect of CRHR1 activation on D1 did not show any treatment effect in extra-amygdala regions e.g. CPu and BNST (Figure 18 D-E, Supplementary material: Tables 5 and 6). Interestingly, i.c.v activation of CRHR1 did not affect D1 expression level in mice lacking CRHR1 in D1-contatining neurons either in the Ilp or in the BLA (Ilp: p=0.205; BLA: p=.648, Figure 18 B-C), demonstrating that stressin I-induced increase of D1-binding sites is due to co-localized receptors.

3.1.3 Mechanistic aspects of D1-CRHR1 receptor interaction

To follow up on our novel findings of CRHR1-D1 mediated anxiety-like responses at a mechanistic level we included next ITC electrophysiology and studies on receptor heteromerization in human embryonic kidney **(**HEK293T27) cells.

3.1.3.1 CRHR1 increases dopamine-induced hyperpolarization in the ITCs

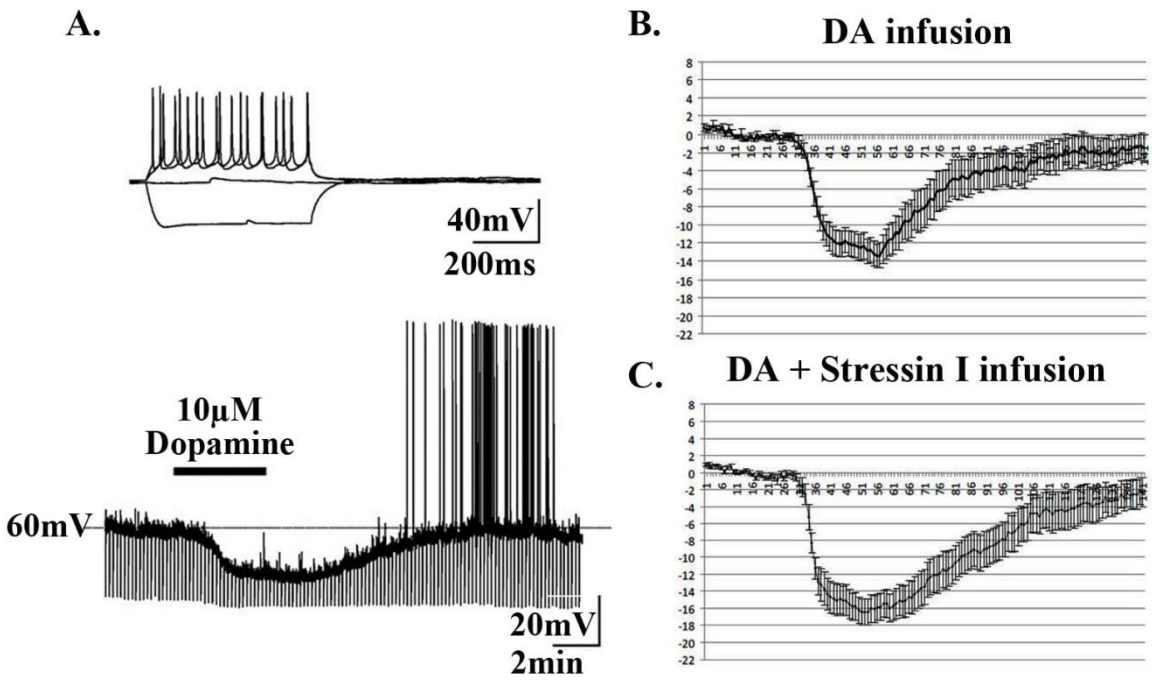

Figure 19: Co-activation of CRHR1 and DA causes an enhanced hyperpolarization of amygdala ITCs. A (upper panel) shows a representative firing pattern recorded from a single D1-containing ITC in response to extra-cellular dopamine (10µM, upper panel) and the time course after bath application of dopamine followed by a hyperpolarization and an increase of firing rate (A, lower panel) using a *Gad67*-GFP reporter line). B-C show a hyperpolarization of ITC-neurons upon infusion of dopamine (10µM, B) alone. This effect is enhanced in amplidude and time in combination with stressin I (C). Data are provided by Drs Jüngling and Pape (Westfälische Wilhelms-University Münster)

A study was performed by Drs Jüngling and Pape (Westfälische Wilhelms-University Münster, Münster, Germany) to determine whether CRHR1-D1 receptor interaction modify dopamine transmission in amygdala ITCs. Whole-cell patch-clamp recordings were performed on acutely prepared amygdala slices of *Gad67*-GFP mice, in which ITC cells can be readily identified. As demonstrated in Figure 19, activation of D1 by infusion of 10 μM dopamine suppresses the excitability of the ITCs. This effect was even more enhanced by co-infusion of stressin I and dopamine, thus indicating that CRHR1 act synergistically with dopamine, determining a hyper-polarization in ITC masses.

3.1.3.2 D1 and CRHR1 form heteroreceptor complex in vitro

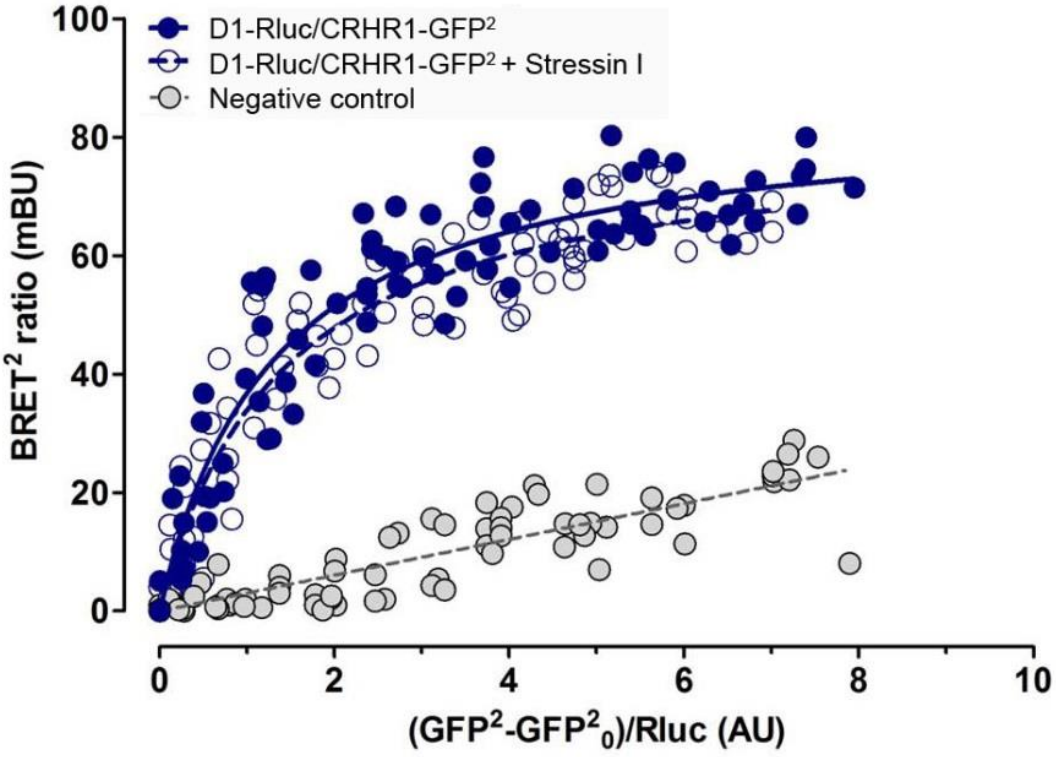

Figure 20: D1 and CRHR1 form heteroreceptor complex in vitro. BRETassay shows specific dopamine D1 and CRHR1 interaction in HEK293T cells co-transfected with a constant amount of D1-Rluc plasmid and increasing amount of the CRHR1-GFP^2 plasmid with or without the presence of 100nM stressin I (blue and white dots, respectively). Cells individually expressing $D_{2L}R^{Rluc}$ or $NTS1R^{GFP2}$were used as a negative control (grey dots). The fluorescence value obtained from the GFP^2, plotted on the X-axis, is normalized with the luminescence value of Rluc expression 10 min after coelenterazine incubation. Data are expressed in mean ± SEM (n= 4, in 8 replicates). The D1-CRHR1 curve fitted better to a saturation curve than to a linear regression as found with mixed pool of cell from cell individually expressing $D1R^{Rluc}$ + $CRHR1^{GFP2}$ (F test ($P < 0.001$). Data are means ± S.E.M.; n = 4. Data are provided by Drs. Borroto-Escuela and Fuxe from the Karolinska Institutet, Stockholm, Sweden

D1-CRHR1 heteroreceptor complexes have been demonstrated using BRET2 saturation curves assay in HEK293T27 cells. Cells were co-transfected with a constant amount of D1-Rluc plasmid and increasing amount of the CRHR1-GFP^2 plasmid. As a negative control, cells expressing either D1-Rluc or either CRHR1-GFP^2 plasmid were mixed together.

Figure 20 shows that there is not difference in BRET^2 ration between vehicle and stressin I (100nM) treated cells. However, D1-CRHR1 curve fitted better to a saturation curve than to a linear regression as found in the negative control (F test: $p < .001$), which revealed a high affinity between the receptors. Furthermore, high $BRET_{max}$ (Mean ± SEM: 84.98 ± 2.95) and low $BRET_{50}$ (Mean ± SEM: 1.34 ± 0.16) values together suggested that the saturation level was reached with a fast rate which confirm the elevated affinity between D1 and CRHR1. Thus, this aspect might represent a plausible explanation to the loss of sensitivity in detecting differences between the treatment with or without stressin I infusion.

3.1.4 Summary

Study 1 showed that the activation of CRHR1 strongly increases D1 binding sites in all the amygdala but not extra-amygdala regions. This pharmacological interaction was accompanied by an anxiety-like response on the EPM test after intra-amygdala stressin I injection which was fully blocked by pretreatment with D1 antagonist SCH23390. The pharmacological interaction is cell-specific, because knockout mice lacking CRHR1 receptors within D1 expressing neurons did not show this effect. Together, these findings strongly suggest that CRHR1 is mediating its anxiogenic-like responses (at least in part) via co-localized D1, emphasizing the role of ITCs in processing emotional responses, and thus point to a novel mechanism of amygdala physiology. Further mechanistic aspects of this interaction are provided by electrophysiological experiments and BRET assays, indicating that CRHR1 acts synergistically with D1 on suppression of amygdala ITCs and most likely via receptor heteromerization.

3.1.5 Behavioral characterization of D1^{Cre}-CRHR1$^{f/f}$ line

A series of behavioral tests was performed to characterize the behavior of D1^{Cre}-CRHR1$^{f/f}$ mice and their littermates during baseline conditions.

Homecage locomotion did not show a genotype effect using D1^{Cre}-Crhr1$^{f/f}$ (n=4) and CRHR1$^{f/f}$ (n=5) mice ($F[1,7]=0.769$, $p>.05$). As expected, mice displayed higher activity during the dark phase (repeated measures analysis showed a main effect for hours ($F[17,119]=39.918$, $p<.001$, Figure 21A) and lower during the light phase.

The total length (cm) travelled during the 15 minutes spent in the Open Field did not show any differences between the genotypes (one-way ANOVA: $F[1,49]=0.4$, $p>.05$, n=22-29 mice/group, Figure 21B). The analysis on the percentage of time spent in the center of the arena, considered an index for anxiety, did not reveal any main effect of genotype (one-way ANOVA: $F[1,49]=0.6$, $p>.05$).

In the EPM test D1^{Cre}-Crhr1$^{f/f}$ mice (n=15) and CRHR1$^{f/f}$ (n=10) did not show any difference in the time spent in the open arm during 5 minutes (one way ANOVA: main effect of genotype: $F[1,23]=0.1$, $p>.05$). The number of total entries did not differ between the genotype (control: 10.84 ± 1.49; knock-out: 12.49 ± 1.43; main effect genotype: $F[1,23]=0.3$, $p>.05$, Figure 21C).

Another paradigm applied to determine the level of anxiety-like behavior represents the dark-light box test, in which D1^{Cre}-Crhr1$^{f/f}$ mice (n=13) and their littermates (n=9) were tested for 5 minutes. One-way ANOVA analysis did not show a genotype effect in the time spent in the light compartment ($F[1,20]=3.18$, $p=.092$, Figure 21D) or in number of total entries ($F[1,20]=0.1$, $p=.726$).

D1^{Cre}-Crhr1$^{f/f}$ mice (n=15) and their Crhr1$^{f/f}$ controls (n=11) were next tested in a conditioned emotional response test, commonly used to investigate the responses to threatening and stressful stimuli. During the acquisition phase the percentage of freezing acquired in the last trial is significantly lower as compared to the first trial (main effect within trial $F[1,24]=101.1$, $p<.001$), demonstrating that all mice learnt to associate the acoustic-cue with the stressful stimuli (foot-shock). However, D1^{Cre}-Crhr1$^{f/f}$ mice and controls reached a similar level of freezing during the last trial (main effect genotype: $F[1,24]=1.8$, $p=.197$).

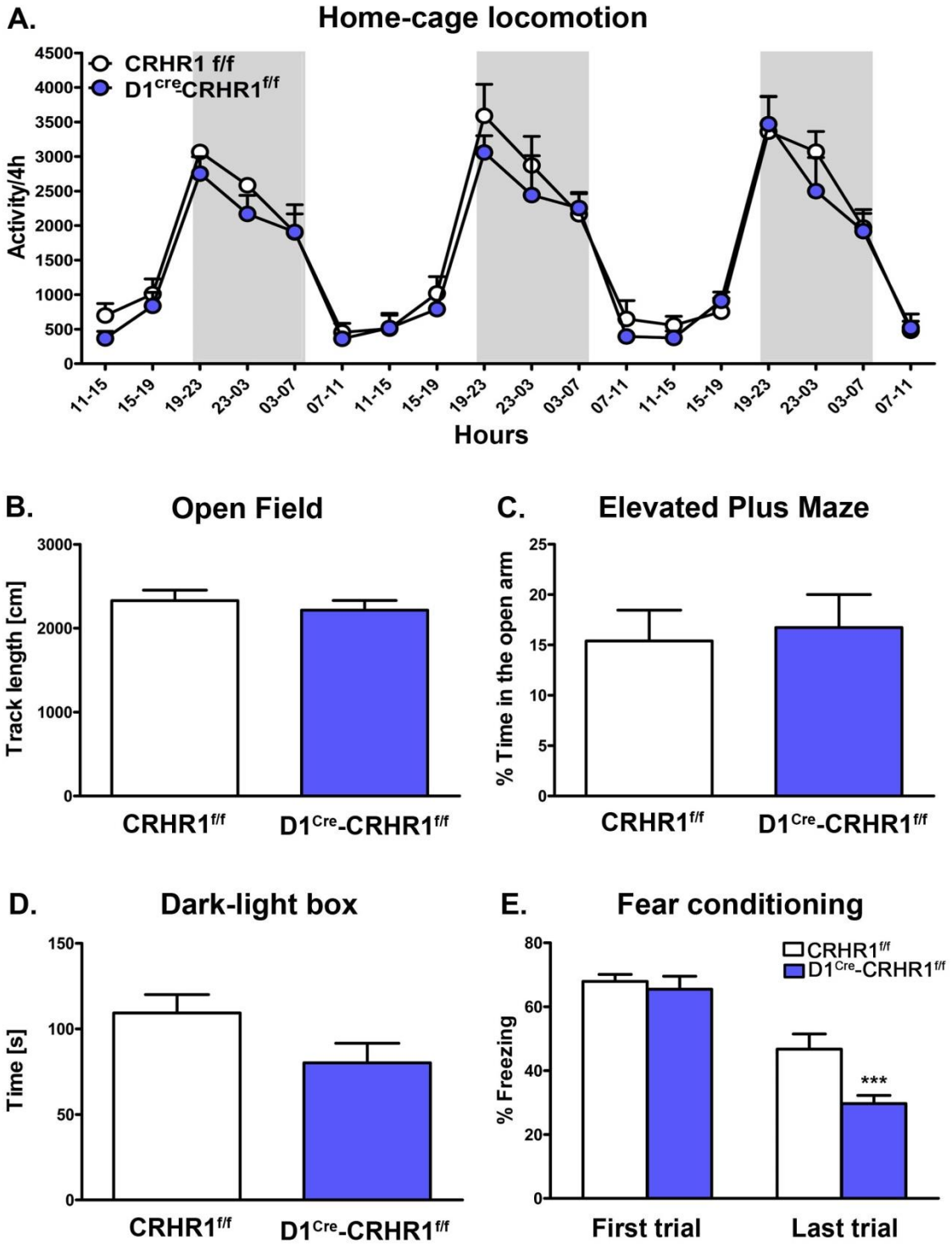

Figure 21: Behavioral characterization of the D1^{Cre}-CRHR1$^{f/f}$ mouse line. A set of behavioral tests were performed to address the basal and stess-induced behavior in control CRHR1$^{f/f}$ mice (white bars) and their specific knock-out D1^{Cre}-CRHR1$^{f/f}$ littermates (violet bars). A: Home-cage locomotion during 3 days. Dark phases are showed with grey background. Data are expressed in activity/4h. B: Total length (cm) covered during 15min of Open Field. C: Percentage of time spent in the open arms during 5min of EPM. D: Time spent in the light compartment during the 5 min in the dark-light box-test (expressed in seconds). E: Cue recall after fear conditioning. Data are expressed in percentage of freezing time during the 6 min test. All data are expressed in mean ± SEM , *p<0.05, **p<0.01, ***p<0.001.

Repeated measures analysis did not show any difference between the genotypes during the context-recall phase ($F[1,24]=0.1$, $p=.740$). During the cue-recall test, the tone was presented without foot-shock for 6 minutes and the percentage of freezing was monitored. Repeated measures analysis of variance revealed a main effect of genotype ($F[1,24]=5.8$, $p=.024$), main effect of minutes ($F[1,24]=87.0$, $p<.001$) and of the interaction minutes x genotype ($F[1,24]=5.7$, $p=.025$). As expected, Newman-Keuls post-hoc test showed a significant decrease of freezing behavior during the last minute of the test for both genotypes ($p<.001$). Interestingly, $D1^{Cre}$-$Crhr1^{f/f}$ mice expressed a significantly lower degree of freezing (-36%, $p=0.002$) compared to $CRHR1^{f/f}$ littermates, as showed in Figure 21E.

3.1.5.1 Summary

In summary, the behavioral characterization shows a normal locomotion activity in both genotypes and no difference in anxiety-like behavior when animals performed the test under stress-free conditions. However, once the CRH system has been activated (i.e. during acquisition of fear conditioning), $D1^{Cre}$-$Crhr1^{f/f}$ mice recovered faster than their controls, which points to a role of D1-CRHR1 interaction in the vulnerability towards stress.

3.2 STUDY 2: IMPACT OF CRHR1-D1 INTERACTION ON ALCOHOL DEPENDENCE

Increasing evidences indicate that amygdala dopamine receptors play a role in the modulation of emotional responses, especially D1 receptor (Stevenson and Gratton, 2003; de la Mora et al., 2010). Alcoholism induces long-term neuro-adaptations that determine a negative emotional state leading to an excessive alcohol consumption aimed to relief the negative emotionality (Koob and Kreek, 2007; Koob and Le Moal, 2008b). Up-regulated CRHR1 within the amygdala was observed during prolonged abstinence in alcohol dependent rats and mice (Sommer et al., 2008; Eisenhardt et al., 2015). The aim of this study is to specify the role of amygdala D1-CRHR1 interaction in alcohol dependence using a $D1^{Cre}$-$Crhr1^{f/f}$ mouse line.

3.2.1 Long lasting decrease of D1 expression level in the amygdala of post-dependent rats

Receptor autoradiography and *in situ* hybridization assays were performed on post-dependent and control Wistar rats in order to identify long-lasting changes in D1 receptor protein and mRNA expression patterns in the amygdala in alcohol dependence.

[^{3}H]-SCH23390 receptor autoradiographies in drug-naïve animals showed a similar D1 protein distribution compared to the immunohistochemistry experiments (Figure 22A). D1 receptor availability was about 1.5-3.5 times higher in the ITC clusters compared to the BLA and CeA, respectively. However, the highest expression level of D1 was observed in the Ivp. Here, D1 binding was more than 6 times higher than in the BLA and 1.6-1.8 times higher than in other ITC clusters. In a similar manner, *in situ* hybridization showed that D1 mRNA was 2-4 and 7-13 times more abundant in ITCs than in the BLA and CeA, respectively. Interestingly, mRNA levels in the Ivp were not markedly different from other ITC clusters as seen on the protein level.

Analysis of [^{3}H]-SCH23390 binding to the D1 showed a significant down-regulation in the BLA (by 15%), CeA (22.5%), Imp (18%), and IN (18%). The effect was strongest in the Ivp where D1 binding sites were reduced by 43%. As shown in Figure 22B, D1 mRNA in post-dependent animals was similarly down-regulated in all analyzed ITC clusters as well as in the BLA (decreased by 23%) and CeA (by 39%) when compared to drug-naïve air-exposed control animals. The strongest reduction of D1 mRNA was detected in the Ivp (38.5%) and IN (36%). All mean (± SEM), F and p values from receptor autoradiography and *in situ* hybridization assays are listed in the Supplementary Tables 7 and 8 respectively.

