Year : 2016

Crtc1$^{-/-}$ mice and mood disorders: Role of the CREB-CRTC1 pathway in depression and antidepressant response

Elsa Meylan

Elsa Meylan, 2016, *Crtc1*$^{-/-}$ mice and mood disorders: Role of the CREB-CRTC1 pathway in depression and antidepressant response

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_DC2C1A04D8901

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Centre de Neurosciences Psychiatriques, Département de Psychiatrie

Crtc1$^{-/-}$ MICE AND MOOD DISORDERS: ROLE OF THE CREB-CRTC1 PATHWAY IN DEPRESSION AND ANTIDEPRESSANT RESPONSE

Thèse de doctorat en Neurosciences

présentée à la

Faculté de Biologie et de Médecine
de l'Université de Lausanne

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Lausanne 2016

Thèse n°165

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

LEMANIC NEURO SCIENCE DOCTORAL SCHOOL

UNIL | Université de Lausanne

UNIVERSITÉ DE GENÈVE

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UNIVERSITÉ DE GENÈVE

Programme doctoral interuniversitaire en Neurosciences des Universités de Lausanne et Genève

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur Prof. Jean-Pierre **Hornung**
Directeur de thèse	Monsieur Dr Jean-René **Cardinaux**
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le Conseil de Faculté autorise l'impression de la thèse de

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intitulée

***Crtc1-/-* MICE AND MOOD DISORDERS:**
ROLE OF THE CREB-CRTC1 PATHWAY IN DEPRESSION AND ANTIDEPRESSANT RESPONSE

Lausanne, le 15 avril 2016

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Jean-Pierre Hornung

Acknowledgments

First of all, I would like to express my deepest gratitude to my thesis director, Dr. Jean-René Cardinaux, for offering me the opportunity to work on this highly interesting project that allowed me to work at both the behavioral and molecular level. I warmly thank him for his supervision and constant availability, but also for the liberty he gave me to fulfill my work. I also extremely appreciated his knowledge and advices, interesting discussions and most importantly, his understanding and humanity.

I would like to greatly thank Prof. Jean-Pierre Hornung, Prof. Johannes Gräff and Dr. Olivier Braissant for kindly agreeing to be part of my thesis committee and for their useful advices and corrections that allowed me to improve this manuscript.

A huge thank goes to my dear colleague Clara Rossetti. It has been a great pleasure to work alongside her during these years. Her precious advices, critical comments, knowledge but mainly her kindness and friendship make her one of the most valuable colleague one can imagine!

I address a special thank to the students that I had the opportunity to supervise: Marina Pagliaro, Suzanne Badoux and Gabriel Vachey. It has been a pleasure to work with you and guide you through your respective projects which contributed to this thesis. A very, very, very special thank and a wink to my dear master student, Sara Dias, for the wonderful year spent with you at the CNP, and for the great work you did through your project. I am glad that you are no longer my student, but my friend!

I would like to thank all members of the CNP and related animal facility, either PI, lab members, students and technical staff, who in a way or another contributed to this work and also provided a nice and welcoming work environment. A special thank to Dr. Jean-Luc Martin and Dr. Fulvio Magara for their advices and critical discussions about my work, as well as for the constantly interesting coffee-machine talks!

A bittersweet thank goes to all the mice used and sacrificed in this study. I sincerely hope it was worthwhile.

I sincerely thank Prof. Pierre Magistretti and the NCCR Synapsy, as well as Prof. Olivier Halfon for the financial support they provided for this project.

Many thanks also to Dr. Ulrike Toepel for her assistance and incredibly efficient coordination of the Lemanic Neuroscience Doctoral School.

A big thank to Irène Riederer for her great lab management, immense kindness and constant assistance, and of course, for the delicious cakes…!

Huge thanks go to all my great close colleagues, past and present at the CNP. In particular, I would like to thank Benjamin, Anthony, Antoine, Zac, Aurélien, Jérôme, Alexandre and Isabel. Thank you for all the nice moments in the lab, and the after-work events! Thank you for the great memories that I will keep. I would like to thank also some people that are no longer close to me, particularly Damien and his family.