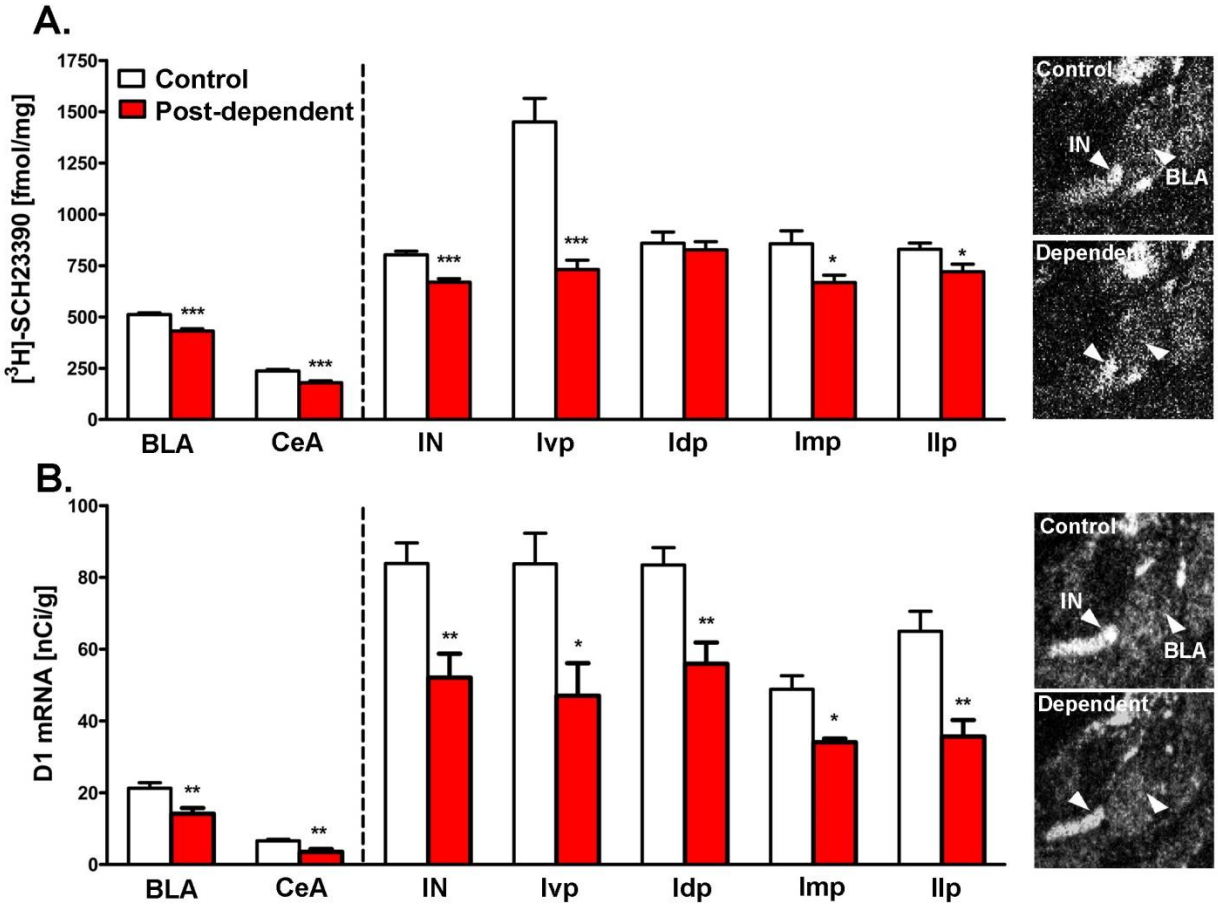

Figure 22: D1 protein and D1 mRNA expression levels are strongly decreased in the amygdala regions of post-dependent rats. A: D1 binding sites (expressed in fmol/mg) were analyzed by 10 nM [^{3}H]-SCH23390 receptor autoradiography. Non-specific binding was measured by adding 1µM SKF (not shown). B: Expression patterns are the same for D1 mRNA *in-situ* hybridization. Non-specific hybridization was determined by a sense-riboprobe (not shown). The panel on the right shows the D1 distribution in the analyzed BLA, CeA and ITC clusters for receptor autoradiography and *in-situ* hybridization assays in controls and alcohol dependent rats. Arrowheads indicate localization of IN and BLA. Data are expressed as means ± SEM (receptor autoradiography: n=3-8/group; *in-situ* hybridization: n=5-8/group). Statistical analysis was performed by one-way ANOVA followed by Fisher's LSD post-hoc test, *p<0.05, **p<0.01, ***p<0.001. BLA: basolateral amygdala; CeA: central amygdala; Idp: dorsal; Ilp: latera paracapsular island; Imp: medial; IN: main nucleus of the intercalated cells; Ivp: ventral.

3.2.2 Alcohol dependent D1^{Cre}-Crhr1$^{f/f}$ mice are less vulnerable to stress as compared to their littermates

In order to investigate the impact of D1-CRHR1 receptor interaction on alcohol dependence, ethanol consumption of D1^{Cre}-Crhr1$^{f/f}$ mice and their littermates was investigated through two-bottle free-choice paradigm. Post-dependent phenotype was induced in the animals by repeated cycles of chronic intermittent exposure (CIE) to ethanol intoxication followed by withdrawal (modified from Becker and Lopez, 2004). To further identify the functional involvement of D1-

CRHR1 interaction in the regulation of stress during alcohol dependence, the CRH system of mice was challenged by repeated forced swim stress and the level of ethanol consumption was monitored during the following days.

Statistical analysis relative to alcohol consumption during the initial habituation phase did not reveal any main effect of genotype ($F[1,29]=0.03$, $p=.849$; n= 15-18 animal/group) as well as in the baseline drinking behavior between the genotypes ($F[1,29]=0.6$, $p=0.443$).

After 4 weeks of CIE procedure, followed by 3 days of withdrawal, $D1^{Cre}$-$Crhr1^{f/f}$ mice and their $CRHR1^{f/f}$ littermates had again access to alcohol and its consumption was monitored daily. Withdrawal scores were assessed in both dependent and control mice, starting from the end of the cycle of ethanol intoxication (time 0) and the measurements were repeated after 4, 8 and 12 hours (Figure 23C). Repeated measures analysis of variance was performed in all mice ($CRHR1^{f/f}$ control: n=11; $D1^{Cre}$-$Crhr1^{f/f}$ control: n=8; $CRHR1^{f/f}$ dependent: n=6; $D1^{Cre}$-$Crhr1^{f/f}$ dependent: n=9) and revealed a strong main effect of treatment ($F[1,30]=114.6$, $p<.001$), hours ($F[3,90]=29.3$, $p<.001$) and their interaction (treatment x hours: $F[3,90]=23.9$, $p<.001$). As expected, dependent mice displayed signs of withdrawal, while controls behaved normally. However, no overall effect for the genotype was found either in controls or dependent animals ($F[1,30]=0.3$, $p=.604$).

Also ethanol metabolism was evaluated at the same time points as for the withdrawal signs: blood alcohol concentration (BAC) immediately after the intoxication correspond to 188.15 ± 2.45 mg/dl in $CRHR1^{f/f}$ mice, while in $D1^{Cre}$-$Crhr1^{f/f}$ mice was 209.45 ± 9.85 mg/dl. During the following 4 and 8 hours, BAC dropped down until reaching the values of 3.90 ± 0.20 and 7.95 ±0.55 mg/dl 12 hours after the last exposure, without showing any differences between the genotypes ($F[1,2]=0.2$, $p=.731$).

As presented in Figure 23D, alcohol intake increased dramatically at during the first day after abstinence. Indeed, mice consumed a total amount of ethanol 3-4 times higher than before CIE treatment. Although at day 2 and 3 alcohol intake slowly decreased the consumption level remained significantly above the baseline (days: $F[3,33]=96.4$, $p<.001$, for Mean ± SEM values see Supplementary materials Table 9). Repeated measures analysis revealed only a trend in the overall effect for genotype ($F[1,11]=5.8$, $p=.06$), while the interaction genotype x days is significant ($F[3,33]=3.6$, $p=.038$), demonstrating that post-dependent $D1^{Cre}$-$Crhr1^{f/f}$ mice consume significantly less alcohol than the CRHR1-floxed littermates during all the days after CIE treatment.

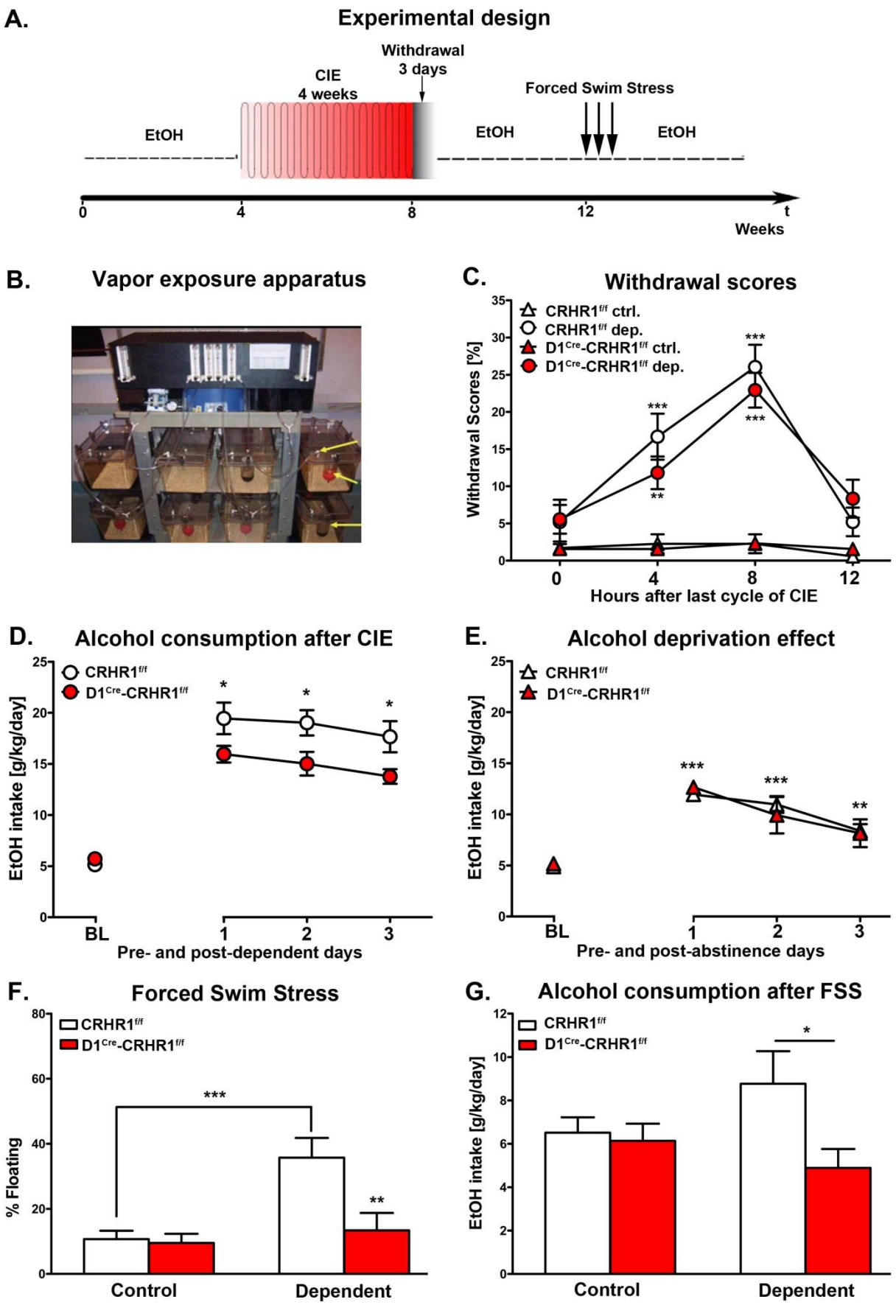
A.
Experimental design
Withdrawal
3 days
CIE
4 weeks
Forced Swim Stress
EtOH
EtOH
EtOH
0
4
8
12
t
Weeks
B.
Vapor exposure apparatus
C.
Withdrawal scores
CRHR1f/f ctrl.
CRHR1f/f dep.
D1Cre-CRHR1f/f ctrl.
D1Cre-CRHR1f/f dep.
Withdrawal Scores [%]
Hours after last cycle of CIE
D.
Alcohol consumption after CIE
CRHR1f/f
D1Cre-CRHR1f/f
EtOH intake [g/kg/day]
BL
Pre- and post-dependent days
E.
Alcohol deprivation effect
Pre- and post-abstinence days
F.
Forced Swim Stress
% Floating
Control
Dependent
G.
Alcohol consumption after FSS

Figure 23: Alcohol dependent D1^{Cre}-CRHR1$^{f/f}$ display a lower alcohol intake and vulnerability to stress compared to their dependent CRHR1$^{f/f}$ littermates. A: experimental design. B: ethanol vapor exposure apparatus used to induce alcohol dependence (Eisenhardt et al., 2015). C: Withdrawal scores measured 0, 4, 8, 12h after the last cycle of alcohol vapor exposure. D: Alcohol consumption (g/kg/day) in CRHR1$^{f/f}$ and D1^{Cre}-CRHR1$^{f/f}$ mice before and after chronic intermittent exposure (CIE). E: Alcohol deprivation effect (ADE) in not exposed animals. Ethanol intake is expressed in g/kg/day. F: percentage of floating during the first day of Forced Swim Stress (FSS) in control and alcohol dependent animals. G: Alcohol consumption during the first day after repeated FSS. All data are showed in means ± SEM.

While half of the mice were treated with CIE, control mice for both genotypes were kept in abstinence for 4 weeks before they were allowed to have again access to alcohol. As showed in Figure 23E, alcohol consumption was increased by 2.4-2.5 times on day 1 compared to baseline, however, on day 2 and 3 alcohol intake decreased. Repeated measures analysis revealed an overall effect only for the days factor ($F[3,51]=34.8$, $p<.001$; for Mean ± SEM values see Supplementary material: Table 9), indicating an effect due to the alcohol deprivation since all days differ from the baseline. No difference was found between the genotypes ($F[1,17]=0.008$, $p=.929$).

After 4 weeks of free access to alcohol, all mice reached a comparable level of daily ethanol intake (two-way ANOVA genotype: $F[1,29]=0.01$, $p=.900$; treatment: $F[1,29]=0.4$, $p=.250$; genotype x treatment: $F[1,29]=0.02$, $p=.9$). Thus, the CRH system of all groups was challenged again using a mild-stress paradigm, Forced Swim Test, repeated for 3 consecutive days. The percentage of floating, during the first day of stress, considered an index of depression-like behavior, was measured in post-dependent mice and their control (Figure 23F). Two-way ANOVA revealed a main effect for all the variances (genotype: $F[1,21]=8.5$, $p=.008$; treatment: $F[1,21]=12.8$, $p=.002$; genotype x treatment: $F[1,21]=6.8$, $p=.016$). Remarkably, the percentage of floating is significantly higher in post-dependent CRHR1$^{f/f}$ mice as compared to their controls ($p=.0002$), while D1^{Cre}-Crhr1$^{f/f}$ dependent mice did not differ from their controls ($p=.518$). Furthermore, the floating percentage of D1^{Cre}-Crhr1$^{f/f}$ dependent mice seemed to be less vulnerable to stress as compared to their littermates, since they spent less time floating ($p=.002$).

A similar pattern was found in alcohol consumption the day after the repeated stress paradigm. Dependent D1^{Cre}-Crhr1$^{f/f}$ mice consumed significantly less alcohol than their littermates ($p=.013$), confirming their resistance to stress compare to their CRHR1$^{f/f}$ controls (Figure 23G). Alcohol consumption was monitored for consecutive 4 weeks and the new baselines on drinking behavior

did not differ between genotypes (two-ways ANOVA genotype: F[1,29]=3.8, p=.060; treatment: F[1,29]=1.2, p=.289; genotype x treatment: F[1,29]=0.04, p=.841, Figure 23G).

3.2.3 Summary

Study 2 demonstrates a strong decrease in D1 expression level in post-dependent rats, which may counterbalance the strong increase in CRHR1 level (Sommer et al., 2008) induced by the chronic intermittent exposure treatment. Furthermore, dependent $D1^{Cre}$-$Crhr1^{f/f}$ mice displayed less vulnerability to stressful stimuli as compared to their littermates, supporting the hypothesis of a maladaptive functionality of the interaction between D1-CRHR1 and its involvements in the hyper-responsivity to stress during dependence.

3.3 STUDY 3: DIFFERENTIAL ROLE OF DOPAMINERGIC- AND DOPAMINOCEPTIVE CRHR1 ON STRESS-INDUCED DRINKING BEHAVIOR

The ventral tegmental area (VTA) plays a pivotal role in the regulation of the rewarding effect of drugs (Koob, 1992; Wise, 1996; Pierce and Kumaresan, 2006). Although it is a highly heterogeneous brain region, the main neuronal population in the VTA consists in dopaminergic projections which innervate the forebrain areas (Sesack and Grace, 2010). However, the presence and the distribution of D1-containing neurons within this region are so far not well established, as well as their impact on the behavior. VTA activity is modulated by CRH and its receptor CRHR1 (Rodaros et al., 2007; Wanat et al., 2008), which place this region in a crucial position in the regulation of the responses to stressful stimuli. However, it is not clear the cell-specific role of CRHR1 within the dopaminergic system in the regulation of stress-induced alcohol-drinking behavior.

Thus, the goal of Study 3 is first to define the distribution of pre and post-synaptic DAergic neurons within the VTA using double immunohistochemistry for D1ir and THir, respectively. We then determine the co-distribution of CRHR1 with TH - and/or D1- containing neurons using a *Crhr1*-GFP reporter line. Furthermore, we aim to establish the role of CRHR1 in dopaminergic and dopaminoceptive neurons on alcohol consumption after repeated stress, using cell-type specific CRHR1 knock-out mice for the dopaminergic system (i.e. $DAT^{CreERT2}$-$CRHR1^{f/f}$ and $D1^{Cre}$-$CRHR1^{f/f}$).

3.3.1 Distribution of TH- and D1- expressing neurons in the VTA

Even though the distribution of dopaminergic (THir) neurons in the VTA is well established, the distribution and characterization of D1-expressing cells is not well established.

As showed in Figure 24 C and D, all TH-ir and D1-ir cells are fully co-localized with NeuN-ir, indicating their neuronal specific origin. Quantitative analysis revealed that 70% of NeuN-positive cells contained TH-ir, while only 23% expressed D1-ir.

TH-ir positive neurons were highly expressed in the substantia nigra pars compacta (SNC) and throughout the entire VTA region, with a stronger expression in the ventral division of the VTA (Figure 24C). On the other hand, D1ir-containing neurons were mostly found in the substantia nigra

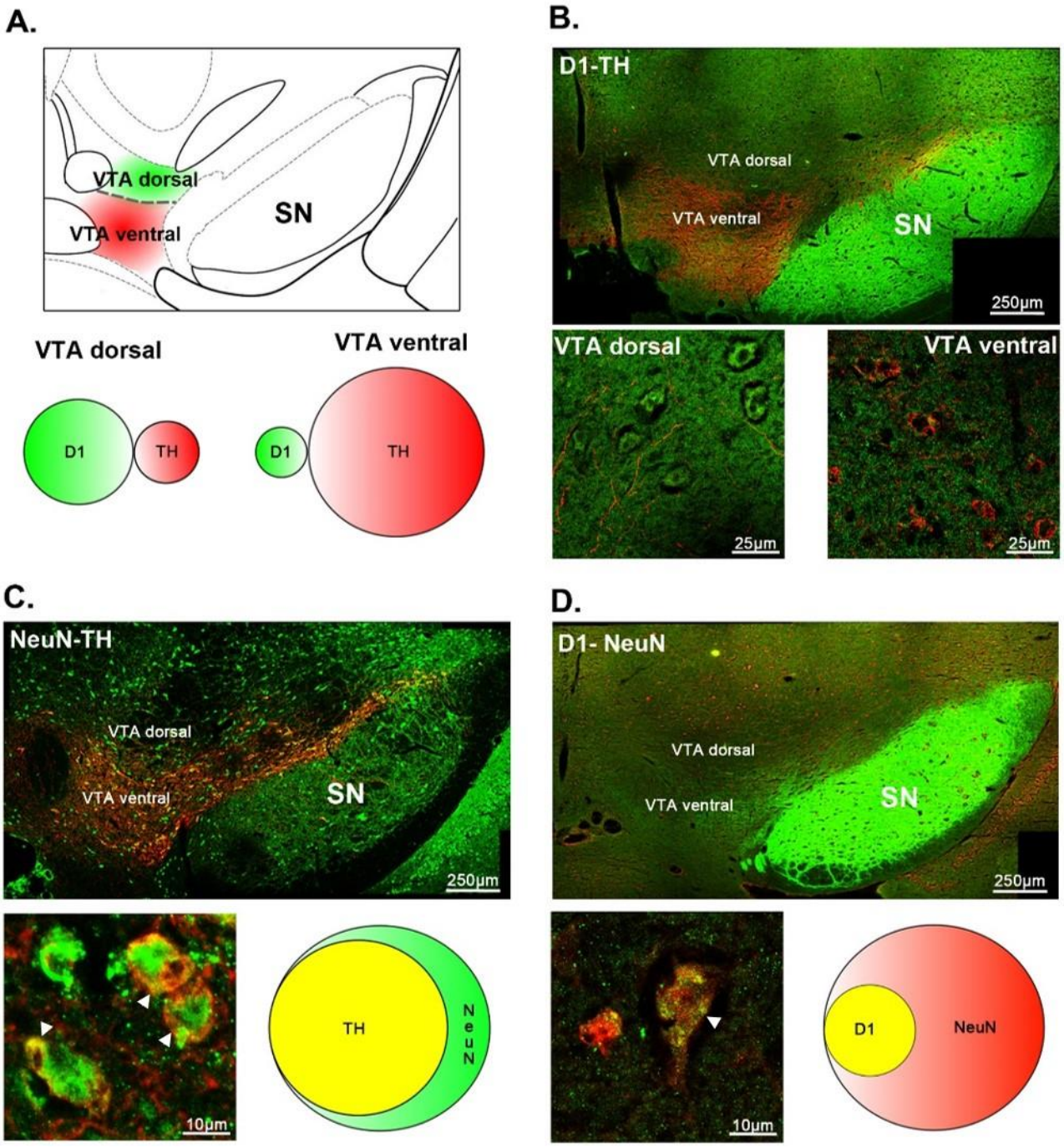

Figure 24: Distribution of DAergic (THir) and dopaminoceptive (D1ir) neurons in the VTA brain region. A-B: schematic representation of the VTA (-3.16mm from Bregma level (Paxinos and Franklin, 2000)) and overview of immunofluorescence staining for D1ir- (green) and THir- (red) expressing neurons with merged channels, indicating a differential distribution of the neurons. Pie charts show the quantification for the dorsal and ventral part of the VTA, while 'zoom in' images showed the absence of co-localized D1-THir cells. C: Overview, zoom in and quantification of the NeuNir- (green) and THir-(red) positive cells. D: Qualitative and quantitative representation of double immunostaining assay for D1ir- (green) and NeuNir- (red) expressing neurons. Yellow: colocalized THir/D1ir, THir/NeuNir, D1ir/NeuNir.

pars reticulata (SNR) and to a lower degree within the dorsal division of the VTA Figure 24D). There was no co-localization of TH-ir and D1-ir in VTA neurons (Figure 24B). However, TH-ir innervations were observed in the proximinity of D1-ir cell-bodies, suggesting that THir-containing fibers in part project to the dorsal division, innervating post-synaptic D1-ir positive VTA neurons. Based on these observations, we suggest an anatomical organization of D1-dopaminoceptive and TH-dopaminergic neurons within the VTA which may also underlie differential functional aspects.

3.3.2 Localization of CRHR1 in dopaminergic and dopaminoceptive neurons of VTA

In order to identify to which extend CRHR1 co-localized with TH-dopaminergic and D1-dopaminoceptive neurons in the VTA, double immunostainings were carried out using the *Crhr1*-GFP reporter line.

Double immunostainings performed with a green fluorescent-label for CRHR1 (GFPir,) and NeuN-ir (red colour) -showed that all CRHR1-GFPir followed a neuronal distribution (Figure 25B). Quantification of the confocal microscopic images revealed that half of the total NeuN-ir cells in the VTA expressed the reporter gene, demonstrating that CRHR1 is distributed through the entire VTA.

Images recorded from the ventral portion of the VTA showed a high degree of co-localization between GFP-ir (visualized in green) and TH-ir (red) neurons. Up to 91% of GFPir-expressing neurons contained THir, suggesting a strong interaction of CRHR1 with the dopaminergic system in the VTA (Figure 25C).

D1ir-CRHR1 (GFP-ir) double immunostaining showed that D1 (labelled in red) was mainly distributed in the SNR, as previously observed. However, quantitative analysis focused on the dorsal division of the VTA revealed that half of CRHR1-GFPir neurons are co-expressed with D1-ir neurons (Figure 25D), and thus implying a role of CRHR1 in D1 VTA neurons.

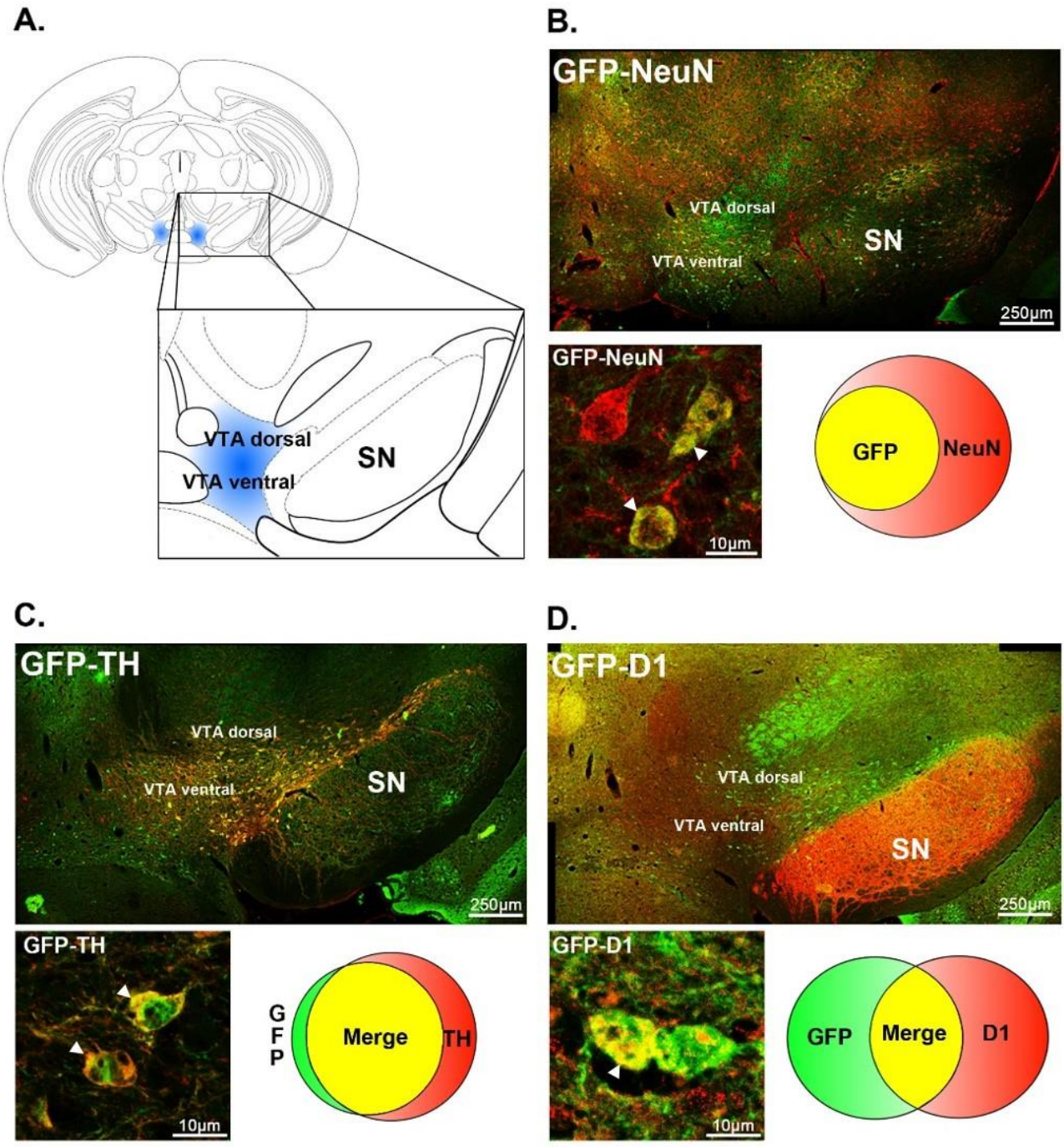

Figure 25: Qualitative and quantitative identification of CRHR1 in TH- and D1- expressing neuron in the VTA. A: schematic representation of the VTA (-3.16mm from Bregma level (Paxinos and Franklin, 2000). B: VTA overview shows immunofluorescence staining for GFP (green) and NeuN (red) with merged channels. The zoomed in image and the pie-chart show that the whole population of GFP-stained cells overlap with NeuN-staining. C: VTA overview and zoom in for GFP and TH- expressing neurons. The graphs shows strong co-localization of GFP+ cells with TH (91%). D: confocal microscope image of double-immunostraining for GFP and D1 positive cells. Half of the CRHR1-expressing neurons co-localized with D1. *Crhr1*-GFP reporter mouse line used for this study was provided by Dr. NJ Justice (Salk Institute for Biological Studies, La Jolla, California, USA).

3.3.3 Impact of CRHR1 neuron-specific expression on stress-induced alcohol drinking behavior

In order to assess the cell-specific role within the dopaminergic system of CRHR1, alcohol drinking behavior after repeated stress, was investigated $DAT^{CreERT2}$-$CRHR1^{f/f}$ and $D1^{Cre}$-$Crhr1^{f/f}$ mouse line. Inducible $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice express a truncated, non-functional CRHR1 in dopaminergic neurons, while the constitutive $D1^{Cre}$-$Crhr1^{f/f}$ mice present a knockout of CRHR1 specifically in D1-positive cells. All animals were first habituated to consume a 2% ethanol solution, then 4, 8 up to 12%. Once their alcohol consumption reached a stable baseline, part of the animals were stressed with 5 consecutive cycles of alcohol vapor intoxication and withdrawal (1 week). Control mice were not exposed to the stress-inducing procedure but received daily i.p. injections of pyrazole (Becker and Lopez, 2004). Only water was available during the treatment for all groups. After three days of withdrawal, chronically stressed mice and their controls had free access to alcohol. Ethanol consumption was monitored for four consecutive days.

During the habituation phase, knockout mice did not differ from their littermates ($DAT^{CreERT2}$-$CRHR1^{f/f}$ line: genotype $F[1,25]=0.06$, $p=.811$; $D1^{Cre}$-$Crhr1^{f/f}$ line: genotype $F[1,17]=1.5$, $p=.230$).

3.3.3.1 Repeated-stress alcohol drinking behavior in $DAT^{CreERT2}$-$CRHR1^{f/f}$ and $D1^{Cre}$-$CRHR1^{f/f}$ mouse lines

$DAT^{CreERT2}$-$CRHR1^{f/f}$ (n=8) and their $CRHR1^{f/f}$ littermates (n=6) significantly increased their alcohol intake after repeated stress (Figure 26A). Repeated measures analysis of variance revealed significant main effect of days ($F[4,48]=18.8$, $p<.001$), but no effect of genotype ($F[1,12]=3.1$, $p=.104$) and interaction effect ($F[4,48]=2.2$, $p=.078$). However, further analysis revealed a difference between the genotypes on the second day of alcohol drinking (Alcohol intake on day 2: g/Kg; mean ± SEM in $DAT^{CreERT2}$-$CRHR1^{f/f}$: 8.85 ± 1.84 and $CRHR1^{f/f}$: 15.07 ± 1.84, $p=.048$)

As for the $DAT^{CreERT2}$-$CRHR1^{f/f}$ mouse line, $D1^{Cre}$-$Crhr1^{f/f}$ mice (n=5) and their $CRHR1^{f/f}$ littermates (n=4) increased the alcohol intake after chronic stress (Figure 26B). Although repeated measures statistics showed a strong main effect of days ($F[4,28]=7.0$, $p<.001$), post-hoc analysis revealed a significant difference between the baseline and the first day after vapor exposure, probably due to the low number of animals per each group. Furthermore, no difference between the genotypes was found during repeated measure analysis (main effect genotype: $F[1,7]=0.1$, $p=.784$).

For both mouse lines, water intake showed an opposite pattern compared to the alcohol consumption, which indicats a voluntary preference towards alcohol. There were no differences

between the groups (repeated measures: for DAT^{CreERT2}-CRHR1$^{f/f}$ line F[1,12]=0.5, p=.482; for D1^{Cre}-Crhr1$^{f/f}$ line: F[1,7]=0.3, p=.614).

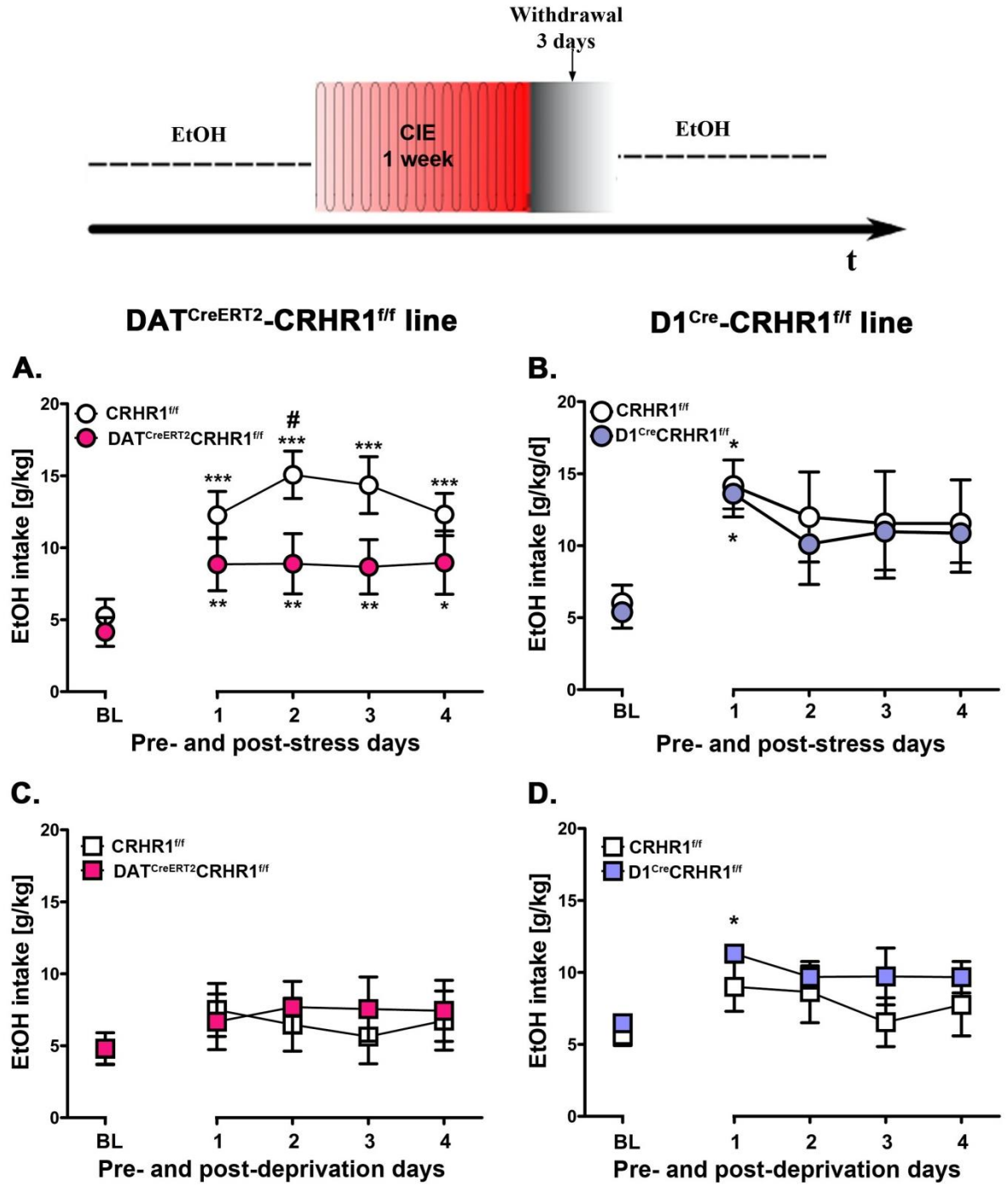

Figure 26: Differential stress-induced alcohol consumption in DAT^{CreERT2}-CRHR1$^{f/f}$ and D1^{Cre}-CRHR1$^{f/f}$ mouse line. A: Alcohol intake in DAT^{CreERT2}-CRHR$^{f/f}$ mice (pink) and their CRHR$^{f/f}$ littermates (white) before and after repeated stress. B: Alcohol ingestion in D1^{Cre}-CRHR$^{f/f}$ mice (violet) and their CRHR$^{f/f}$ littermates (white) before and after repeated stress. C-D: EtOH consumption before and after one week of deprivation in DAT^{CreERT2}-CRHR$^{f/f}$ and D1^{Cre}-CRHR$^{f/f}$ mice, respectively. All data are shown as g/kg/day (means±SEM). Upper part of the figure showed the timeline for the stress-induced alcohol drinking experiment.

3.3.3.2 Effect of alcohol deprivation in DAT^{CreERT2}-CRHR1$^{f/f}$ and D1^{Cre}-CRHR1$^{f/f}$ mouse lines

Treatment controls for the animals exposed to repeated stresswere kept in alcohol abstinence during the treatment. After one week, bottles containing 12% alcohol solution and water were available *ad libitum* and their drinking behavior was monitored daily.

DAT^{CreERT2}-CRHR1$^{f/f}$ (n=7) and their CRHR1$^{f/f}$ littermates (n=6) showed a slight increase of alcohol intake after ethanol deprivation (Figure 26C). Repeated measures analysis of variance revealed a significant main effect for days (F[4,44]=3,5 p=.015), but no effect of genotype (F[1,11]=0.6, p=.808) and in the interaction between genotype x days (F[4,44]=1.0, p=.415).

Also D1^{Cre}-Crhr1$^{f/f}$ mice (n=5) and their CRHR1$^{f/f}$ littermates (n=5) increased the alcohol intake after abstinence (Figure 26D). Repeated measures statistics showed a main effect of days (F[4,32]=4.8, p=.004), while post-hoc analysis revealed a significant difference on day 1 only for the knockout animals. All animals consume a comparable amount of alcohol (genotype effect: F[1,8]=1.3, p=.294; genotype x day: F[4,32]=0.5, p=.755).

As previously observed for the stress-induced drinking behavior, water intake follows an opposite pattern compared to the alcohol consumption which indicated a voluntary preference towards ethanol.

3.3.4 Summary

In the first part of Study 3, immunohistochemistry assays revealed a net anatomical separation within the VTA between TH-ir and D1-ir neurons, since TH-expressing cells are mainly distributed in the ventral portion of the VTA, while D1-containig population is located in the dorsal area. Furthermore, double immunostaining performed on VTA sections of CRHR1-GFP reporter line, revealed the CRHR1 is distributed within the entire region and it is co-expressed in both TH-ir and D1-ir cells, suggesting a cell-specific impact in the regulation of the dopaminergic system.

For this reason, in the second part of this study, we demonstrated that CRHR1 in DAT- and D1-containing neurons are differentially involved in alcohol related behavior using cell-specific CRHR1 knockout mice. Thus, CRHR1 in dopaminergic neurons seem to be mediating stress-induced drinking, while CRHR1 in dopaminoceptive neurons seem not to affect this behavior at all.

3.4 STUDY 4: αCAMKII-DEPENDENT CRHR1 MODULATES STRESS-INDUCED ALCOHOL SEEKING BEHAVIOR IN THE CENTRAL NUCLEUS OF THE AMYGDALA

The central nucleus of the amygdala (CeA), a region highly enriched of CRH-containing neurons, is deeply involved in the regulation of anxiety and stress-induced responses during alcohol dependence (Gilpin et al., 2015). The CRH system, together with its receptor CRHR1 is a key mediator of endocrine, autonomic and behavioral reactions to stressful stimuli (Smagin et al., 2001). The CRH system plays a pivotal role also in many aspects of alcohol addiction e.g. development of dependence, withdrawal and alcohol-seeking behavior (Koob and Kreek, 2007). Alpha calcium/calmodulin-dependent protein kinase II (αCaMKII) plays a crucial role in the modulation of synaptic function by its calcium-specific activation (Yamauchi, 2005) and it is considered a marker for excitatory transmission (Benson et al., 1992; Jones et al., 1994), although recent studies suggest the involvement of αCaMKII in amygdala inhibitory transmission as well (Huang et al., 2014). Furthermore, it has been recently shown that alcohol induces adaptation in αCaMKII of the amygdala (Salling et al., 2014).

Thus, in this study, we first identified the co-localization of CRHR1 and αCaMKII by double-immunostaining performed on amygdala brain section of Crhr1-GFP reporter mouse line. Then, the involvement of CRHR1 in αCaMKII-expressing neurons of the CeA was investigated by combining AAV mediated gene transfer approach and genetically modified rat line (αCaMKII^{CreERT2}). Alcohol-mediated behavior was assessed in αCaMKII^{CreERT2} rats over-expressing CRHR1 in the CeA and their respective controls, by an operant conditioning paradigm.

Dr. Kai Schönig and Dr. Dusan Bartsch (Central Institute of Mental Health, Mannheim, Germany) provided the CaMKII^{CreERT2} rat line; Dr George von Jonquieres and Dr. Matthias Klugmann (University of New South Wales, Australia) generated and tested *in vitro* the CRHR1-overexpression AAV virus. Stefanie Uhrig supported the behavioral experiments.

3.4.1 Co-localization between CRHR1 and αCaMKII in the CeA

In order to estimate if and to which extent CRHR1 is co-localized with αCaMKII, double fluorescence immunostainings were performed on coronal amygdala brain section of *Crhr1*-GFP reporter mice. Different amygdala levels were recorded by confocal microscope and co-located GFPir and αCaMKII-ir containing cells of amygdala region were quantitatively analyzed. As shown in Figure 27, GFPir-positive neurons were mainly distributed along the intercalated cells clusters

and the lateral nucleus of the CeA (CeL), while αCaMKII-ir neurons are highly expressed in the BLA and the CeL. Merging images showed that αCaMKIIir- and GFPir- positive cells are distributed mainly in the CeA. Further quantification revealed that 48% of αCaMKIIir-positive neurons co-expressed CRHR1-GFPir.

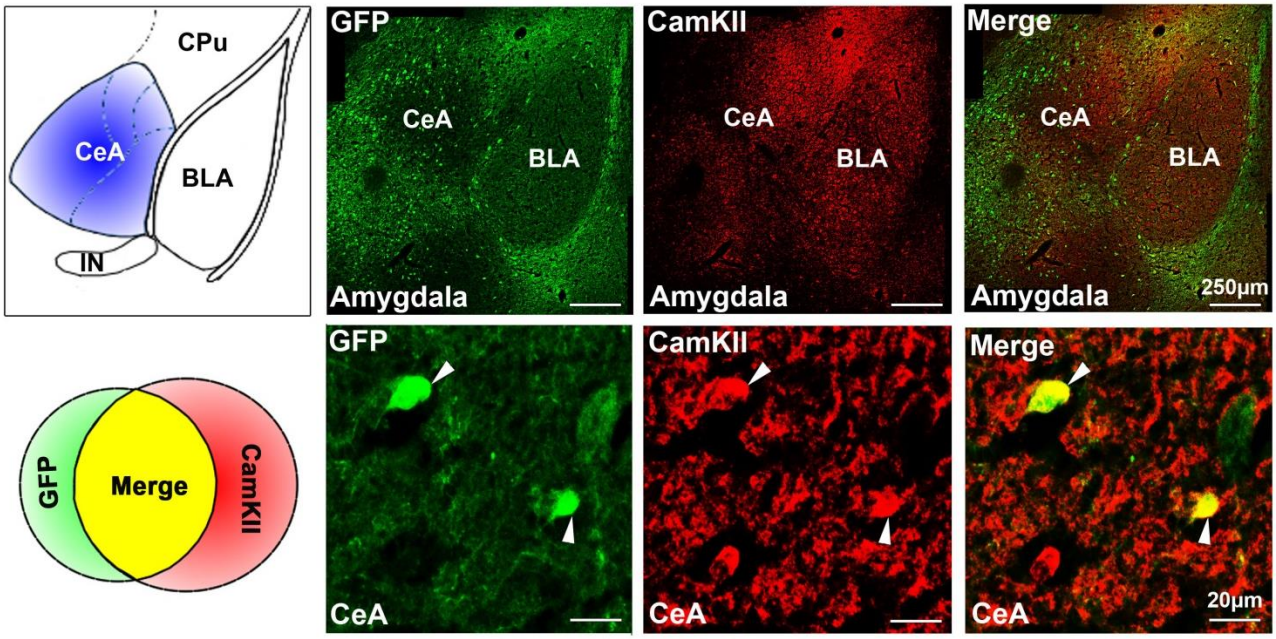

Figure 27: Identification of co-located CRHR1-GFPir- and CamKIIαir- in the CeA. Upper panel shows the overview of the amygdala from the mouse atlas (Paxinos and Frankling, 2000) and from immunofluorescence staining for GFPir (green), αCamKII ir (red) and with merged channels (yellow) in *Crhr1*-GFP reporter mouse line. Lower panel left shows quantification of co-located GFPir- and αCamKII ir -positive cells. White arrows indicate to GFPir and αCamKII ir co-localization. BLA: basolateral amygdala; CeA: central amygdala.

3.4.2 *In vitro* validation of Cre-dependent activation of AAV-*Crhr1* overexpression

To confirm conditional CRHR1 expression in vitro, human embryonic kidney (HEK293) cells were transfected with a plasmid in which the *Crhr1* cDNA was preceded by transcriptional termination signals that were flanked by Cre recombinase recognition sequences (loxP sites). Immunocytochemical analysis showed that the absence of Cre recombinase prevented, whereas co-transfection with a Cre plasmid activated CRHR1 expression (Figure 28).