And last, but far from least, thank you from the bottom of my heart to the people who are dearest to me. Thank you my beloved Gion, for your constantly incredible support, understanding and most of all, love, which helped me much more than you know. And I thank my incredible family: my wonderful parents and my great sister for their infallible love and support since the beginning, and for providing me the experience and education that have allowed me to be where and who I am now. This work would not have been possible without you, and it is without hesitation dedicated to all of you!

Thank you all!

Summary

Mood disorders, which include major depressive disorders and bipolar disorders, are complex neuropsychiatric diseases and are among the leading causes of disability worldwide. Studies over the past decades have highlighted the heterogeneity of these troubles and suggest that they result from complex interactions between genetic or molecular alterations and environmental stressors. Numerous findings have shown the importance of neuroplasticity-related processes, and it is hypothesized that a defective and dysfunctional neurocircuitry would underlie these diseases.

The pleiotropic transcription factor CREB has been widely involved in mood disorders, as it is crucial for the expression of genes underlying synaptic plasticity, such as brain-derived neurotrophic factor (BDNF). The expression of this neurotrophin mainly relies on CREB, but also on CREB-regulated transcription coactivator 1 (CRTC1), a potent activator of CREB-dependent transcription involved in mechanisms such as synaptic plasticity and long-term potentiation.

To further investigate the role of CRTC1 in the brain, our group generated a CRTC1-deficient mouse line that presented several behavioral and molecular phenotypes related to mood disorders.

The aim of the present thesis was to further investigate the involvement of CRTC1 in mood disorders etiology, as well as in antidepressant response, through the use of the *Crtc1*$^{-/-}$ mice model. We found that these animals are resistant to the therapeutic effects of classical antidepressants. We indeed showed that CRTC1 was necessary for the antidepressant-induced upregulation of neurotrophic genes. Correspondingly, by pharmacologically acting on epigenetic gene regulation, we were able to restore *Bdnf* expression in *Crtc1*$^{-/-}$ mice, which was paralleled by a partial rescue of their depressive-like phenotype. We also demonstrated that *Crtc1*$^{-/-}$ mice present impairments of several systems, including the agmatinergic system which importantly contributes to their phenotype. Finally, we provided evidence that agmatine might be a new fast-acting antidepressant.

Altogether, the results presented here confirm CRTC1 as a potent mediator of several molecular pathways, impairments of which participate in the establishment of mood disorders. Furthermore, they also provide new insights into the role of CRTC1 in such troubles, as well as in antidepressant response.

Résumé

Les troubles de l'humeur, comprenant la dépression et les troubles bipolaires, sont des troubles neuropsychiatriques complexes, et font partie des principales causes d'invalidité dans le monde. Les recherches menées durant les dernières décennies mettent en évidence l'hétérogénéité de ces troubles, et suggèrent qu'ils résultent d'interactions complexes entre altérations génétiques ou moléculaires et stress environnemental. De nombreuses études ont montré l'importance de la neuroplasticité et des mécanismes qui lui sont liés, car il semblerait que ces maladies découlent d'une circuiterie neuronale altérée et dysfonctionnelle.

Le facteur de transcription CREB est très impliqué dans les troubles de l'humeur. Il est en effet crucial pour l'expression de gènes liés à la plasticité synaptique, tels que BDNF (brain-derived neurotrophic factor). L'expression de cette neurotrophine est principalement régulée par CREB, mais également par CRTC1 (CREB-regulated transcription coactivator 1), un important co-activateur de CREB. Il est aussi impliqué dans des mécanismes tels que la plasticité synaptique et la potentialisation à long terme. Afin d'étudier plus en détails le rôle de CRTC1, notre groupe a généré une lignée de souris déficientes en CRTC1 qui présentent plusieurs altérations comportementales et moléculaires liées aux troubles de l'humeur.