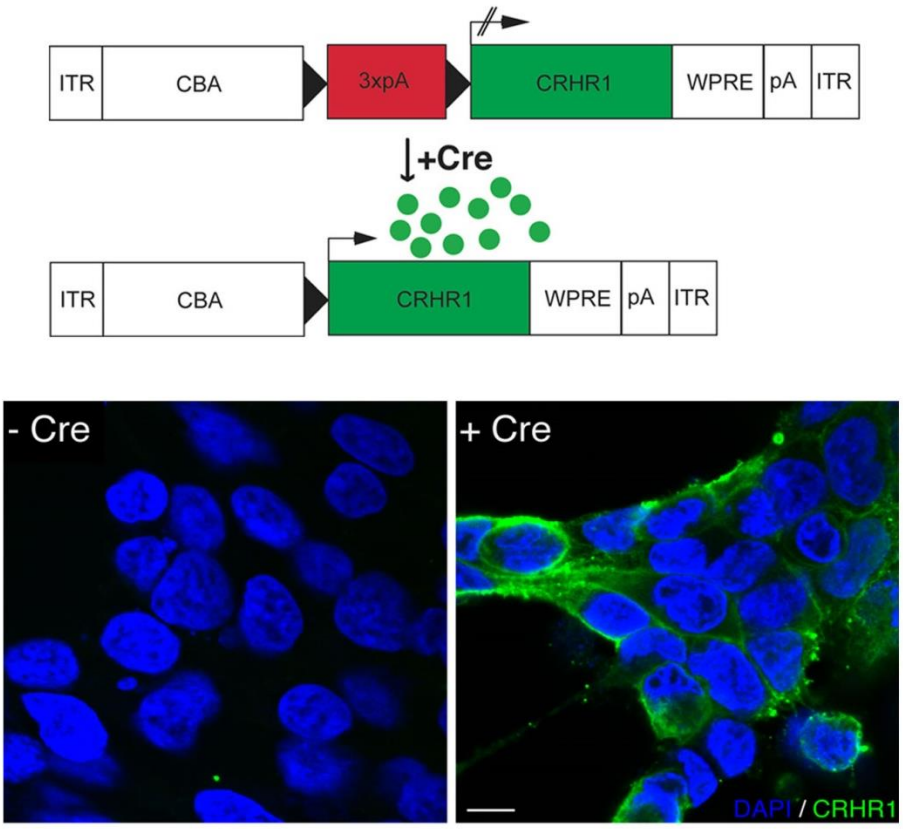

Figure 28: *in vitro* validation of AAV-CRHR1 overexpression. HEK cells were co-transfected with pAAV-Stop-CRHR1 and a control plasmid (left panel) or pAAV-Cre (right panel). Transgene expression detected by immunocytochemistry for CRHR1 is completely silenced in the absence of Cre following Cre-dependent activation of transgene expression CRHR1 immunoreactivity can be detected at the cell surface. Blue: cell nucleus staining with DAPI, Green:CRHR1. Bar scale: 10µm

3.4.3 *In vivo* validation of Cre-dependent activation of AAV-*Crhr1* overexpression

Rats were treated with bilateral virus injections to induce CRHR1 mRNA overexpression in the CeA of αCaMKII^{CreERT2} rats. *In situ* hybridization showed the localization of the virus (by identifying WPRE-sequence) and revealed a strong, 24-fold increase of CRHR1 mRNA compared to animals injected with control virus, in the targeted area ($F[1,23]=31.6$; $p<.001$, Figure 29). The rats were also tested in the Open Field to determine any effect of the CRHR1 overexpression on the locomotion activity under baseline conditions. Animals with increased Crhr1 mRNA in the CeA did not show any significant changes in locomotor activity, which excludes the possibility of this factor to influence ethanol drinking experiments (Figure 29D).

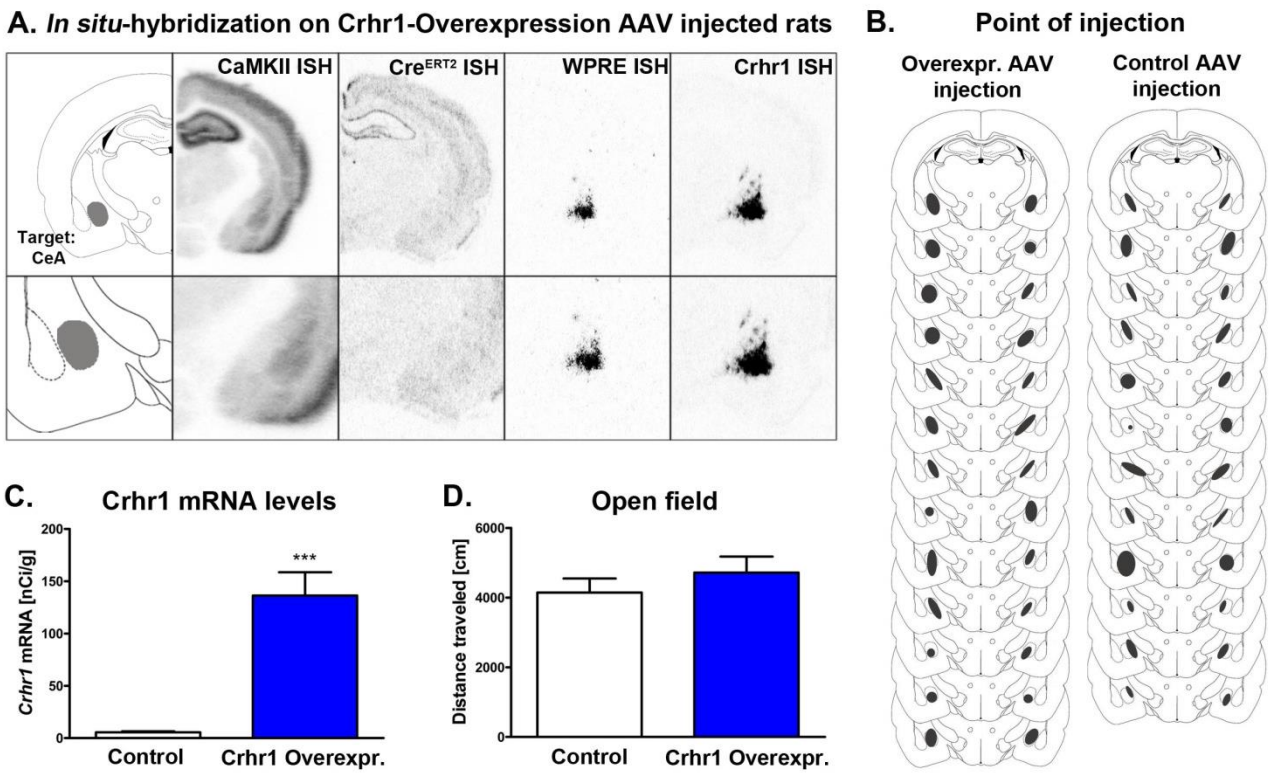

Figure 29: *in vivo* validation of AAV-CRHR1 overexpression and map of the injection sites. A: Schematic outline of the target region (Bregma -2.8 mm, according to (Paxinos et al, 1998), and representative images of in situ hybridization for αCaMKII, Cre, WPRE and Crhr1 mRNA following tamoxifen treatment. B: Map of the injection sites. C. Crhr1 mRNA expression in the CeA. Bar graphs are expressed as mean ± SEM (nCi/g) D. Basic behavioral assessment of rats with intra-amygdala Crhr1 overexpression by open field test. Graphs show distance travelled (cm) during 15 min of testing. Statistical analysis for both *in situ* hybridization and Open Field test was performed by one-way ANOVA, n=12-13/group, p values: ***p<0.001.

3.4.4 Yohimbine- but not Cue-induced Reinstatement is increased by intra-amygdala *CRHR1* overexpression

αCaMKII^{CreERT2} rats were trained to self-administer 10% ethanol by pressing a lever in the operant apparatus. After a saccharine-fading procedure rats went through 10 training sessions and reached a baseline of 77.0±11.7 (controls) and 84.1 ±10.1 (designated CRHR1 overexpression group) active lever presses (Figure 30B). Two-way ANOVA did not find any significant difference between the genotypes either in the active lever presses (F[10,14]=0.8, p=.649) or in the inactive lever presses (F[10,14]=0.7, p=.744). Both groups got the same amount of reinforces during the training phase

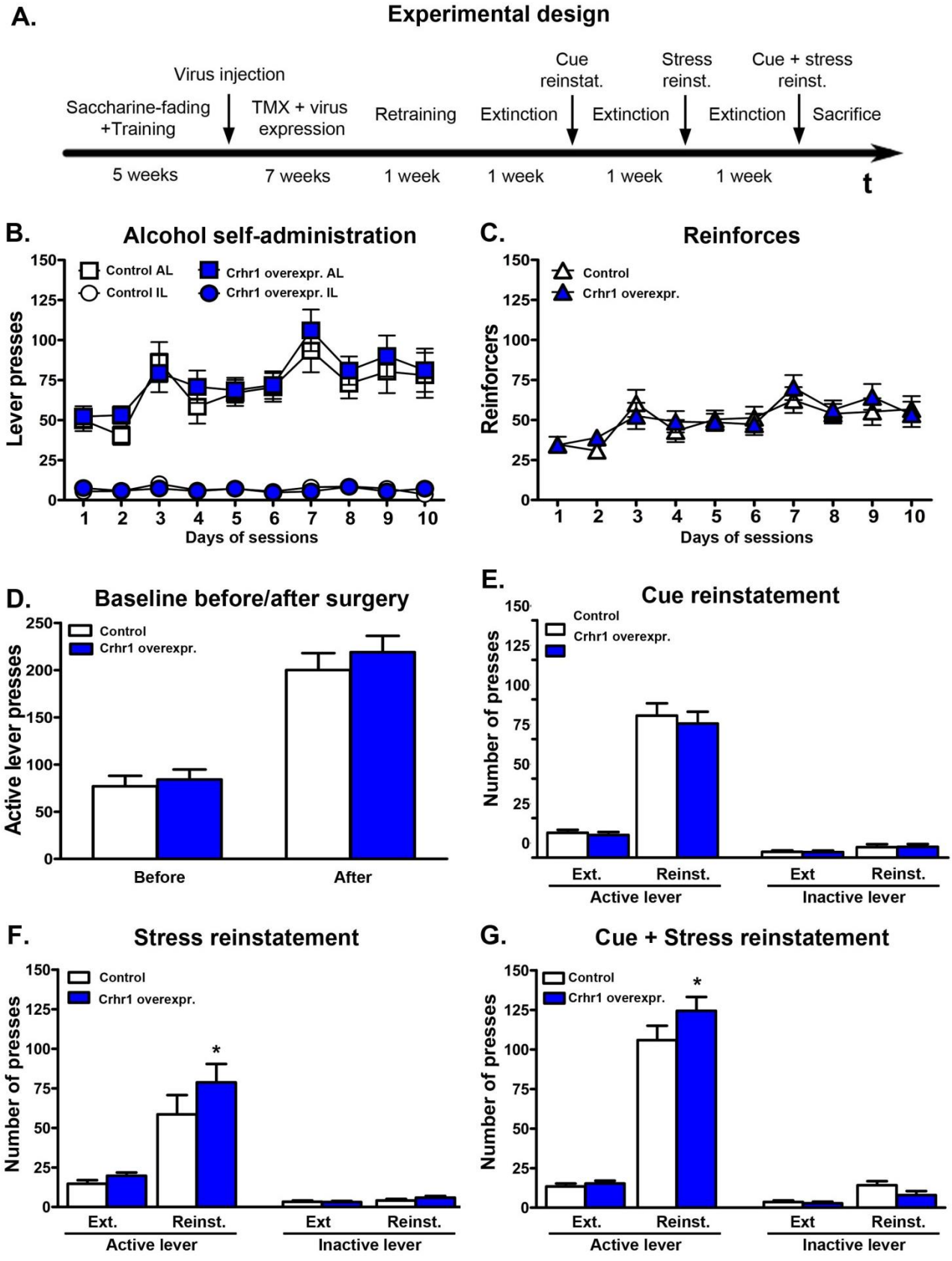
A.
Experimental design
Virus injection
Saccharine-fading +Training
TMX + virus expression
Retraining
Extinction
Cue reinstat.
Extinction
Stress reinst.
Extinction
Cue + stress reinst.
Sacrifice
5 weeks
7 weeks
1 week
1 week
1 week
1 week
t
B.
Alcohol self-administration
Control AL
Control IL
Crhr1 overexpr. AL
Crhr1 overexpr. IL
Lever presses
Days of sessions
C.
Reinforces
Control
Crhr1 overexpr.
Reinforcers
Days of sessions
D.
Baseline before/after surgery
Control
Crhr1 overexpr.
Active lever presses
Before
After
E.
Cue reinstatement
Control
Crhr1 overexpr.
Number of presses
Ext.
Reinst.
Ext
Reinst.
Active lever
Inactive lever
F.
Stress reinstatement
Control
Crhr1 overexpr.
Number of presses
*
Ext.
Reinst.
Ext
Reinst.
Active lever
Inactive lever
G.
Cue + Stress reinstatement
Control
Crhr1 overexpr.
Number of presses
*
Ext.
Reinst.
Ext
Reinst.
Active lever
Inactive lever

Figure 30: Stress-induced alcohol seeking behavior in CaMKII^{CreERT2} rat injected with CRHR1-overexpression virus and controls. Experimental design (A). Active and inactive lever presses (B.) and number of reinforcers (C.) during training sessions with conditioned and environmental cue. Animals have not been injected with any virus but are already designated to their respective groups. D. Baselines of the last three training sessions before and after intra-amygdala injection of Crhr1 overexpression virus. E-G: Cue (E), stress (F) and combined cue + stress (G) -induced reinstatement of ethanol-seeking behavior. Bar graphs illustrate number of lever presses of control rats (white bars) and rats with Crhr1 overexpression in the CeA (dark-blue bars). Stress was induced by i.p. injection of yohimbine. Statistical analysis was performed by repeated measures ANOVA, followed by Newman-Keuls post-hoc test if appropriate; n=12-13; p values: *p<0.05 control vs. Crhr1 overexpression.

(repeated measures: treatment F[10,14]=0.9, p=.549, Figure 30C). A period of 6 weeks was given to the animals to recover from virus injection and tamoxifen treatment. The new baseline obtained after 1 week of alcohol self-administration was increased by 2.6 times in both groups (two-way ANOVA: time F[1,23]=121.2, p<.001). However, it is important to underline that there was no treatment effect, either before or after the surgery (F[1.23]=0.6, p=.455, Figure 30D).

After extinction, the rats were tested for cue, stress, and a combined cue-stress reinstatement of ethanol seeking (Figure 30 E-G). Both groups showed a significant reinstatement in all tests (repeated measures analysis day effect: Cue reinstatement: F[1,23]=200.1, p<.001; Stress reinstatement: F[1,23]=39.2, p<.001; Cue and Stress reinstatement: F[1,23]=287.3, p<.001). Furthermore, overall effect on lever showed that animals clearly distinguished between reward- and not reward-associated lever (repeated measures analysis lever effect: Cue reinstatement: F[1,23]=23.9, p<.001; Stress reinstatement: F[1,23]=84.1, p<.001; Cue and Stress reinstatement: F[1,23]=292.3, p<.001). CRHR1-overexpression in the CeA did not significantly influence cue reinstatement, however post-hoc analysis revealed a slight increment of the active lever presses during stress-induced reinstatement compared to control rats (p=.025), as well as the combination of cue and stress-induced reinstatement (p=.0108).

3.4.5 Summary

Taken together, these results confirmed the specific involvement of CeA CRHR1 on stress-induced reinstatement. Furthermore, the methodological tools applied, warranted to disentangle the functional effect of CRHR1 on the modulation of CeA- αCaMKII neurons in responses to stress-induced alcohol seeking behavior.

3.5. STUDY 5: OPPOSITE EFFECTS OF CRHR1 ON DAT AND D1 NEURONS IN COCAINE CUE-INDUCED REINSTATEMENT

Drug craving and subsequent relapse following abstinence remain a major challenging aspect to the treatment of drug addiction in humans. Relapse can be caused by one of several factors, including exposure to drug-related cues or the drug itself, as well as stress. Hyper-function of CRHR1 has been implicated in the pathology of chronic stress, including the development of anxiety and mood disorders. Furthermore, there is high comorbidity of anxiety and mood disorders with substance use disorders in humans and stress plays an important role in increasing the vulnerability and motivation to abuse drugs in addicted individuals (Koob and Le Moal, 2001).

The purpose of the current study was to examine the role of neuronal selectivity of CRHR1 receptors on cocaine-mediated behaviors. This study was performed in collaboration with Dr. Rick E. Bernardi, who performed cocaine self-administration behavioral experiments and Dr. Jan Deussing, who provided $DAT^{CreERT2}$- and $D1^{CreERT2}$-$CRHR1^{f/f}$ mice.

3.5.1 Home cage locomotion in $DAT^{CreERT2}$-$CRHR1^{f/f}$ and $D1^{CreERT2}$-$CRHR1^{f/f}$ mouse lines

$DAT^{CreERT2}$-$Crhr1^{f/f}$ and controls did not differ in their home cage activity during 3 days of recording with e-motion devices (genotype: $F[1,19]=0.075$, $p=.787$, n=10-11 animal/group, Figure 31A). A similar pattern was observed in $D1^{CreERT2}$-$CRHR1^{f/f}$ mice and their control littermates. No difference between the genotypes was found ($F[1,20]=0.010$, $p=.922$, n=11 animal/group, Figure 31B). Repeated measures analysis of variance revealed for both lines a main effect of hours ($DAT^{CreERT2}$- $CRHR1^{f/f}$ line: $F[17,323]=19.937$, $p<<.001$; $D1^{CreERT2}$-$CRHR1^{f/f}$ line: $F[17,340]=73.195$, $p<<.001$). This effect was expected: indeed during the dark phases (grey sections in Figure 31 the locomotion activity increased compared to the light phase, due to the fact that rodents are nocturnal animals.

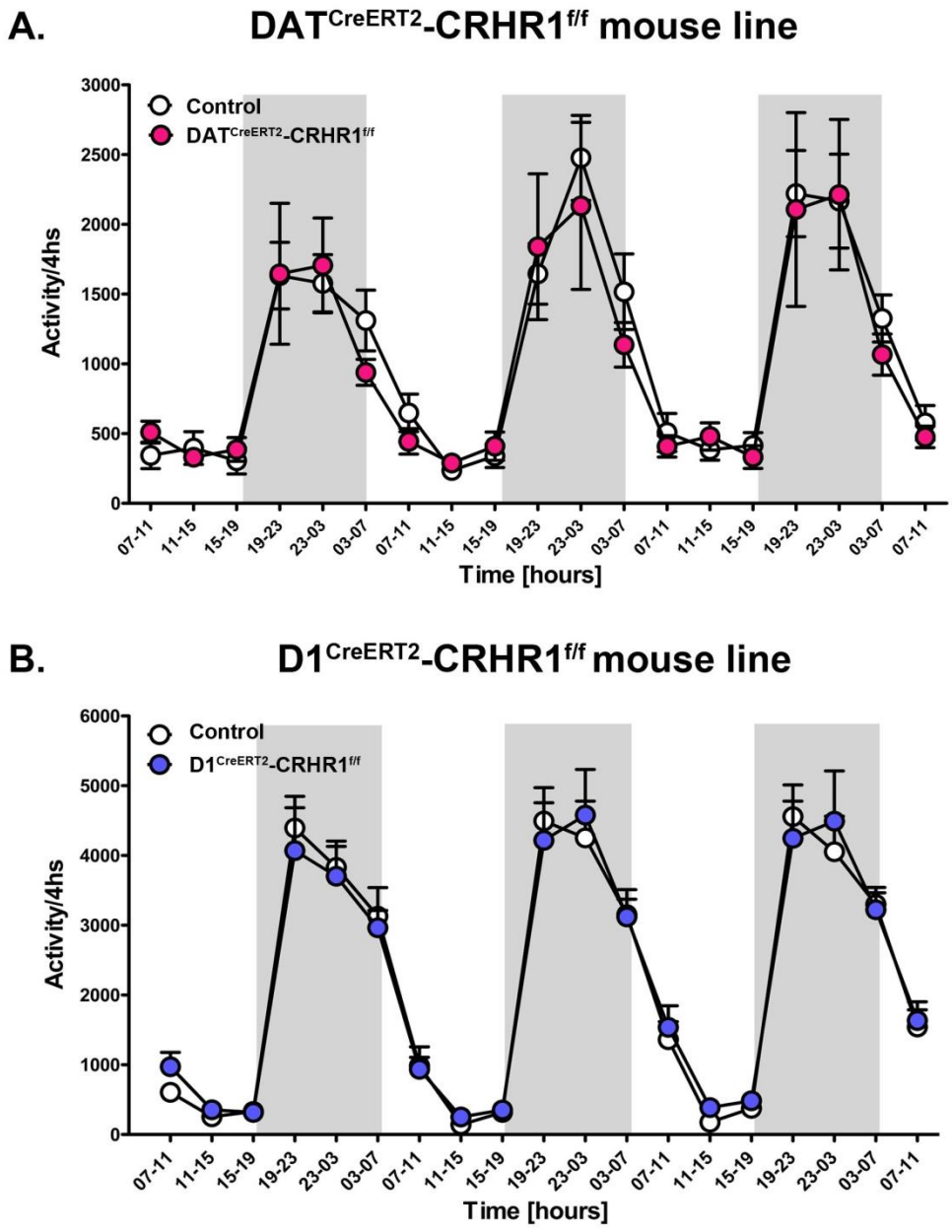

Figure 311: Home cage locomotion in DAT^{CreERT2}-CRHR1$^{f/f}$ and D1^{CreERT2}-CRHR1$^{f/f.}$ Home cage activity was monitored for 3 consecutive days. Dark phases are showed with grey background. Data are expressed in activity/4h. DAT^{CreERT2}-CRHR1$^{f/f}$ mice are represented in pink, while D1^{CreERT2}-CRHR1$^{f/f}$ in violet.