L'objectif de cette thèse était d'investiguer plus en profondeur l'implication de CRTC1 dans l'étiologie des troubles de l'humeur ainsi que dans la réponse aux antidépresseurs, par l'utilisation des souris *Crtc1*$^{-/-}$. Nous avons observé que ces animaux ne répondent pas aux antidépresseur classiques. En effet, nous avons vu que CRTC1 était nécessaire pour l'expression de gènes liés à la neuroplasticité induite par les antidépresseurs. En conséquences, nous avons démontré qu'en agissant sur le système épigénétique de ces souris, nous avons pu restaurer l'expression de *Bdnf*, en parallèle d'une restauration partielle de leur comportement. Nous avons également démontré que les souris *Crtc1*$^{-/-}$ présentent une dysfonction de certains systèmes physiologiques, incluant le système agmatinergique qui contribue grandement à leur phénotype. Finalement, nous avons aussi montré que l'agmatine pourrait être un nouvel antidépresseur à action rapide.

D'une manière générale, ces résultats confirment que CRTC1 est un régulateur-clé de nombreux processus cellulaires dont la dysfonction pourrait contribuer à l'établissement des troubles de l'humeur. De plus, ils amènent également de nouvelles informations sur le rôle de CRTC1 dans ces troubles, ainsi que dans la réponse aux antidépresseurs.

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List of abbreviations

5HT	Serotonin
AC	Adenylate cyclase
ACTH	Corticotrophin
ADC	Arginine decarboxylase
Agm	Agmatinase
AMPAR	AMPA receptor
ATF-1	Activating transcription factor 1
BD	Bipolar disorders
BDNF	Brain-derived neurotrophic factor
bZIP	Basic leucine zipper
cAMP	Cyclic AMP
CART	Cocaine and Amphetamine regulated transcript
CBP	CREB binding protein
CR	Calretinin
CREB	cAMP-response element-binding protein
CREM	cAMP-response element modulator
CRF	Corticotrophin-releasing hormone/factor
CRH	Corticotrophin-releasing hormone/factor
CRTC	CREB-regulated transcription coactivator
CUMS	Chronic unpredictable mild stress
DBS	Deep brain stimulation
DNMT	DNA methyltransferase
ECS	Electroconvulsive seizures
eEF2	Eukaryotic elongation factor 2
ENaC	Epithelium Na^+ channel
FST	Forced-swim test
GABA	Gamma-aminobutyric acid
GAD 1-2	Glutamate decarboxylase 1 and 2

GPRC	G protein-coupled receptor
GR	Glucocorticoid receptor
GSK-3	Glycogen synthase kinase 3
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HPA	hypothalamic-pituitary-adrenal
IDO	Idoleamine 2,3-dioxygenase
IFNγ	Interferon γ
IL-1β	Interleukine 1β
IL-6	Interleukine 6
IMPase	Inositol monophosphatase
LH	Learned helplessness
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAOI	Monoamine oxidase inhibitor
MeCP2	Methyl-CpG-binding protein 2
MDD	Major depressive disorders
NA	Noradrenalin
NAc	Nucleus accumbens
NIH	Novelty-induced hypophagia
NMDAR	NMDA Receptor
NO	Nitric oxide
NPY	Neuropeptide Y
NR4As	Orphan nuclear receptors
NRIs	Noradrenalin reuptake inhibitors
OSFS	Open-space forced swim test
PFC	Prefrontal cortex
PI	Phosphoinositol
PKA	Protein kinase A
PV	Parvalbumin

PVN	Paraventricular nucleus
SAHA	Suberoylanilide hydroxamic acid
SIK	Salt inducible kinase
SNRI	Selective noradrenalin reuptake inhibitor
SNRIs	Serotonin and noradrenalin reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitor
SST	Somatostatin
TCA	Tricyclic antidepressant
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TNFα	Tumor necrosis factor α
TORC	Transducer of regulated CREB activity
TRKB	Tropomyosin receptor kinase B, also known as Tyrosine receptor kinase B
TS	Tail suspension
VPA	Valproate