3.5.2 Cocaine-induced locomotor sensitization

DAT^{CreERT2}- CRHR1$^{f/f}$ and controls did not differ in the locomotor response to saline (n=7 and n=9 for DAT^{CreERT2}- CRHR1$^{f/f}$ and control mice, respectively) or the acute and sensitized locomotor response to cocaine (n=8 and n=9 for DAT^{CreERT2}- CRHR1$^{f/f}$ and control mice, respectively). Figure 32 shows the locomotor activity to saline or cocaine on acute (day 1) and chronic (day 5) treatment

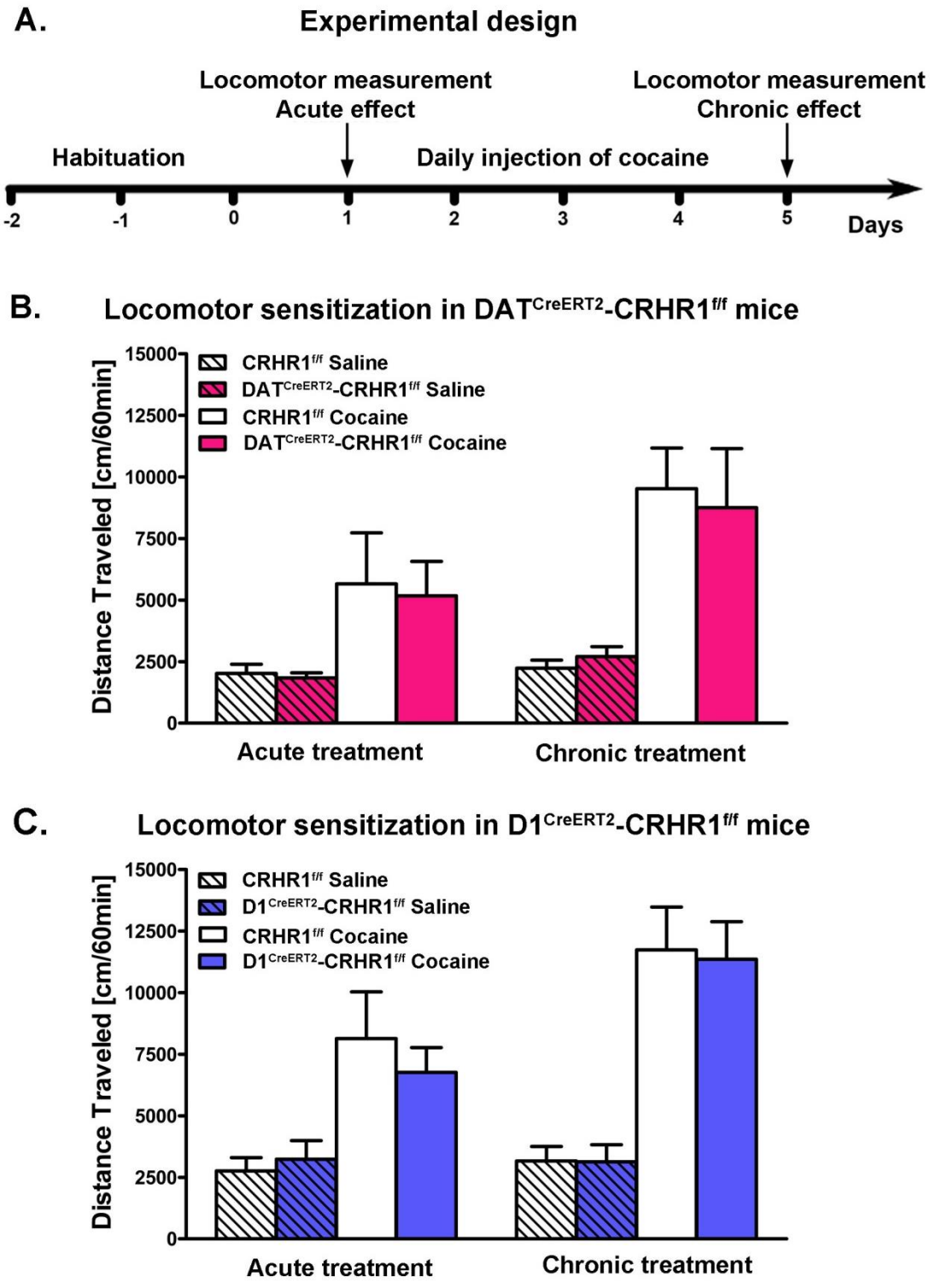

Figure 322: **Cocaine-induced locomotor sensitization in DAT^{CreERT2}-CRHR1$^{f/f}$ and D1^{CreERT2}-CRHR1$^{f/f.}$** Upper panel shows the time course (days) for cocaine-induce locomotor sensitization. B. Sensitization in DAT^{CreERT2}-CRHR1$^{f/f}$ (pink) and their CRHR1$^{f/f}$ littermates (white). Panel C reveals cocaine sensitization in D1^{CreERT2}-CRHR1$^{f/f.}$ (violet) and their littermates (white). Control saline-treated groups are represented with lined bars. All data are showed as mean activity (± SEM) during the 60-min locomotor activity trial on acute and chronic cocaine/saline treatment. Statistical analysis were performed as repeated measures ANOVA, with Fisher's post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001.

for each group. Data represent mean (± SEM) distance traveled (cm) across the 60-min activity trials. A repeated measures analysis revealed significant main effects of both treatment ($F[1,27]=15.7$, $p<.01$) and day ($F[1,27]=29.7$, $p<.01$) and a significant treatment x day interaction ($F[1,27]=16.6$, $p<.001$), but no other effects. Both $DAT^{CreERT2}$- $CRHR1^{f/f}$ mice showed a significant increase in locomotion from acute to chronic cocaine-treatment, confirming sensitization, while saline-treated $DAT^{CreERT2}$- $CRHR1^{f/f}$ and controls littermates did not reveal any treatment effect on locomotion (Figure 32B).

A similar pattern was observed in the cocaine-induced locomotor sensitization in $D1^{CreERT2}$-$CRHR1^{f/f}$ mouse line (Figure 32 C). Indeed, as showed in figure, $D1^{CreERT2}$-$CRHR1^{f/f}$ and their $CRHR1^{f/f}$ littermates did not differ in the locomotor response to saline (n=9 and n=10 for $D1^{CreERT2}$-$CRHR1^{f/f}$ and control mice, respectively) or the acute and sensitized locomotor response to cocaine (n = 9 each for $D1^{CreERT2}$-$CRHR1^{f/f}$ and control mice). However, a repeated measures analysis revealed significant main effects of both treatment ($F[1,34]= 29.1$, $p<.001$) and day ($F[1,34]= 48.2$, $p<.001$) and a significant treatment x day interaction ($F[1,33]= 41.7$, $p<.001$), but no other effects. Paired t-tests confirmed both cocaine-treated $D1^{CreERT2}$-$CRHR1^{f/f}$ mice and controls revealed a strong increase in locomotion from acute to chronic cocaine treatment, demonstrating a cocaine-induced locomotion sensitization. As expected, such increase was not found in saline-treated $D1^{CreERT2}$-$CRHR1^{f/f}$ mice and controls littermates.

3.5.3 Cocaine self-administration and cue-induced reinstatement in $DAT^{CreERT2}$-$Crhr1^{f/f}$ mice

$DAT^{CreERT2}$- $CRHR1^{f/f}$ (n=7) mice and controls (n=8) did not differ in cocaine self-administration. Figure A shows the mean (± SEM) responding on the active and inactive levers during the 2 hours sessions performed for 8 consecutive days. Repeated measures analysis revealed significant main effects of lever ($F[1,13]=221.1$, $p<.001$) and a significant main effect of day ($F[2,25.6]=3.4$, $p<.05$), indicating a distinction between the active and inactive levers and a fast learning ability (Figure 33A). However, there was no difference between the genotypes, both in the number of presses and in reinforces during the self-administration sessions (Figure 33 B).

Figure 33C shows the mean (± SEM) responding on the active and inactive levers during the final 2 days of extinction sessions: Two-way ANOVA analysis demonstrated a significant main effect of

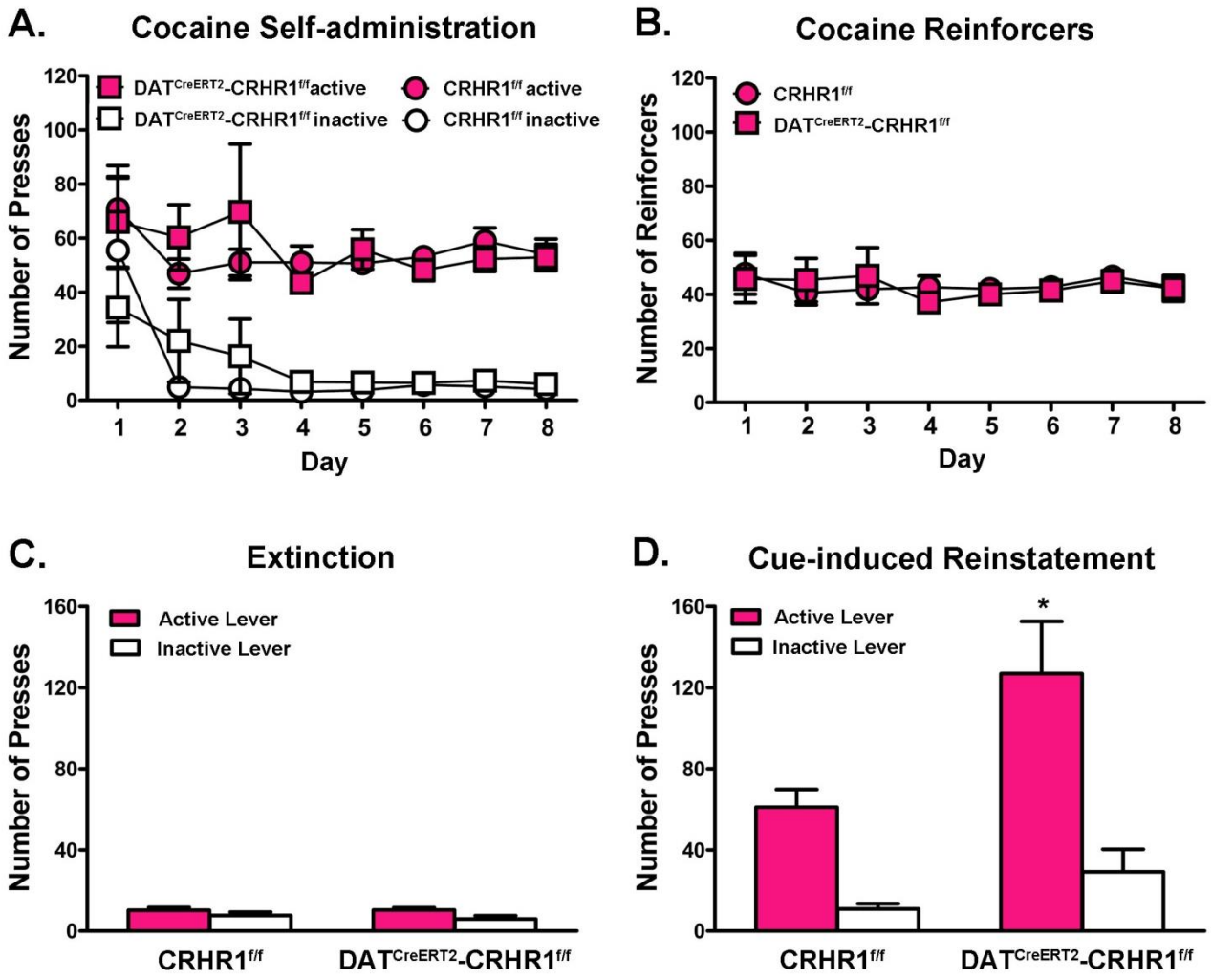

Figure 333: Cocaine self-administration and cue reinstatement in DAT^{CreERT2}-CRHR1$^{f/f}$ mice. A: Both DAT^{CreERT2}-CRHR1$^{f/f}$ (pink bars) and controls (white bars) demonstrated similar acquisition of the task. B: Number of cocaine reinforcers achieved during the acquisition phase. C: No difference in responding in DAT^{CreERT2}-CRHR1$^{f/f}$ mice and controls during the final two days of extinction trials. D: DAT^{CreERT2}-CRHR1$^{f/f}$ mice demonstrated increased cue-induced reinstatement relative to controls, as indicated by an increase in responding on the active lever without a corresponding increase in responding on the inactive lever. All data represent mean number of presses (± SEM) on the active and inactive levers during a single 2hr session of cue reinstatement; *p<0.05, **p<0.01, ***p<0.001.

lever (F[1,13]=21.9, p<.001), but no main effect of genotype (F[1,13]=0.2, p=.682)and no interaction (F[1,13]=1.6 p=.234).

During cue-induced reinstatement, DAT^{CreERT2}- CRHR1$^{f/f}$ mice pressed two times more the cocaine-associated active lever compare to their littermates (p<.05, Figure 333 D), but the groups did not differ on the number of inactive lever presses, indicating a selective increase in responding on the cocaine-associated lever by DAT^{CreERT2}- CRHR1$^{f/f}$ mice relative to controls. Statistical

analysis detected a main effects of genotype (F[1,13]=6.1, p=.027) and lever (F[1,13]=53.3, p<.001), but more importantly, a significant lever x genotype interaction (F[1,13]=5.5, p=.035).

3.5.4 Cocaine self-administration and cue-induced reinstatement in D1^{CreERT2}- CRHR1$^{f/f}$ mice

Self-administration phase did not reveal any difference between D1^{CreERT2}-CRHR1$^{f/f}$ mice (n=9) and their CRHR1$^{f/f}$ controls (n=10) (Figure 34A). As for DAT^{CreERT2}-CRHR1$^{f/f}$ mice, three-way ANOVA revealed a significant main effects of lever (F[1,15]=140.6, p<.001), indicating a distinction between the active and inactive levers, and a significant day x lever interaction (F[4.1,69.8]=9.5, p< .001). However, there was no main effect of genotype or genotype x lever interaction, and no other main effects or interactions Figure 34B shows the mean (± SEM) number of cocaine infusions received during the 8 days of cocaine self-administration. A two-way ANOVA (day x genotype) showed a significant main effect of day (F[3.7,62.4]=3.7, p<.05), but no significant main effect of genotype or day x genotype interaction (Fs<1, p>.05), indicating no difference in number of reinforcers based on genotype.

Responding at the conclusion of extinction did not differ between D1^{CreERT2}-CRHR1$^{f/f}$ mice and controls (Figure 34C), showing a significant main effect of lever (F[1,17]=43.0, p<.001), but no main effect of genotype (F[1,17]=0.5, p=.484) and no interaction (F[1,17]=1.0, p=.320). During cue reinstatement, D1^{CreERT2}-CRHR1$^{f/f}$ mice demonstrated pressed the cocaine-associated active lever 42% less than the CRHR1$^{f/f}$ mice (Figure 34D). Statistical analysis demonstrated a main effects of genotype (two-way ANOVA: F[1,17]=8.6, p=.009), lever (F[1,17]=74.3, p<.001) and a significant lever x genotype interaction (F[1,17]=4.8, p=.042). These results indicated a selective decrease in responding on the cocaine-associated lever by D1^{CreERT2}-CRHR1$^{f/f}$ mice relative to their controls.

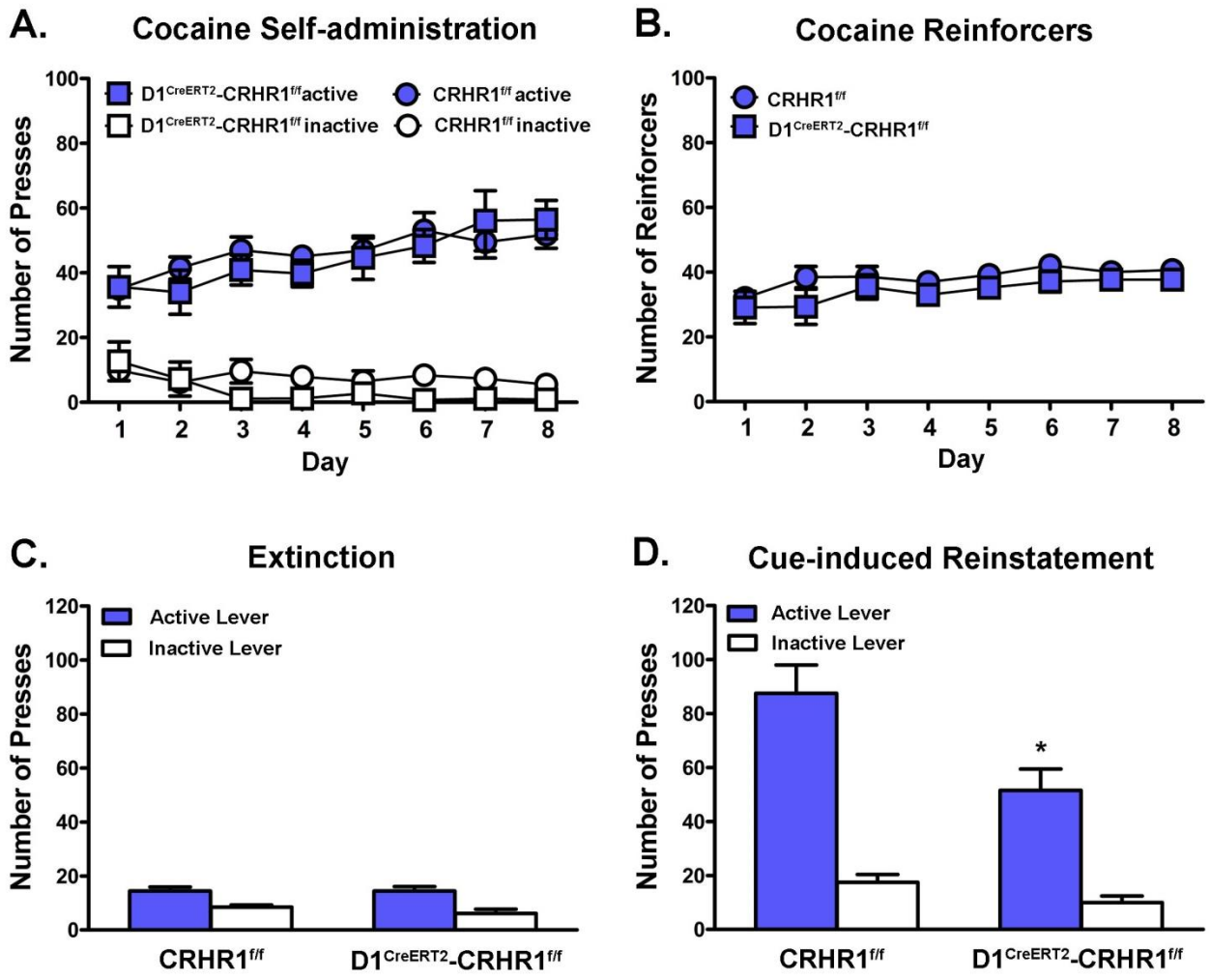

Figure 34: Cocaine self-administration and cue reinstatement in D1^{CreERT2}-CRHR1$^{f/f}$ mice. A: Both D1^{CreERT2}-CRHR1$^{f/f}$ (violet bars) and controls (white bars) demonstrated similar acquisition of the task. B: Number of cocaine reinforcers achieved during the acquisition phase. C: No difference in responding in D1^{CreERT2}-CRHR1$^{f/f}$ mice and controls during the final two days of extinction trials. D: D1^{CreERT2}-CRHR1$^{f/f}$ mice demonstrated increased cue-induced reinstatement relative to controls, as indicated by an increase in responding on the active lever without a corresponding increase in responding on the inactive lever. All data represent mean number of presses (± SEM) on the active and inactive levers during a single 2hr session of cue reinstatement; *p<0.05, **p<0.01, ***p<0.001.

3.5.5 Summary

These data demonstrate the involvement of CRHR1 in cue-induced reinstatement following cocaine self-administration, and implicate CRHR1 in presynaptic negative feedback and postsynaptic feed-forward roles controlling dopamine signaling and subsequent cocaine-mediated behaviors. These results implicate a bi-directional control of CRHR1 in DAT- and D1-containing neurons related to relapse-like behavior.

PART IV: DISCUSSION

4.1 DISCUSSION STUDY 1: CROSS-TALK OF CRHR1 AND D1 ON ANXIETY-RELATED BEHAVIOR

The cross-talk between receptors is considered a dynamic driving force by which different brain systems synergistically assimilate, regulate and integrate signals during the responses to external stimuli. In the last years, many studies demonstrated the crucial role of the CRH as well as the dopaminergic system in the responses to stressful stimuli (Kormos and Gaszner, 2013; Belujon and Grace, 2015). A pivotal region involved in stress-mediated behavior is the amygdala which receives input in its basolateral portion from cortical regions (LeDoux et al., 1991; Strobel et al., 2015), integrates and filtrates the information through the intercalated cells (ITCs) (McDonald, 1985; Ehrlich et al., 2009), and regulates the responses to the stimuli by the CeA-mediated activation of downstream brain regions (e.g. BNST, PFC and Acb) (LeDoux, 2000).

According to the work from Justice (2008) and Jacobsen (2006), CRHR1 and D1 seem to be co-expressed within the amygdala region. Thus, the aim of this study was to demonstrate and characterize a potential interaction of both receptors in emotional responses to stressful stimuli. This was achieved by identification of D1 expression level through receptor autoradiography assays performed on brain tissues of animals previously treated with CRHR1 agonists alone and/or in combination with D1 antagonist.

Using the *Crhr1*-GFP reporter line, double immunostainings demonstrated for the first time a high degree of co-localized CRHR1 and D1 in the amygdala nuclei e.g. BLA, CeA and in the main nucleus of the ITCs (IN, Figure 15).

I.c.v. injection of CRHR1-specific stressin I agonist, induced an increase in D1 binding sites exclusively in the amygdala nuclei (Figure 16). Notably, the effect obtained by CRH treatment is not as strong as by stressin I. This might be explained through differential affinities of the compounds for the CRH receptors family. CRH as natural neuropeptide, binds both CRHR1 (Ki=0.95 nM) and CRHR2 (Ki=13 nM) (Donaldson et al., 1996), while stressin I displays specifically high affinity for CRHR1 (Ki=1.5 nM) versus CRHR2 (Ki=224 nM) (Rivier et al., 2002; Rivier et al., 2007). Thus, CRHR2 may affect the action of CRHR1 on D1 by competing for the ligand or interfering on the transductional level (Grammatopoulos, 2012), according to its role in

attenuating the emotional responses to stress (Bale et al., 2000; Kishimoto et al., 2000; Valdez et al., 2003).

Site-specific injection of stressin I induced a similar increase of D1 binding sites in the amygdala region as shown in the previous experiment (Figure 17). Interestingly, the upregulation of D1 binding after stressin I was fully blocked by co-treatment with the D1 antagonist (SCH23390), thus pointing to a strong impact of CRHR1 in the stimulation of D1 activiy. Functional evidence of a CRHR1-D1 crosstalk on anxiety-like behavior was given by the elevated plus maze test. As expected, amygdala specific stimulation of CRHR1by stressin I induced an anxiogenic behavior which is in agreement with a variety of studies (Smagin et al., 2001; Bruchas et al., 2009). More intriguing, co-treatment of stressin I with D1 antagonist SCH23390 fully blocked anxiogenic responses after CRHR1 activation alone. These results are in line with a recent study published from De la Mora and colleagues (2005), in which D1 blockade by intra-amygdala injection of SCH23390 displayed an anxiolytic effect. D1 activation on anxiety-related behavior has been further shown in further studies applying different behavioral paradigms like e.g. fear-potentiated acoustic startle response (Lamont and Kokkinidis, 1998), conditioned freezing response (Guarraci et al., 1999; El-Ghundi et al., 2001) or dark-light box (de la Mora et al., 2005). Although the dopamine system is also involved in the regulation of motor behavior, the site-specific blockage of D1 in the amygdala did not alter the locomotion activity (Figure 17). Indeed, the number of total entries during the elevated plus maze test did not differ between the treatment groups. Thus, our results demonstrate that stimulation of CRHR1 mediates the responses to stressful stimuli at least in part by synergistic activation of D1 in the amygdala.

As a final proof, we used double transgenic mice, in which CRHR1 was deleted in D1 neurons ($D1^{Cre}$-$CRHR1^{f/f}$ mice). Here, i.c.v. injection of stressin I did not augment the level of D1 binding sites in the amygdala regions, while in control mice ($CRHR1^{f/f}$) stressin I increased D1 similar as shown in the previous rat experiment (Figure 18).

Behavioral characterization of the $D1^{Cre}$-$CRHR1^{f/f}$ line under baseline conditions did not show any behavioral differences between the genotypes as assessed by homecage locomotion and Open Field, as well as in anxiety-related test i.e. EPM, Dark-Light box (Figure 21). However, during the cue-recall phase of fear conditioning test, an anxiety-like paradigm mainly related to the amygdala circuitry, $D1^{Cre}$-$CRHR1^{f/f}$ mice displayed less percentage of freezing relative to their control littermates ($CRHR1^{f/f}$) and thus, less anxiety. These findings strongly suggest the presence of an

interaction between CRHR1 and D1 receptor in the amygdala region which requires the stimulation of the CRH system.

Functional evidence clarifying the mechanism underling D1-CRHR1 receptor interaction in the ITCs, was provided by electrophysiological whole-cell patch-clamp recordings in the amygdala using a *Gad67*-GFP reporter mouse line (Figure 19). According to the results obtained by Marowski and colleagues (2005), dopamine induced a hyperpolarization of the ITCs (Marowsky et al., 2005). Remarkably, co-treatment with dopamine and stressin I further enhanced and prolonged the hyperpolarization in these cells, suggesting that under stressful conditions, the activation of both CRHR1 and D1 disinhibits the BLA and the CeA, leading to an enhanced response to a stressful stimulus.

Although this study demonstrated a synergistic interaction between CRHR1 and D1 in mediating anxiety-like behaviors, the molecular mechanism underlie this cross-talk remains unknown. The effects on increased D1 upon CRHR1 stimulations were obtained in a time interval of one hour after the treatment, which most likely are due to a regulation at the protein level, involving phosphorylation/ recycling and potential receptor heteromerization processes. Hauger and co-workers demonstrated *in vitro* the role of CRHR1 phosphorilation and desensitization (homologous or heterologous) as an adaptive mechanism to stress (Hauger et al., 1997; Hauger et al., 2000; Dautzenberg et al., 2001; Hauger et al., 2003) involving β-arrestins. The recruitment of this protein triggers different mechanism as e.g. receptor internalization processes (Bonfiglio et al., 2013), followed by degradation or reuptake of the receptor, desensitization (Dautzenberg et al., 2001; Teli et al., 2005) and also signaling propagation (Punn et al., 2006). In our study, the BRET assays provided for the first time a strong evidence of a high affinity between D1 an CRHR1, which determines a fast formation of heterocomplex (Figure 20). The functional relevance of GPCRs homo- or heteromerization was already assessed in different studies demonstrating its impact on the receptor trafficking (Terrillon and Bouvier, 2004), the affinity to the ligand (El-Asmar et al., 2005) and the signaling pathway (Lohse, 2010).

Summary

Our study demonstrated that co-localized CRHR1 mediates anxiogenic responses via D1 through an amygdala specific pathway. Functionally, CRHR1 increases the dopamine-induced hyperpolarization of D1 ITCs, which leads to a disinhibition of the CeA. The nature of D1-CRHR1

interaction may be driven by heteromerization of the two receptors. Thus, D1-CRHR1 interaction represents a **novel mechanism** of anxiety-like responses and our study provides an important mechanistic insight into amygdala and ITCs functioning under physiological conditions and anxiety-like behavior.

4.2 DISCUSSION STUDY 2: IMPACT OF CRHR1-D1 INTERACTION ON ALCOHOL DEPENDENCE

Numerous clinical and preclinical evidences revealed a very close link between anxiety and alcohol use disorders (Kushner et al., 2000; Roberts et al., 2000; Weiss et al., 2001). Indeed, alcohol dependence is defined as a chronic relapsing disease (Heilig and Egli, 2006) and many studies demonstrate the involvement of stress in trigger relapse and chronic negative affective states (Koob and Kreek, 2007; Sinha and Li, 2007; Zorrilla et al., 2014). Research in alcoholic patients and animal models implicates dysregulation of amygdala function in this process determined by excessive activity of the CRH system and its receptor CRHR1 (Gehlert et al., 2007; Sommer et al., 2008; Zorrilla et al., 2014). On the other hand, the role of dopamine and D1 receptor is mainly related to the positive reinforcing effects of alcohol and maintenance of voluntary alcohol consumption (El-Ghundi et al., 1998; Spanagel and Weiss, 1999; Liu and Weiss, 2002). The results previously presented in Study 1 have demonstrated a functional interaction between D1 and CRHR1 in the amygdala and provided evidence on ITCs regulation of the emotional responses to stress. Since there is no knowledge available regarding the involvement of D1-CRHR1 crosstalk in alcohol dependence, the aim of this study was to investigate D1 expression level in the amygdala nuclei in an established rat model of alcohol dependence. Here, we established the functional impact of D1-CRHR1 interaction in the regulation of alcohol consumption and dependence using $D1^{Cre}$-$CRHR1^{f/f}$ mice.

As previously mentioned, the so called "post-dependent state" is a well-established model for dependence characterized by long-lasting behavioral changes as e.g. increased voluntary alcohol consumption, high vulnerability to stress, and long-lasting neuroadaptation that persist during protracted abstinence (Rimondini et al., 2002; Sommer et al., 2008).

In situ hybridization for D1 mRNA revealed a strong and long-lasting down-regulation in all ITC clusters, the BLA, and CeA of post-dependent rats compared to controls (Figure 22B). This effect was translated to protein level as measured by [^{3}H]-SCH23390 autoradiography (Figure 22A). Interestingly, a similar down-regulation of D1 was observed in the accumbal regions of post-dependent rats due to increased extra-cellular level of dopamine in these regions after three weeks of abstinence (Hirth et al., 2016, *"Convergent evidence from alcohol dependent humans and rats for a hyperdopaminergic state during prolonged abstinence"*, in press). Furthermore, previous studies reported an upregulation of CRHR1 at both transcriptional and protein level in the amygdala nuclei of post-dependent rats (Sommer et al., 2008) and mice (Eisenhardt et al., 2015), which

underlie the high vulnerability to stress and elevated alcohol consumption following emotionally stressful situations (Overstreet et al., 2004; Valdez et al., 2004; Breese et al., 2005; Sommer et al., 2008). Thus, during protracted abstinence, the strong upregulation of CRHR1, together with an assumed hyper-dopaminergic state (Hirth et al., 2016, in press), induces a strong decrease of D1 in the amygdala as part of a probably compensatory mechanism.

In order to investigate the impact of D1-CRHR1 interaction on alcohol dependence, alcohol consumption of $D1^{Cre}$-$CRHR1^{f/f}$ mice and their littermates ($CRHR1^{f/f}$) was investigated through a two-bottle free-choice paradigm. The post-dependent phenotype was induced in mice using a similar paradigm as for rats, constituted by chronic intermittent cycles of intoxications and withdrawals (modified from Becker and Lopez, 2004). After three days of withdrawal, all alcohol vapor exposed mice increased their daily alcohol intake, according to previous studies applying the CIE paradigm in mice (Lopez and Becker, 2005; Gilpin et al., 2009). However, mice lacking CRHR1 in D1-containing neurons consumed significantly less alcohol than their genotype-controls (Figure 23). To further identify the functional involvement of D1-CRHR1 interaction in the regulation of stress during alcohol dependence, the CRH system was again challenged by repeated forced swim stress and the alcohol intake was monitored during the following days. Post-dependent $D1^{Cre}$-$CRHR1^{f/f}$ mice displayed a reduced percentage of floating-time during the test and subsequent less stress-induced alcohol intake, clearly indicating a diminished vulnerability to stress compared to their $CRHR1^{f/f}$ littermates. These results together confirm the crucial role of CRHR1 in the regulation of alcohol dependence (Hansson et al., 2006; Sommer et al., 2008). Moreover, as demonstrated in previous studies, CRH-driven hypersensitivity is crucial in the development of alcohol dependence and withdrawal, causing relapse and negative affective states (Heilig and Koob, 2007; Sommer et al., 2008). Our study further confirms this concept, since alcohol intake, did not differ between the groups during baseline conditions and, after repeated stimulation of CRHR1 by forced swim test, all non-dependent animals as well as the post-dependent $D1^{Cre}$-$CRHR1^{f/f}$ mice did not increased their alcohol consumption. Thus post-dependent $D1^{Cre}$-$CRHR1^{f/f}$ mice are less vulnerable to stress and are more efficient to cope with aversive stimuli during alcohol dependence.

Based on the findings provided in Study 1, ITCs are highly involved in the regulation of emotional responses to stress due to D1-CRHR1 interaction. However, the current study demonstrated that during protracted abstinence the strong increase of CRHR1 induces a counter-adaptive down-regulation of D1. Thus, when stressful stimuli occur, ITCs functionality is abolished, leading to a deep disinhibition of the amygdala, in particular the CeA. Under healthy conditions, the

homeostasis is quickly reestablished once the stressful event is terminated. Conversely, during alcohol dependence, the physiological conditions cannot be further restored due to the long-lasting neuroadaptations of CRHR1 and D1 receptors. This permanent activation of the amygdala leads, in turn, to the high vulnerability to stress in alcohol addiction as well as to the augmented consumption of alcohol as an attempt to mitigate the negative affective state developed during abstinence.

Summary

This study demonstrated that during protracted abstinence, post-dependent rat displayed a strong downregulation of D1 mRNA and protein level in the amygdala, which is a probable compensatory effect due to the strong upregulation of CRHR1 (Sommer et al., 2008) together with an hyper-dopaminergic state (Hirth et al., 2016, in press). Furthermore, alcohol drinking behavior monitored in post-dependent $D1^{Cre}$-$CRHR1^{f/f}$ mice and controls, revealed that CRHR1-D1 receptor interactions in amygdala neurons are involved in the hyper-responsiveness to stress that leads to a chronic negative affective states and thus, perpetuates the vicious cycle of alcohol addiction.

4.3 DISCUSSION STUDY 3: DIFFERENTIAL ROLE OF DOPAMINERGIC- AND DOPAMINOCEPTIVE CRHR1 ON STRESS-INDUCED DRINKING BEHAVIOR

The ventral tegmental area (VTA) is a heterogeneous brain region that plays a crucial role in motivation and reward processing (Nestler and Carlezon, 2006; Fields et al., 2007; Wise and Morales, 2010; Jennings et al., 2013). Although it is constituted by a mixture of neurons, the major component of the VTA consists of dopaminergic cells (Nair-Roberts et al., 2008; Sesack and Grace, 2010). Dopamine projections originating in this nucleus innervate the striatal, cortical, limbic and hypothalamic regions. Through these pathways, dopamine regulates different physiological functions, such as the control of coordinated movements and hormone secretion, as well as motivated and emotional behaviors (Hornykiewicz, 1966; Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012). Lots of efforts have been spent to provide a common organization of the multitude of neuronal populations of the VTA based not only on the functional and anatomical characteristics, but also on the projecting target regions and the micro-circuitries within the VTA (Lammel et al., 2012; Stamatakis and Stuber, 2012; Lammel et al., 2014b). Even though the functional role of the dopaminergic neurons in the VTA is well characterized, the presence and distribution of D1-expressing neurons within this region is so far not well established. Thus, the aim of this study is to disentangle the anatomical organization of the dopaminergic system within the VTA. In order to achieve this aim, we performed double-immunostainings on coronal C57Bl/6 mouse midbrain sections for tyrosine hydroxylase (TH), an enzyme mainly involved in the synthesis of dopamine, and D1.

D1-ir was found close to cell bodies, while TH-ir was located on both cell bodies and fiber tracts. Quantitative analysis of the double immunostainings with NeuN-ir revealed that 70% of the neuronal population in the VTA expressed TH-ir (Figure 24). This finding is in line with previous studies demonstrating (Nair-Roberts et al., 2008; Sesack and Grace, 2010). We further demonstrated that about 23% of the neurons within the VTA express D1-ir. A more intriguing observation was that D1-ir is highly enriched in neurons of the dorsal portion, while TH-ir is mainly found in the ventral part of the VTA. TH-ir and D1-ir cells seemed not to be co-located in the same cells. Furthermore, TH-ir fiber tracts were observable close to D1-ir containing cell-bodies, suggesting that D1 is located post-synaptically to TH-expressing neurons. Thus, the differential distribution of TH-ir and D1-ir suggests an anatomical organization of dopaminergic and dopaminoceptive neurons within the VTA. Phillipson and colleagues (1979) identified in the dorsal

part of the VTA a region called parabrachial nucleus (PBP) (Phillipson, 1979), which later on was associated to the regulation of taste-aversion (Reilly, 1999; Li and Cho, 2006; Hajnal et al., 2009), food reward (Reilly and Trifunovic, 2000; Hajnal et al., 2009), pain (Missig et al., 2014) and fear (Sato et al., 2015). Thus, the organization of dopaminergic and dopaminoceptive neurons may also have an impact on VTA micro-circuitry and function.

The release of dopamine from VTA dopaminergic neurons can be regulated by different neuropeptides, including CRH (Holly et al., 2015). Indeed, it was shown that CRH is released into the VTA during foot-shock (Wang et al., 2005) and social defeat stress (Boyson et al., 2014), forming inhibitory and excitatory synapses onto VTA dendrites through its receptors(Tagliaferro and Morales, 2008). In this way CRH flux into the VTA during stress regulates VTA dopamine neurons projecting to the Acb and the mPFC. However, it is not well established to which extent CRHR1 is expressed in VTA neurons. For this reason, we quantified the level of CRHR1 in dopaminergic (TH-positive) and dopaminoceptive (D1-positive) neurons using the *Crhr1*-GFP reporter line (Figure 25). Analysis revealed that half of the cell population within the VTA expresses CRHR1 (GFP-ir), confirming the high involvement of the CRH system on the regulation of VTA functionality (Boyson et al., 2014; Polter and Kauer, 2014). Furthermore, double-immunostaining performed on the ventral portion of the VTA, previously shown to be highly enriched of THir-containing neurons, revealed a strong co-localization between CRHR1 and TH, further suggesting a functional impact of CRHR1 on dopaminergic projections in this region. Although, GFP-ir cells were recorded in less amount cells in the dorsal area of the VTA, almost half of them co-localized with D1-ir, These findings not only confirm the pivotal role of the CRH system in the activity of the VTA, but demonstrate for the first time the specific co-localization of CRHR1 within the dopaminergic system in different subareas, suggesting that CRHR1 may have a different impact on the behavior, depending on cell-type specific activation.

Regulation of the dopaminergic system in reward-related behaviors has received a great deal of attention because of the serious consequences of dysfunction in this circuitry, such as alcohol addiction and anxiety (Baik, 2013; Polter and Kauer, 2014). During acute stress, VTA dopaminergic neurons are quickly activated (Anstrom and Woodward, 2005; Anstrom et al., 2009; Brischoux et al., 2009), determining a strong increase of extracellular dopamine in the mPFC and Acb shell (AcbSh) (Abercrombie et al., 1989; Imperato et al., 1989). Recent studies applying mice lacking functional CRHR1, demonstrated the role of the receptor in mediating alcohol-drinking behavior after repeated forced swim stress (Sillaber et al., 2002) and social defeat stress (Sillaber et

al., 2002; Molander et al., 2012). Furthermore, intra-VTA injection of CRHR1 antagonist reduces alcohol intake in stressed mice (Hwa et al., 2015). However, the specific influence of CRHR1 on dopaminergic or dopaminoceptive neurons of the VTA in stress-induced alcohol seeking remains to be characterized.

In the current study $DAT^{CreERT2}$-$CRHR1^{f/f}$ and $D1^{Cre}$-$CRHR1^{f/f}$ mouse lines were used to determine the cell-specific involvement of CRHR1 in dopaminergic and D1neurons upon repeated stress. Here, one week of repeated cycles of ethanol vapor exposure was applied as a paradigm for chronic stress and the subsequent voluntary alcohol consumption was considered an index for anxiety-like behavior (Figure 26). After three days of withdrawal, all stressed mice increased their alcohol-intake. However, $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice maintained a lower level of ethanol consumption in respect to their $Crhr1^{f/f}$ littermates. This finding thus demonstrates the impact of dopaminergic CRHR1 in stress induced alcohol intake, giving a further support to the crucial role of CRHR1 dopaminergic neurons of the VTA in the modulation of stressful events. Furthermore, the latter vulnerability to stress displayed by $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice as compared to their controls, is in line with the study from Refojo and colleagues showing a lower level of anxiety in the same animals (Refojo et al., 2011).

Although in Study 2 $D1^{Cre}$-$CRHR1^{f/f}$ mice consumed significantly less alcohol than their $CRHR1^{f/f}$ littermates after three days of withdrawal, here both genotypes consumed a comparable amount of alcohol. The discrepancy between these data can be easily explained. Firstly, the behavioral paradigm, i.e. the CIE procedure, was reduced to 1 week of stress-exposure, which might activate different neuronal pathways involving different mechanisms. Secondly, unlike $D1^{Cre}$-$CRHR1^{f/f}$ mice used in the previous study, these animals did not show a post-dependent phenotype. Thus, the animals had not yet developed long-lasting neuroadaptations observable in alcohol dependence (e.g. upregulation of CRHR1, down-regulation of D1, withdrawal signs), thus in certain extend, they were still able to cope with stress. Our results indicated that CRHR1 in D1 neurons of the VTA does not play a role in stress-induced alcohol-drinking behavior, probably due to the low and defined D1 expression level in this region. However, since the dorsal portion of the VTA anatomically overlaps with the PBP, further studies might be directed to disentangle the role of CRHR1 in D1-neurons within this region in response to pain (Missig et al., 2014) and gustatory regulation (Reilly and Trifunovic, 2000).

Summary

Immunohistochemical assays revealed two different neuronal populations within the VTA: the dopaminergic TH-ir neurons, mainly distributed in the ventral portion and the dopaminoceptive D1-ir neurons mainly expressed in a small division of the dorsal part of the VTA. CRHR1 is expressed in both neuronal populations, suggesting a differential role of CRHR1 in these neurons. Using cell-type specific CRHR1 knockout mice we found that alcohol consumption after repeated stress, is mainly driven by CRHR1 expressed in dopaminergic, but not dopaminoceptive neurons.

4.4. DISCUSSION STUDY 4: αCAMKII-DEPENDENT CRHR1 MODULATES STRESS-INDUCED ALCOHOL SEEKING BEHAVIOR IN THE CENTRAL NUCLEUS OF THE AMYGDALA

The CeA is the main output nucleus of the amygdala and its activity is strongly influenced by the CRH which, by triggering the CRHR1, modulates the responses to stressful stimuli (Arborelius et al., 1999; LeDoux, 2000) not only in naïve, but also in drug dependent animals (Shaham et al., 1998; Erb et al., 2005; Gilpin, 2012; Gilpin and Roberto, 2012).

The calcium/calmodulin-dependent protein kinase II (αCaMKII) plays a crucial role in the modulation of synaptic function by its calcium-specific activation The alpha subunit is the predominant isoform out of 28 different isoforms in mammals brain (Hudmon and Schulman, 2002) and is expressed throughout the forebrain (Benson et al., 1991; Benson et al., 1992; Jones et al., 1994; Liu and Murray, 2012). αCaMKII is considered as a marker for glutamatergic neurons within the hippocampus and cortex region (Benson et al., 1991). Based on *in situ* hybridization studies αCaMKII is express in GABAergic neurons of the thalamic reticular nucleus, the globus pallidus, cerebellar Purkinje cells and the commissural nucleus of the stria terminalis (Benson et al., 1992). Furthermore, recent studies suggested αCaMKII expression in GABAergic cells of the amygdala, in particular the intercalated cell masses (ITCs) (Meins et al., 2010; Huang et al., 2014) and the CeA (Beckerman et al., 2013). Thus, αCaMKII is expressed in both glutamatergic and inhibitory cells. Although the CeA consist mainly in GABAergic neurons, our *in situ* hybridization for αCaMKII and Cre mRNA showed expression also in the CeA (Figure 29). Furthermore, double immunostaining for CRHR1 (GFP-ir) and αCaMKII-ir using a *Crhr1*-GFP reporter line demonstrated that almost half of αCaMKII-positive neurons co-localized with CRHR1 in CeA (Figure 27).

The aim of the present study is to determine the role of CRHR1 in the αCaMKII-expressing cells of the CeA during alcohol-mediated behavior. To achieve this objective, CRHR1 over-expression was induced by adeno-associated virus (AAV) injection in the CeA of rats expressing inducible Cre^{ERT2}-recombinase under the promotor of αCaMKII ($αCaMKII^{CreERT2}$ rats) (Schonig et al., 2012).

Animals were first trained to self-administer alcohol, and then their alcohol-seeking behavior was assessed by cue-, stress- and cue plus stress-induced reinstatements, followed by subsequent extinction sessions (Figure 30). Our results showed that $αCaMKII^{CreERT2}$ rats over-expressing CRHR1 in the CeA increased their responses to stress-induced reinstatement compared to their

controls, but not during cue-induced reinstatement. These results not only confirmed the tight comorbidity between stress and relapse in alcohol dependence, but give also a further insight into the role of CRHR1 in αCaMKII-expressing cells within the CeA in the regulation of alcohol seeking behavior. Remarkably, our findings were obtained using a novel technique that combines advanced genetically modified rats and viral vector gene transfer. These useful tools allow targeting a precise brain region and help to disentangle the role of a receptor within a specific neuronal population in a certain brain region.

Although the method applied was innovative, the results obtained were not completely surprising, since it is well established that CRHR1 within the amygdala nuclei mediates stress-induced reinstatement. Indeed, previous studies demonstrated that injection of CRHR1 antagonists (e.g. antalarmin, d-phe-CRH and CP-154526) inhibits stress-induced reinstatement of alcohol seeking (Rassnick et al., 1993; Le et al., 2000; Hansson et al., 2006; Marinelli et al., 2007; Cippitelli et al., 2012), while activation of the CRH system by CRH site specific injection mediates increased anxiety-like responses, alcohol intake during withdrawal or other stressful conditions (Koob and Kreek, 2007; Huang et al., 2010; Wills et al., 2010). Furthermore, constitutive limbic CRHR1 knockout mice displayed lower anxiety compared to their controls (Muller et al., 2003); similar results on anxiety-like behaviors were obtained in rats treated with a RNA-interference technique in order to specific knock-down the CRHR1 in CeA (Callahan et al., 2013).