1. INTRODUCTION

1.1. Mood disorders

1.1.1. Prevalence and burden of mood disorders

Mood disorders are among the most prevalent and debilitating psychiatric illnesses worldwide. These complex and chronic multifactorial diseases mainly comprise two major disorders: major depressive disorders (MDD) and bipolar disorders (BD) [1]. As of 2005, MDD and BD had a lifetime prevalence of respectively 16.6% and 4.4% in the U.S.A. [2]. While BD has an overall equivalent prevalence in men and women, MDD is nearly twice more frequent in women (10% to 25%) than in men (5% to 12%)[2,3]. The varying rates depend greatly on age, ethnicity, residential area, health condition and general social environment [1,2,4]. Mood disorders are a serious health concern, as MDD is predicted to become the second leading cause of disability worldwide by 2020 [5]. In addition to health and social life deterioration, mood disorders also have a severe economic impact. In 2010, total cost of mood disorders was estimated around 113.4 billion € in Europe [6]. These troubles also exhibit high comorbidity with anxiety disorders, but also with aggressive behaviors and weight imbalance[7–9], thus worsening their social, health and economic burden. Furthermore, mood disorders are life-threatening, as they are strongly associated with suicidal behavior. It has been indeed observed that around 60% of committed suicides are related to mood disorders [10], thus urging the need to extensively investigate these illnesses.

1.1.2. Major depressive disorders

MDD, also known as depression or unipolar depression, are the most frequent of mental troubles as around 17% of the population is affected at some point in life[11] . Depression is a heterogenic and complex disease with both genetic and environmental components[12,13]. According to the diagnostic and statistical manual of mental disorders (DSM V), MDD are characterized by the presence of one or more depressive episodes. A depressive episode is defined as a period of two weeks at least, during which the patient exhibit a depressed mood and/or anhedonia (loss of pleasure), plus at least four symptoms of the list summarized in **Table 1** [14] . In addition, depressive episodes are often recurring, as 80% of depressed people display multiple episodes [15]. Finally, a subset of the depressive population (34%) also present increased irritability and anger attacks [8].

1.1.3. Bipolar disorders

In BD, also known as bipolar depression or manic-depressive disorders, patients alternate between depressive episodes (as described above) and manic or hypomanic episodes. A manic episode is characterized by a period of two weeks at least, during which the patient exhibits an elevated, euphoric and irritable mood, in addition with some other manic symptoms summarized in **Table 1**[14] . A hypomanic episode is a milder and shorter form of manic episode. It lasts 4 days at least and is defined by a less severe elevated and euphoric mood. BD type I are characterized by a rapid cycling between depression and mania, while in BD type II, depressive and hypomanic episode often co-occur in mixed episodes[14].

Depressive episode	Manic Episode
Presence of at least five of the following symptoms during at least two weeks. Symptoms 1 and /or 2 must be present. **(1)** Depressed mood most of the day, nearly every day. **(2)** Diminished interest or pleasure in most activities (anhedonia), nearly every day. **(3)** Significant weight gain or loss. **(4)** Insomnia or hypersomnia. **(5)** Psychomotor retardation or agitation. **(6)** Fatigue, tiredness or loss of energy. **(7)** Feeling of worthlessness and guilt. **(8)** Cognitive impairments. **(9)** Recurrent thought of death, suicidal ideation, suicide plan or attempt.	Presence of at least 3 of the following symptoms during at least two weeks. **(1)** Elevated and euphoric mood. **(2)** Irritability, anger or aggression. **(3)** Increased self-esteem and grandiosity. **(4)** Decreased need for sleep. **(5)** More talkative than usual. **(6)** Flight of ideas or racing thoughts. **(7)** Distractibility. **(8)** Increase in goal-directed activity or psychomotor agitation. **(9)** Excessive involvement in pleasurable activities with high potential for painful consequences. **(10)** Hallucinations and delusions.

Table 1.1 : Major features and diagnostic criteria for depressive and manic episodes. Adapted from the DSM V [14].

1.2. Neurobiological pathways involved in the etiology of mood disorders

As indicated above, mood disorders are complex heterogenic diseases. There have been several lines of evidence that genetic factors play a major role in the etiology of these troubles [16–18]. However, genetic susceptibility is not solely responsible for the disease, as it would only account for 37% for the occurrence risk of depression[19]. Thus, it is now widely accepted that mood disorders originate from both genetic and/or environmental causes. Numerous hypotheses have been and are being developed regarding the etiology of mood disorders, as numerous physiological systems were found to be disturbed in these troubles. This chapter will review some of these said hypotheses, with a special focus on the various systems that were studied in this thesis.