In our study, alcohol seeking behavior was induced by the pre-treatment with yohimbine, an alpha-2-adrenoceptor antagonist. Previous studies demonstrated that the effect of yohimbine on alcohol reinstatement is stable and reliable (Shepard et al., 2004; Le et al., 2005) and is reversed by CRHR1 antagonists (Marinelli et al., 2007; Cippitelli et al., 2012). It also induces CRH mRNA expression level in the BNST and the CeA (Funk et al., 2006), demonstrating the interaction between the noradrenergic and the CRH system. Based on neuroanatomical and functional interactions between these two systems in stress-induced relapse-like behavior it is likely that noradrenaline, arising from the lateral tegmental nuclei (LTN) and the locus coeruleus (LC) acts on CRH-containing neurons in in the CeA (Quirarte et al., 1998; Erb et al., 2001).

Anatomical evidences showed that the lateral portion of the CeA (CeL) receives excitatory afferents from cortical areas and thalamus, while the medial part of the CeA (CeM), receives excitatory projections from the BLA as well as inhibitory input from the ITCs and CeL (Pitkanen et al., 2000; Ciocchi et al., 2010; Haubensak et al., 2010). Both subnuclei of the CeA project to the BNST (Sun et al., 1991). However, it is not well understood which synaptic connections drive emotion and

alcohol-related behavior (Ehrlich et al., 2009; Pape and Pare, 2010). Beckerman et al. (2013) determined the structural distribution of CRH and CRHR1 in NMDA-expressing and projecting neurons of the CeA to the BNST (Beckerman et al., 2013). Their results revealed extensive expression of NMDA receptors in the soma and dendrites of CRH-ir neurons as well as in the post-synaptic CRHR1-expressing cells receiving input from CRH-positive axon terminals. Based on the knowledge that αCaMKII is a key modulator of NMDA receptors signaling, it is likely to infer that both αCaMKII and NMDA receptor follow a similar distribution (Benson et al., 1992). Thus, during stress-induced reinstatement, αCaMKII-containing neurons of the CeA received glutamategic input directly from the BLA (Pitkanen et al., 1997), the limbic cortex (McDonald, 1998), the hippocampus (Canteras et al., 1992), the thalamus (Li and Kirouac, 2008) and indirectly from the BLA by the activation of CRH-releasing neurons from the lateral part of the CeA (Eliava et al., 2003; Asan et al., 2005). The high expression of CRHR1 in these neurons might modulate the release of CRH in the BNST which in turn regulates the alcohol-seeking behavior via the activation of the Acb (Figure 3535).

Although the over-expression of CRHR1 is restricted to a neuron-specific population of a single brain region, the behavioral effect was detectable. These results confirm the role of CRHR1 in the CeA and reveal for the first time the impact of CRHR1 containing αCaMKII-cells on stress-induced alcohol seeking behavior. However, further investigation would be necessary in order to understand whether αCaMKII is expressed exclusively within excitatory neurons or not. The specific role CRHR1 pre- and post-synaptically in the neurons of the CeA need further investigation as well.

Summary

Our study further demonstrated the role of CeA CRHR1 in the regulation of the alcohol-seeking behavior under stressful conditions. Moreover, by using an innovative combination of transgenic rat line and region- and cell-type specific AAV overexpression of CRHR1, we demonstrate that αCaMKII-CRHR1 in CeA mediates at least in part stress-induced reinstatement to alcohol seeking behavior. The role of αCaMKII-CRHR1 during cue-induced reinstatement will be the aim of future investigation which requires CRHR1-overexpression in αCaMKII-neurons in brain regions such as e.g. BLA on alcohol-mediated seeking behavior.

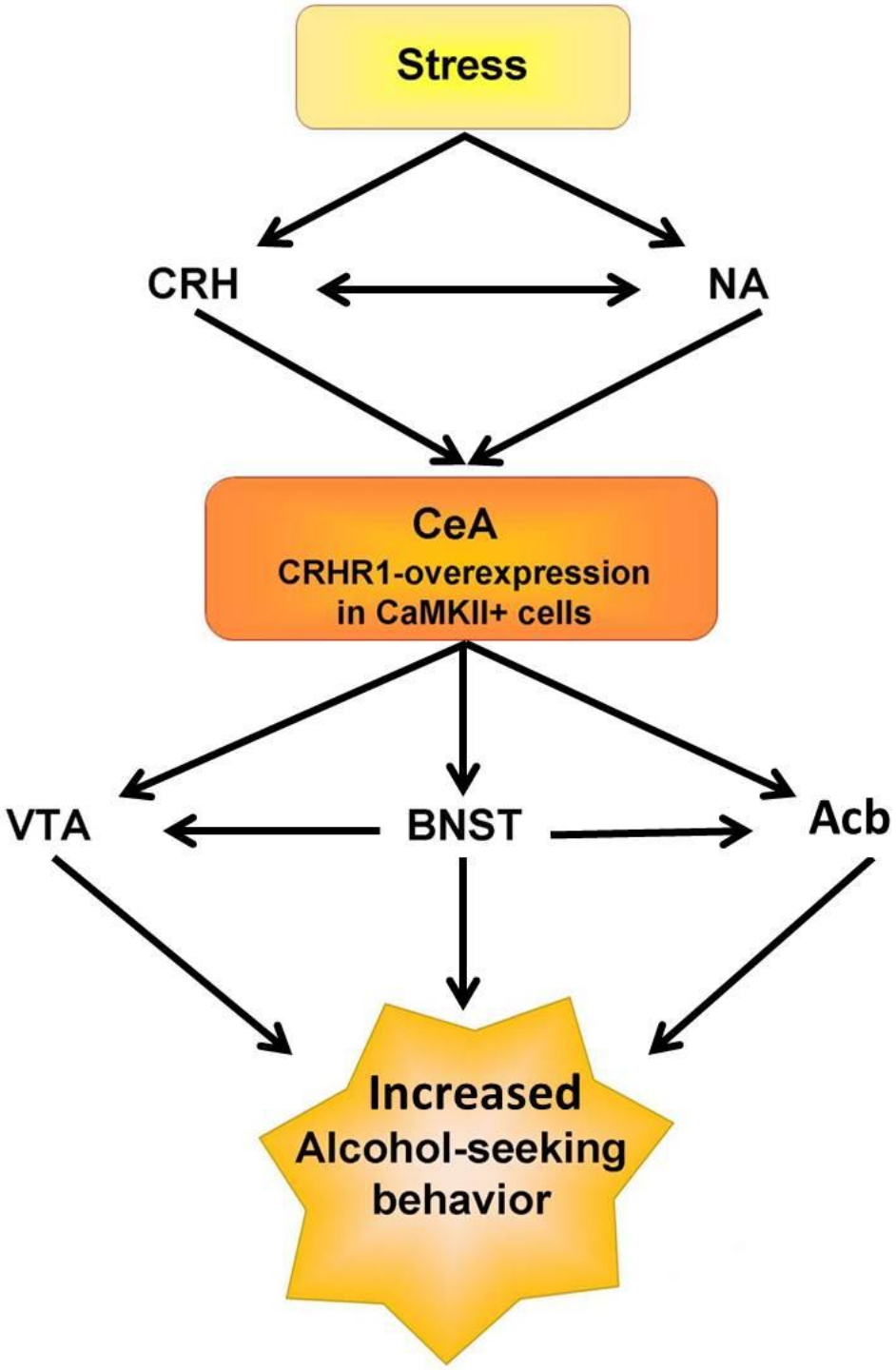

Figure 3535: Schematic representation of the mechanism underlie the increased alcohol-seeking behavior in rats overexpressing CRHR1 in their CeA. A stressful stimulus, here constituted by yohimbine injection (1.25 g/kg, i.p.), activates the CRH and the noradrenergic (NA) systems. Together these systems activate the central amygdala (CeA) of αCaMKII^{CreERT2} rats previously treated with Crhr1-overexpressing virus. The enhanced functionality of the CeA, further activates downstream regions i.e. BNST, VTA and Acb, determining an increased alcohol seeking behavior in αCaMKII^{CreERT2} rats compare to their controls.

4.5 DISCUSSION STUDY 5: OPPOSITE EFFECTS OF CRHR1 ON DAT AND D1 NEURONS IN COCAINE CUE-INDUCED REINSTATEMENT

The role of CRHR1 is well established in the regulation of the mesolimbic dopaminergic system and in response to drug of abuse. However, since CRHR1 is expressed in both dopaminergic and D1 neurons (Study 3), the aim of this study was to determine CRHR1 cell-specific impact on cocaine-related behavior using $D1^{CreERT2}$-$CRHR1^{f/f}$ and $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice. Locomotor sensitization, cocaine self-administration and cue-induced reinstatement for cocaine were tested in both lines.

$D1^{CreERT2}$- $CRHR1^{f/f}$, $DAT^{CreERT2}$-$CRHR1^{f/f}$ and their corresponding $CRHR1^{f/f}$ (controls) littermates showed an increase in locomotor activity after acute and chronic treatment of cocaine, indicating that CRHR1 in D1- and DAT- expressing cells are not implicated in the regulation of acute or sensitized locomotor response to cocaine (Figure 32). Furthermore, the number of active lever presses and reinforcers during cocaine self-administration did not differ between the knockout lines and their respective controls, suggesting that CRHR1 is not involved in the regulation of the dopaminergic system during cocaine self-administration (Figure 33 A-B and Figure 34 A-B).

The most intriguing results were obtained during the expression of cocaine-seeking behavior, since $D1^{CreERT2}$-$CRHR1^{f/f}$ mice displayed a lower number of active lever presses during cue-induced reinstatement compared to their controls (Figure 34D), while $DAT^{CreERT2}$-$CRHR1^{f/f}$ knockout mice increased their cocaine-seeking response relative to controls (Figure 33D). These findings suggested that the impact of CRHR1 on the modulation of relapse-like behavior for cocaine is related to the cell-type on which the receptor is specifically expressed.

The attenuation in the response to the conditioned cue in $D1^{CreERT2}$-$CRHR1^{f/f}$ mice is consistent with previous studies in which peripheral administration of CRHR1 antagonists attenuates drug-seeking behavior during cue- (Goeders and Clampitt, 2002; Cosme et al., 2015), stress- (Shaham et al., 1998; Marinelli et al., 2007; Zislis et al., 2007) and drug-induced reinstatement (Przegalinski et al., 2005) of different drugs. More in detail, peripherally injected CRHR1 antagonists determined a reduction of the response to the cue-induced reinstatement also in animals previously exposed to cocaine (Gurkovskaya and Goeders, 2001; Goeders and Clampitt, 2002; Smith and Aston-Jones, 2011). Furthermore, many studies demonstrated a diminution of cue-induced reinstatement due to the blockade of CRHR1 upon i.c.v. (Moffett and Goeders, 2007) or site-specific treatments with receptor antagonists in the insular cortex (Cosme et al., 2015).

In Study 1, double-immunostaining performed on sections of the *Crhr1*-GFP reporter mouse line revealed the co-localization between CRHR1 and D1 receptors in the BNST, in different nuclei of the amygdala (e.g. BLA, CeA), in the intercalated cell masses and in less extend in the VTA (Bernardi et al., 2015). Furthermore, Studies 1 and 2 demonstrated the existence of a strong interaction between CRHR1 and D1 receptors in the amygdala and their function within this region in mediating the responses to stressful stimuli and alcohol addiction. Interestingly, the amygdala, in particular the BLA, plays a crucial role in conditioned cue-induced drug-seeking behavior (Kalivas and McFarland, 2003; Kalivas and Volkow, 2005; See, 2005) by receiving dopaminergic afferent from the VTA and projecting both into the PFC and, directly or indirectly, into the Acb (Di Ciano and Everitt, 2001; Stefanik and Kalivas, 2013). The CRH neuropeptide, acting via CRHR1, has been demonstrated to potentiate D1 modulation of BLA-to-PFC pathway (Orozco-Cabal et al., 2008), since systemic pretreatment with the D1 antagonist SCH23390 blocked i.c.v. CRH-induced reinstatement of cocaine seeking (Brown et al., 2012). Thus, the loss of CRHR1 in D1 neurons in the BLA impedes the complete activation of glutamatergic projections to the PFC and Acb. The BLA-driven mechanisms might explain the attenuated cocaine-seeking behavior of $D1^{CreERT2}$-$CRHR1^{f/f}$ mice. Although D1 and CRHR1 have displayed a strong amygdala-specific interaction in the responses to aversive stimuli and alcohol dependence (Studies 1 and 2), the crucial regions involved in D1-CRHR1 interaction in cocaine-mediated behavior has to be further investigated. Nevertheless, our findings obtained with the inducible $D1^{CreERT2}$-$CRHR1^{f/f}$ line demonstrated that CRHR1 is involved in the regulation of conditioned cue-induced cocaine-seeking by interacting with D1.

The augmented response during cue-induced reinstatement displayed by $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice relative to their control group, is surprising since CRHR1 activation with CRH administration into the VTA has been demonstrated to enhance cocaine self-administration (Blacktop et al., 2011). Thus, a loss of CRHR1 in VTA should result in attenuated dopamine release involved in cocaine seeking (Ritz et al., 1987; Anderson and Pierce, 2005). Indeed, our findings are in contrast with a study showing a decrease in cue-mediated drug-seeking behavior induced by a lentiviral knockdown of CRHR1 in the VTA (Chen et al., 2014). However, it is crucial to underline that our experimental design included extinction training before the reinstatement, while the work provided by Chen and colleagues is based on an abstinence paradigm in which drug-seeking was not preceded by extinction. Although both designs have been widely applied to understand the neurobiological mechanisms mediating drug-related behaviors, it has been showed that they deeply differ mechanistically. Extinction induces plasticity changes in glutamatergic afferents from the

PFC to the Acb causing a blunted long-term depression, which in turn serve to inhibit cue-induced drug-seeking (Sutton et al., 2003; Kauer and Malenka, 2007; Knackstedt et al., 2010). Furthermore, functional inactivation of the BLA or PFC impairs explicit cue- and context-induced reinstatement of cocaine seeking after extinction but not after abstinence (McLaughlin and See, 2003; Fuchs et al., 2005; Fuchs et al., 2006b), demonstrating the crucial role of BLA and PFC in the extinction learning process. Conversely, abstinence paradigm involves mainly the dorsolateral caudate–putamen (dlCPu), a region required for habit learning (Jog et al., 1999; Fuchs et al., 2006a), which may underlie compulsive drug seeking behavior (Gerdeman et al., 2003). Remarkably, microdialysis performed in $DAT^{CreERT2}$-$CRHR1^{f/f}$ mouse line, have previously demonstrated a decreased stress-induced dopamine response in the PFC (Refojo et al., 2011). Thus, an increase in cue-induced reinstatement due to a loss of CRHR1 in DAT-containing neurons may be due to decreased dopamine release in the PFC (Gurkovskaya et al., 2005; Refojo et al., 2011), and a subsequent loss of enduring extinction-related glutamatergic plasticity in the Acb that modulates drug-seeking responses. This dysregulation in extinction-induced plasticity may also be affected by alterations in glutamatergic signaling in other dopamine terminal regions, such as the amygdala, which is also important for cue-induced reinstatement (See, 2005).

It is worth to mention another mechanism which might underlie the increase of reactivity of $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice during cue-induced reinstatement. The lack of CRHR1 on dopaminergic neurons determines a higher extracellular concentration of the neuropeptide which might be recruited by the CRH binding protein (CRH-BP). This protein activates CRHR2, facilitating calcium flow by a NMDA-mediated pathway and determining in turn the burst firing of the cell (Overton and Clark, 1997; Ungless et al., 2003; Wang et al., 2005). Furthermore, CRHR2 regulates glutamate release onto VTA dopamine neurons by disinhibition of the GABAergic suppression on glutamatergic cells (Williams et al., 2014). All together, these studies support the hypothesis that explains the increase of cue-induced reinstatement in $DAT^{CreERT2}$-$Crhr1^{f/f}$ mice in terms of compensatory mechanism: the loss of CRHR1 on DAT-expressing neurons, determines an increase in burst firing of the dopaminergic neurons and thus, dopamine is released into downstream regions like amygdala, PFC and Acb, which leads to a stronger expression of relapse-like behavior. The concerted actions between pre- and postsynaptic CRHR1 in the VTA, as well as the role of CRHR2 in drug-seeking behaviors need further evaluations during cue-conditioned reinforcement. Nonetheless, our findings and those previously reported indicate a strong impact of midbrain CRHR1 on drug-seeking behaviors.

Summary

Here we report a bi-directional control of CRHR1 in DAT- and D1-containing neurons during cue-induced reinstatement, following cocaine self-administration. $DAT^{CreERT2}$-$CRHR1^{f/f}$ mice displayed a strong increase of level pressing in cue-induced reinstatement, while $D1^{CreERT2}$-$CRHR1^{f/f}$ mice demonstrated a decrease in cue-induced reinstatement, indicating cell-type selectivity of CRHR1 receptors in the control of relapse-like behavior. Thus, in addition to its well-documented role in stress-induced reinstatement of drug-seeking, CRHR1 also plays an important role in conditioned reinforcement based on neuronal localization.

4.6 GENERAL DISCUSSION

A large amount of effort has been spent characterizing the involvement of the CRH system and its CRHR1 receptor in anxiety and addiction disorders. Despite promising results obtained in preclinical studies, several CRHR1 antagonists used for the treatment of depression and anxiety have not shown the expected efficacy in clinical trials (Zobel et al., 2000; Holsboer, 2014). The first reported clinical trial with the CRHR1 antagonist R121919 demonstrated some efficacy in the treatment of patients with major depression (Zobel et al., 2000). Although the experimental design has been largely criticized, it generated interest in the role of CRHR1 as a target for pharmacological treatments for anxiety and depression. To date, few results of clinical studies are published: however, several CRHR1 antagonists are currently undergoing phase I and phase II trials (e.g. R121919, verucerfont (Protocol #CRS106139), NCT00514865 (Zorrilla and Koob, 2010)). So far none have completed a successful phase III trial (Koob and Zorrilla, 2012).

The development of clinically-relevant compounds to treat drug disorders is challenging, as drug addiction is a dynamic process characterized by an alteration of the CRH system, which leads to the development of maladaptive behaviors. In recent years, several approaches have been investigated to help alcoholic patients to control not only alcohol drinking but also alcohol craving and relapse (Volpicelli et al., 1992; Monti et al., 1993). Pharmacological therapies such as naltrexone, acamprosate, and disulfiram are currently prescribed for the treatment of alcohol dependence (Volpicelli et al., 1992; Monti et al., 1993). However, despite some promising results, none of these medications is sufficiently effective. Pexacerfont, a CRHR1 antagonist, which showed a strong anxiolytic effect in preclinical experiments (Gilligan et al., 2009; Zhou et al., 2012) failed to exert an effect on behavioral, neuroimaging, and neuroendocrine outcomes in alcohol-dependent patients during early abstinence (Kwako et al., 2015). Thus, understanding the neurobiology of alcoholism will strongly contribute to the development of clinically valid compounds.

One possible explaination for the lack of effect during clinical trials is the fact that the majority of the compounds developed have been directed against specific receptors or proteins. Alternatively, intra-membrane receptor-receptor interactions in the central nervous system are considered a dynamic driving force by which different brain systems concertedly regulate, filtrate and integrate signals in response to external stimuli. Thus, an extensive characterization of the unique pharmacological and functional properties of receptor-receptor heteromerization may lead to the identification of new strategies for the development of novel medications to treat anxiety-related disorders and drug dependence.

In this regard, the findings presented in **Study 1** have provided solid evidence of an interaction between D1 and CRHR1 in the amygdala and ITCs. Thus, D1-CRHR1 cross-talk represents a **novel mechanism** of anxiety-like responses. Furthermore, behavioral and molecular experiments demonstrated the involvement of this interaction in the modulation of the response to stress during physiological conditions and alcohol dependence. D1-CRHR1 cross-talk may play a role in the mechanisms that underlie the negative affective state and relapse, both characteristics of alcohol dependence (**Study 2**).

Under physiological conditions, the BLA receives, integrates and projects sensory inputs to the CeA by innervating the intercalated cell masses (ITCs). These GABAergic clusters of interneurons tonically inhibit the activity of the CeA, which innervates efferent brain regions in a steady state manner (Figure 36A). When a stressful stimulus occurs, the BLA receives stronger innervations and CRHR1, mainly distributed within the ITCs, are activated. Via the enhancement and the recruitment of D1 expression on the cell-membrane, CRHR1 and D1 form heterocomplexes which induce a hyperpolarization of the ITCs. As a consequence, the CeA is temporary disinhibited and thus is free to orchestrate the responses to stress by the activation of downstream regions (e.g. BNST, VTA and Acb) (Figure 36B). Once the threatening stimulus disappears, the steady state is restored. In post-dependent animals, the strong recruitment of the CRH system leads to an increase of CRHR1 expression levels within the amygdala nuclei and thus, as a counteradaptive mechanism, D1 levels are downregulated. These neuroadaptations not only modify the molecular composition of the amygdala, but also alter its functionality. The strong disruption of ITC activity leads to a complete and long-lasting disinhinibition of the CeA (Figure 36C). This mechanism underlies, in part, the hyper-responsiveness to stressful inputs and the increased anxiety that result in increased alcohol consumption as relief from a negative affective state during alcohol dependence.