1.2.1. Role of the monoaminergic system

This first hypothesis focus on the involvement of the monoaminergic system (specifically serotonin, noradrenaline and dopamine) in mood disorders. It is based on the mechanisms of actions of the first antidepressants that were discovered in the early 1950's[20]. Initially developed for the treatment of other diseases, these drugs were found to have strong antidepressant effects and to provoke an overall increase in monoaminergic signaling[21]. These drugs are now classified as tricyclic antidepressants (TCA) and monoamine oxidase inhibitors (MAOI). Their mechanisms of action rely respectively on the increase of monoamines reuptake and on the inhibition of the monoamine catabolic enzyme [22]. Later on, other types of antidepressants were developed and all of them focused on the monoaminergic system as well. These include selective serotonin or noradrenaline reuptake inhibitors (respectively SSRI and SNRI) and tetracyclic antidepressants. All these drugs were found to significantly increase monoamine availability, therefore the monoaminergic hypothesis of depression was developed. This latter proposes that mood disorders are caused by a deficiency in monoamine signaling, which would be reversed upon antidepressant treatment [21,23–25].

There are several pieces of evidence supporting this theory. Serotonin (5HT) and noradrenaline (NA) are important neurotransmitters implicated in several behaviors and functions that are impaired in mood disorders. These include mood regulation, appetite, arousal, sexual function, sleep and cognitive functions. In human, 5HT decrease has been related with suicidal behaviors in mood disorders patients[26–29] , and plasma levels of NA were

found to be higher in bipolar manic patients than bipolar depressed patients [30,31]. Clinical studies have also shown that MDD patients display a decreased sensitivity of the serotonin receptor 1A (5-HT1A), thus leading to a dysfunction of 5HT signaling[26]. Interestingly, post-mortem studies have highlighted the role of p11 (a protein enhancing serotonin 1B receptor activity) in mood disorders, as it is decreased in the brain of depressive patients. Furthermore, p11 knock-out mice exhibit a depressive-like behavior and reduced response to antidepressants[32].

Secondary messengers of the monoamines signaling have also been studied, such as the cyclic AMP (cAMP) and the phosphatidylinositol pathways. Post-mortem brains of persons who committed suicide exhibit reduced levels of inositol, which was further observed in MDD patients by magnetic resonance spectroscopy experiments[33,34]. Molecules acting downstream of these pathways have also been investigated, such as the cAMP response element-binding protein (CREB), whose role in mood disorders has been extensively investigated and will be developed in a later section.

However, all available antidepressants require weeks of treatment to produce a clinical response. This indicates that the acute (and rapid) monoamines neurotransmission enhancement produced by antidepressants is not sufficient to explain their effects. Instead, studies point to long-lasting changes in neuronal plasticity (see chapter 1.2.3). Nevertheless, the monoamine hypothesis remains a strong theory because of its predictive power and its derivation from the mechanisms of action of all currently available antidepressants[23,35].

1.2.2. Involvement of stress and HPA axis regulation

Stress has been associated for a long time with mood disorders, as they often (but not always) occur following traumatic life events or chronic stress[36,37]. The brain reacts to acute and chronic stress through the activation of the hypothalamic-pituitary-adrenal axis (HPA axis) (**Fig 1.1**). In this pathway, neurons of the paraventricular nucleus (PVN) of the hypothalamus secrete vasopressin and corticotrophin-releasing hormone (CRH or CRF) in response to stress. Through its receptors in the anterior pituitary, CRH stimulates the synthesis and the release of corticotrophin (ACTH) in the systemic circulation. This will stimulate the adrenal cortex to release cortisol (corticosterone in rodents), which, besides its peripheral actions to restore