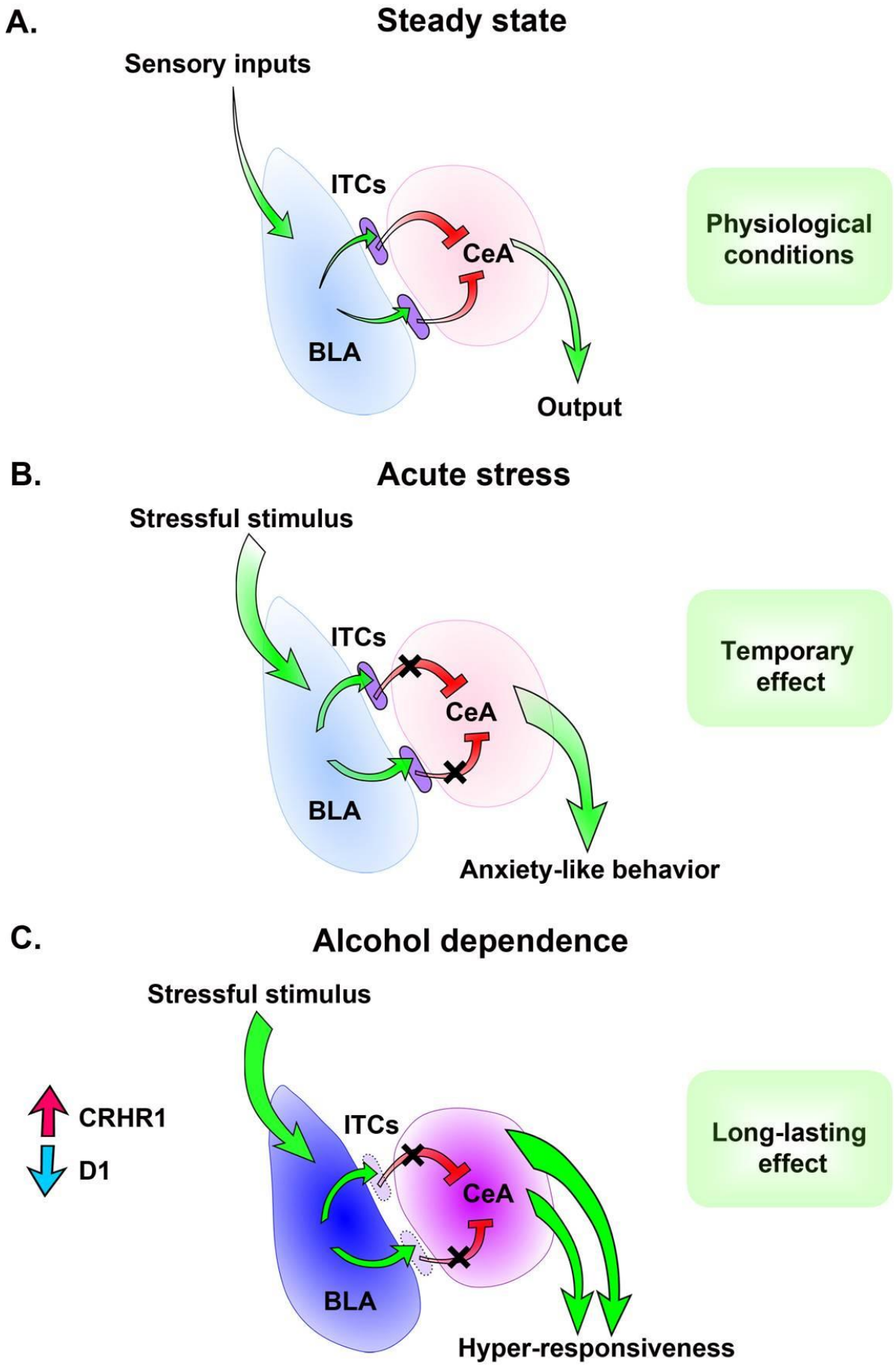
A.
Steady state
Sensory inputs
ITCs
CeA
BLA
Output
Physiological conditions
B.
Acute stress
Stressful stimulus
ITCs
CeA
BLA
Anxiety-like behavior
Temporary effect
C.
Alcohol dependence
Stressful stimulus
CRHR1
D1
ITCs
CeA
BLA
Hyper-responsiveness
Long-lasting effect

Figure 36: Involvement of D1-CRHR1 interaction in the regulation of emotional responses to stressful stimuli under healthy conditions and alcohol dependence. Under physiological conditions, intercalated cell masses (ITCs) tonically inhibit the activity of the CeA (A). During acute stress, CRHR1 activation within the ITCs, due to the strong BLA innervations, recruits D1 and together form heterocomplexes. Temporary hyperpolarization of the ITCs disinhibits the CeA which regulates the responses to the stressdul stimulus (B). During alcohol dependence, the CRHR1 upregulation, together with a decreased in D1 expression (couteradaptive mechanism), disrupt the activity of the ITCs (C). This leads to a long-lasting disinhinibition of the CeA, causing hyper-responsiveness to stressful inputs. BLA: Basolateral amygdala; CeA: Central amygdala; ITCs: interacalted cells

Further studies investigating the interaction of CRHR1 with the dopaminergic system in stress-induced alcohol consumption (**Study 3**) and cocaine-mediated behavior (**Study 5**) showed a cell-specific impact of CRHR1 when expressed pre- or post-synaptically on the dopaminergic neuron. Using mice lacking the CRHR1 in DAT- or D1-containing neurons, we demonstrated that alcohol consumption following repeated stress is mainly driven by CRHR1 expressed in dopaminergic, but not dopaminoceptive neurons (**Study 3**). Furthermore, **Study 5** provided evidence of a dualistic opposite control of CRHR1 in DAT- and D1-containing neurons in cocaine-mediated behavior. These data demonstrated the involvement of CRHR1 in cue-induced reinstatement following cocaine self-administration, and implicate presynaptic and postsynaptic CRHR1 in negative feedback and postsynaptic feedforward mediation, respectively, of dopamine signalling and subsequent cocaine-mediated behaviors.

Advanced genetically modified animals in combination with viral vector gene transfer were applied in **Study 4** to disentangle the role of CRHR1 in specific neuronal populations in a defined brain region such as CeA. The findings confirmed the role of CeA CRHR1 in the regulation of alcohol-seeking behavior under stressful conditions. Moreover, we demonstrated that αCaMKII-CRHR1 in the CeA mediates, at least in part, stress-induced reinstatement to alcohol seeking behavior.

In conclusion, the work presented in this PhD thesis demonstrates the functional impact of the cross-talk between CRHR1 and the specific neuronal populations of the dopaminergic system in the response to stressful stimuli under physiological conditions and in alcohol dependence. The development of compounds acting on either CRHR1 receptors in a neuron-specific manner or receptor-heterocomplexes represent two valid strategies for novel pharmacological therapies to treat anxiety-related disorders and stress-induced relapse in drug addiction.

SUMMARY AND OUTLOOK

By combining molecular and behavioral techniques, as well as different animal models, this PhD thesis provides important mechanistic insight into the regulation of the response to stressful stimuli during anxiety and alcohol dependence.

We first provided solid evidence of an interaction between D1 and CRHR1 in the amygdala and ITCs, establishing a **novel mechanism** of anxiety-like responses. Behavioral and molecular experiments demonstrated the involvement of this interaction in the modulation of the response to stress during physiological conditions and alcohol dependence (Study 1 and Study 2). D1-CRHR1 interaction may be involved in the mechanisms that underlie the negative affective state and relapse, both characteristics of alcohol dependence. Furthermore, we investigated the cell- and region-specific function of CRHR1 during stress-induced alcohol-seeking (Studies 3 and 4) and cocaine-mediated behavior (Study 5). These results were achieved by the use of different cell-type specific Cre-dependent transgenic animals.

Thus, this PhD thesis established the role of CRHR1 within the dopaminergic system, providing functional evidence of the involvement of this cross-talk in regulation of emotional responses to stress and alcohol dependence. The development of compounds acting on either the CRHR1 receptor in a neuron-specific manner or receptor-heterocomplexes represents a valid strategy to treat anxiety and drug addiction.

However, further studies will be necessary in order to:

- Identify the impact of D1-CRHR1 heteromerization on signaling, desensitization and βarrestin-mediated internalization processes during acute stress
- Characterize morphological changes within the ITCs that may influence their function during alcohol dependence
- Determine the region-specific role of CRHR1 in stress-induced alcohol-seeking behavior by inducing a cell-specific (e.g. αCaMKII) overexpression of CRHR1 in different brain areas (e.g. BNST and VTA).
- Investigate the role of αCaMKII-CRHR1 interaction during cue-induced reinstatement by CRHR1-overexpression in αCaMKII-neurons in other brain regions (e.g. BLA).
- Investigate the functional impact of dopaminergic- and dopaminoceptive CRHR1 in stress-induced cocaine-mediated behavior

In a broader perspective, it would be of relevant interest to investigate:

- The impact of CRHR1 on other brain systems (e.g. glutamatergic, opioid, serotonergic systems) during alcohol dependence, using cell-specific CRHR1 knockout mice
- A possible interaction of CRHR2 and the dopaminergic system in anxiety-related behavior and drug dependence (i.e. alcohol and cocaine)

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SUPPLEMENTARY MATERIALS

Study 1

Suppl. Table 1: Mean ± SEM of [^{3}H]-SCH23390 receptor autoradiography on brain tissues in intra-cerebroventricular injected Wistar rats

Region		CSF		CRH		Stressin I	
		Mean ± SEM [fmol/mg]	**n**	**Mean ± SEM [fmol/mg]**	**n**	**Mean ± SEM [fmol/mg]**	**n**
Amygdala	**IN**	431.55 ± 32.02	6	654.24 ± 79.29	6	746.38 ± 746.38	4
	Ivp	911.54 ± 18.07	5	1297.15 ± 38.03	6	1391.83 ± 97.10	6
	Imp	731.29 ± 34.72	4	1199.18 ± 194.42	5	1012.28 ± 93.94	5
	Idp	489.09 ± 27.93	5	699.76 ± 38.67	5	895.95 ± 61.30	5
	Ilp	706.04 ± 34.70	5	818.71 ± 16.16	3	787.86 ± 59.05	3
	BLA	339.35 ± 26.33	6	538.72 ± 25.21	5	611.55 ± 57.40	6
	CeA	100.34 ± 8.51	6	159.62 ± 11.90	5	210.05 ± 9.55	6
	BMA	142.58 ± 29.22	6	289.38 ± 4.21	5	321.48 ± 19.80	6
	MeA	113.26 ± 11.25	6	185.98 ± 16.90	5	180.38 ± 9.55	6
Extra-amygdala	**CPu**	2045.81 ± 56.03	6	1753.41 ± 164.10	5	2092.08 ± 60.89	5
	Cg	69.11 ± 10.35	4	71.02 ± 3.96	5	63.20 ± 11.75	4
	PrL	94.83 ± 13.36	5	104.67 ± 8.17	5	93.54 ± 6.41	6
	IL	162.40 ± 12.00	4	145.57 ± 19.73	5	135.28 ± 15.56	4
	M1	27.29 ± 3.94	5	27.70 ± 3.44	5	24.28 ± 5.13	3
	BNST	97.78 ± 5.73	5	94.72 ± 11.27	5	89.67 ± 5.75	5
	SN	1113.36 ± 148.20	5	874.39 ± 151.41	5	1153.58 ± 277.01	6

Suppl. Table 2: F- and P- values of [^{3}H]-SCH23390 receptor autoradiography in intra-cerebroventricular injected Wistar rats

Region		F-value	P-value	CSF vs CRH	CSF vs Stressin I	CRH vs Stressin I
Amygdala	**IN**	[2, 13]=7.71	0.006	0.012*	0.003**	0.303
	Ivp	[2, 14]=14.48	0.0004	0.001**	0.00015***	0.305
	Imp	[2, 11]=2.83	0.006	0.037*	0.181	0.335
	Idp	[2, 12]=20.58	0.0001	0.006**	0.00003***	0.009**
	Ilp	[2, 8]=2.34	0,159	0.078	0.180	0.634
	BLA	[2, 14]=12.50	0.0008	0.004**	0.0003***	0.236
	CeA	[2, 14]=32.70	0.00001	0.00096***	0.00000***	0.003**
	BMA	[2, 14]=19.96	0.00008	0.0004***	0.00003***	0.325
	MeA	[2, 14]=10.82	0.001	0.001**	0.001**	0.759
Extra-amygdala	**CPu**	[2, 13]=3.16	0.076	-	-	-
	Cg	[2, 10]=0.22	0,807	-	-	-
	PrL	[2, 13]=0.41	0,672	-	-	-
	IL	[2, 10]=0.73	0,506	-	-	-
	M1	[2, 10]=0.17	0,844	-	-	-
	BNST	[2, 12]=0.26	0,774	-	-	-
	SN	[2, 13]=0.48	0,627	-	-	-

Suppl. Table 3: Mean ± SEM of [^{3}H]-SCH23390 receptor autoradiography on brain tissues obtained from intra-amygdala injected Wistar rats

Region		CSF		Stressin I		Stressin I + SCH23390	
		Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n
Amygdala	IN	447.23 ± 14.20	8	544.74 ± 12.42	7	431.35 ± 14.61	6
	Ivp	368.55 ± 8.25	8	457.35 ± 13.98	7	346.48 ± 6.90	6
	Imp	351.32 ± 7.90	8	430.44 ± 12.12	7	342.58 ± 6.36	6
	Idp	354.95 ± 8.87	8	394.09 ± 15.32	6	352.92 ± 12.53	6
	Ilp	349.69 ± 8.34	8	445.39 ± 9.83	6	335.16 ± 5.06	6
	BLA	242.06 ± 7.80	8	291.74 ± 7.17	6	250.69 ± 3.30	6
	CeA	66.34 ± 2.90	8	91.20 ± 5.10	7	65.30 ± 2.29	6
	BMA	122.90 ± 4.48	7	196.83 ± 6.85	7	127.31 ± 2.81	6
	MeA	79.86 ± 4.75	8	104.95 ± 6.13	7	85.05 ± 3.01	6

Suppl. Table 4: F- and P-values of [^{3}H]-SCH23390 receptor autoradiography from intra-amygdala injected Wistar rats

Region		F-value	P-value	CSF vs Stressin I	CSF vs Stressin I + SCH23390	Stressin I vs Stressin I + SCH23390
Amygdala	IN	[2, 18]=19.22	0.0000	0.00007***	0.433	0.00003***
	Ivp	[2, 18]=31.274	0.0000	0.00000***	0.152	0.00000***
	Imp	[2, 18]=26.717	0.0000	0.00000***	0.519	0.00000***
	Idp	[2, 17]=3.544	0.0517	0.031*	0.904	0.033*
	Ilp	[2, 17]=49.847	0.0000	0.00000***	0.219	0.00000***
	BLA	[2, 17]=14.725	0.0002	0.00007***	0.377	0.0009***
	CeA	[2, 18]=15.670	0.0001	0.0001***	0.846	0.0001***
	BMA	[2, 17]=65.728	0.0000	0.00000***	0.562	0.00000***
	MeA	[2, 18]=7.315	0.005	0.002**	0.474	0.014*

Suppl. Table 5: Mean ± SEM of [^{3}H]-SCH23390 receptor autoradiography performed on intra-cerebroventricular injected D1^{Cre}-CRHR1$^{f/f}$ mice and their littermates

Region		CRHR1f/f				D1^{Cre}-CRHR1$^{f/f}$			
		CSF		Stressin I		CSF		Stressin I	
		Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n
Amygdala	IN	936.33 ± 25.15	6	1116.55 ± 29.76	5	1046.02 ± 36.41	6	1057.75 ± 22.88	7
	Imp	1014.58 ± 11.26	6	1151.32 ± 25.92	5	1072.55 ± 30.05	6	1094.37 ± 13.27	7
	Ilp	737.15 ± 6.84	6	935.14 ± 31.58	4	801.72 ± 11.77	7	827.87 ± 14.37	7
	BLA	349.34 ± 21.27	6	407.99 ± 11.71	5	378.56 ± 11.59	7	387.12 ± 8.79	7
	CeA	212.35 ± 7.39	6	243.29 ± 7.19	5	221.41 ± 7.66	7	216.09 ± 9.03	6
	BMA	251.41 ± 10.79	6	346.17 ± 3.91	5	326.20 ± 9.68	7	307.07 ± 7.06	7
	MeA	227.80 ± 6.78	6	285.41 ± 6.36	5	244.92 ± 11.50	5	260.28 ± 7.04	7
Extra-amygdala	CPu	3591.54 ± 46.45	6	3697.13 ± 16.81	5	3637.43 ± 77.55	7	3640.86 ± 93.45	7
	Cg	337.49 ± 23.27	6	312.52 ± 6.44	5	342.57 ± 16.44	7	315.83 ± 12.22	7
	PrL	371.39 ± 11.84	6	364.13 ± 12.14	5	370.60 ± 14.97	7	347.70 ± 14.36	7
	IL	290.44 ± 9.87	6	280.92 ± 9.48	5	293.80 ± 7.49	7	266.59 ± 10.09	7
	M1	204.30 ± 16.82	6	174.26 ± 5.41	5	205.83 ± 15.39	7	189.15 ± 10.81	7
	AcbC	2430.23 ± 89.04	6	2411.14 ± 134.64	5	2396.03 ± 80.04	4	2421.70 ± 94.12	6
	AcbS	2722.65 ± 71.92	5	2602.43 ± 257.77	4	2568.43 ± 56.97	5	2651.82 ± 110.78	6
	BNST	286.57 ± 6.59	6	293.59 ± 11.04	5	282.52 ± 5.42	7	285.26 ± 12.12	7
	VTA	118.06 ± 11.13	6	124.46 ± 6.77	4	128.31 ± 8.41	6	137.12 ± 8.75	5
	SN	1029.78 ± 13.03	6	1062.26 ± 41.76	5	1010.05 ± 9.48	6	1007.45 ± 18.97	5

Suppl. Table 6: F- and P-values of [^{3}H]-SCH23390 receptor autoradiography performed on intra-cerebroventricular injected D1^{Cre}-CRHR1$^{f/f}$ mice and their littermates

Region		Genotype effect	Treatment effect	Genotype x Treatment effect
Amygdala	**IN**	F[1, 20]=0.778 p=0.388	F[1, 20]=11.069 p=0.003**	F[1, 20]=8.528 p=0.008**
	Imp	F[1, 20]=0.000 p=0.981	F[1, 20]=14.370 p=0.001**	F[1, 20]=7.550 p=0.012*
	Ilp	F[1, 20]=1.860 p=0.187	F[1, 20]=51.320 p=0.0000***	F[1, 20]=30.170 p=0.0000***
	BLA	F[1, 21]=0.089 p=0.768	F[1, 21]=5.769 p=0.026*	F[1, 21]=3.203 p=0.088
	CeA	F[1, 20]=1.284 p=0.270	F[1, 20]=2.560 p=0.125	F[1, 20]=5.130 p=0.035*
	BMA	F[1, 21]=4.173 p=0.054	F[1, 21]=18.737 p=0.0003***	F[1, 21]=42.482 p=0.00000***
	MeA	F[1, 19]=0.248 p=0.624	F[1, 19]=20.588 p=0.0002***	F[1, 19]=6.905 p=0.017*
Extra-amygdala	**CPu**	F[1, 21]=0.005 p=0.944	F[1, 21]=0.555 p=0.465	F[1, 21]=0.487 p=0.493
	Cg	F[1, 21]=0.065 p=0.801	F[1, 21]=2.480 p=0.130	F[1, 21]=0.003 p=0.957
	PrL	F[1, 21]=0.378 p=0.545	F[1, 21]=1.160 p=0.294	F[1, 21]=0.313 p=0.582
	IL	F[1, 21]=0.342 p=0.565	F[1, 21]=3.824 p=0.064	F[1, 21]=0.888 p=0.357
	M1	F[1, 21]=0.363 p=0.553	F[1, 21]=2.936 p=0.101	F[1, 21]=0.240 p=0.629
	AcbC	F[1, 17]=0.013 p=0.911	F[1, 17]=0.001 p=0.975	F[1, 17]=0.046 p=0.833
	AcbS	F[1, 16]=0.161 p=0.693	F[1, 16]=0.020 p=0.889	F[1, 16]=0.609 p=0.447
	BNST	F[1, 21]=0.443 p=0.513	F[1, 21]=0.275 P=0.605	F[1, 21]=0.053 p=0.820
	VTA	F[1, 17]=1.439 p=0.247	F[1, 17]=0.633 p=0.437	F[1, 17]=0.016 p=0.900
	SN	F[1, 18]=2.734 p=0.115	F[1, 18]=0.439 p=0.516	F[1, 18]=0.605 p=0.447

Study 2

Suppl. Table 7: D1 receptor autoradiography in the amygdala nuclei of alcohol-dependent rats and their controls

Region	Control		Post-dependent		F-value	P-value
	Mean ± SEM [fmol/mg]	n	Mean ± SEM [fmol/mg]	n		
IN	802.48 ± 18.31	7	668.96 ± 17.18	8	[1,13]=28.264	0.0001***
Ivp	1450.23 ± 115.08	6	731.26 ± 45.76	6	[1,10]=33.699	0.0002***
Idp	859.38 ± 54.85	8	827.11 ± 39.15	7	[1,13]=0.217	0.649
Imp	856.27 ± 63.17	7	667.50 ± 35.65	8	[1,13]=7.242	0.018*
Ilp	829.84 ± 30.67	7	720.29 ± 37.36	7	[1,12]=5.137	0.043*
BLA	512.04 ± 8.48	7	431.63 ± 10.92	6	[1,11]=0.188	0.0001***
CeA	236.68 ± 7.46	8	179.71 ± 8.95	7	[1,13]=24.299	0.0003***

Suppl. Table 8: *In situ* hybridization for D1 mRNA expression in the amygdala nuclei of alcohol-dependent rats and their controls

Region	Control		Post-dependent		F-value	P-value
	Mean ± SEM [nCi/g]	n	Mean ± SEM [nCi/g]	n		
IN	83.89 ± 5.70	8	52.04 ± 6.69	7	[1,13]=13.291	0.003**
Ivp	83.78 ± 8.50	7	47.04 ± 9.05	6	[1,11]=8.734	0.013*
Idp	83.48 ± 4.78	7	55.95 ± 5.93	5	[1,10]=13.283	0.004**
Imp	48.85 ± 3.72	7	34.06 ± 1.02	4	[1,9]=8.439	0.017*
Ilp	64.98 ± 5.59	6	35.63 ± 4.63	5	[1,9]=15.477	0.003**
BLA	21.23 ± 1.55	8	14.18 ± 1.58	8	[1,14]=10.099	0.007**
CeA	6.61 ± 0.43	6	3.50 ± 0.79	5	[1,9]=13.053	0.006**

Suppl. Table 9: Mean ± SEM values relative to alcohol consumption during baseline and the 3 consecutive days after either abstinence (control) or CIE treatment (post-dependent) in $D1^{Cre}$-$CRHR1^{f/f}$ mice and their littermates. (*) Indicates the difference vs the baseline; (#) Indicetes the difference between the genotypes.

	Control		**Post-dependent**	
	$CRHR1^{f/f}$ (N=12)	**$D1^{Cre}$-$CRHR1^{f/f}$** (N=7)	**$CRHR1^{f/f}$** (N=6)	**$D1^{Cre}$-$CRHR1^{f/f}$** (N=7)
Baseline	4.88 ± 0.56	5.17 ± 0.35	5.14 ± 0.24	5.71 ± 0.61
Day 1	11.95 ± 0.42***	12.66 ± 0.62***	19.45 ± 1.54***	15.95 ± 0.81***#
Day 2	10.97 ± 0.82***	9.92 ± 1.79***	19.02 ± 1.24***	15.02 ± 1.16***#
Day 3	8.38 ± 0.66***	8.15 ± 1.36***	17.66 ± 1.52***	13.77 ± 0.71***#