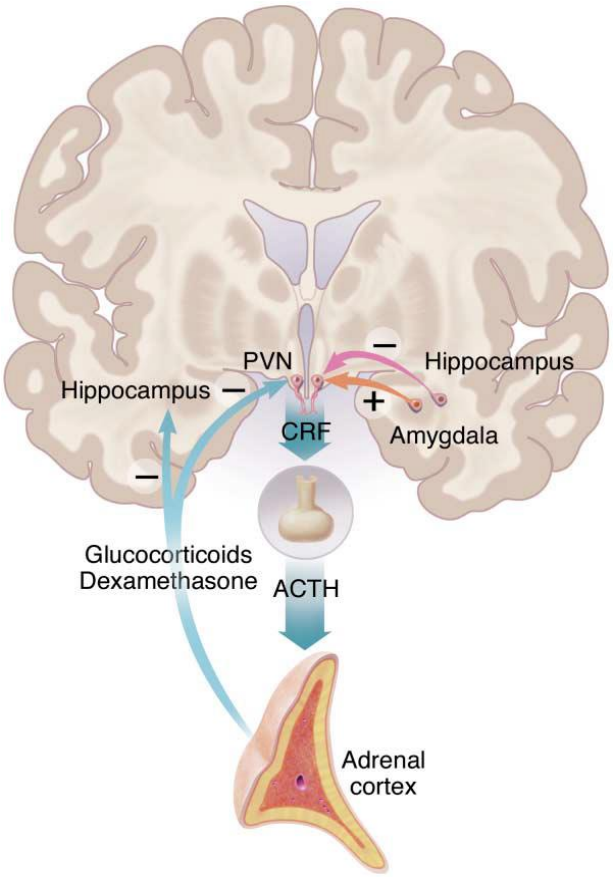

Figure 1.1 : The HPA axis. PVN neurons secrete corticotrophin releasing hormone (CRH or CRF) in response to stress. CRH will then stimulate corticotrophin (ACTH) release in the blood from the anterior pituitary. In response to ACTH signaling, the adrenal cortex will secrete glucocorticoids which will reduce CRH production, in a negative feedback response. The amygdala has an enhancing effect on the HPA axis, whereas the hippocampus has an inhibitory effect. Taken from [23].

homeostasis, will decrease the hypothalamic secretion of CRH through a feedback inhibition[38,39].

Several brain structures control the activity of the HPA axis, such as the hippocampus and prefrontal cortex (PFC) , which have an inhibitory effect on the hypothalamic CRH secretion, whereas the amygdala has an enhancing effect[23,39].

Several pieces of evidence have shown a dysfunction of the HPA axis in mood disorders. Post-mortem studies have pointed out that CRH levels are increased in the brain of depressive persons, who also exhibit higher cortisol levels in plasma[23,40,41]. Furthermore, HPA axis hyperactivity has been observed in untreated depressed and bipolar patients with an absence of the feedback response, and these features were corrected by antidepressant treatment[42–44]. Similar observations could be done in rodents, as animals that underwent early maternal separation presented abnormal HPA axis function[45,46].

While normal levels of glucocorticoids seem to promote the hippocampal inhibition of the HPA axis, chronic high levels (in the occurrence of chronic stress) could cause damages on hippocampal neurons, a phenomenon that could contribute to the establishment of mood disorders[23,35]. In line with this hypothesis, it has been observed that the size of the adrenal cortex and the anterior pituitary was increased in depressed patients[47], while the size of the hippocampus was decreased, possibly because of cellular damages and death, as well as reduced neurogenesis caused by high cortisol levels[48–50] . Indeed, chronic stress has been shown to produce atrophy and death of hippocampal neurons, and magnetic resonance

imaging (MRI) studies have revealed reduced hippocampal volumes in patients with post-traumatic stress disorder[51] and rodent studies have confirmed these findings[52–54] . In parallel, antidepressants and mood stabilizers were found to stimulate neurogenesis in the hippocampus, which could then explain their action[53–56].

To summarize, the stress hypothesis proposes that hyperactivation of the HPA axis (due to recurrent stress) causes hypercortisolism and enhanced CRH and glucocorticoid signaling, thus causing hippocampal damages. This would induce impairments in neuronal circuitry, therefore leading to the disease[51].

Although there is undeniable evidence of the implication of stress in mood disorders, its role is however not totally clear. Indeed, mood disorders also occur in the absence of early-life or chronic stress, and not all patients show dysfunction of the HPA axis[35,57]. Therefore, the link between stress and mood disorders is not completely established.

1.2.3. The network hypothesis of mood disorders

As explained above, the monoamine and stress hypotheses of mood disorders are pertinent propositions regarding the etiology of mood disorders, yet they are not sufficient. Thus, the network hypothesis allows to combine these two hypotheses into a new one. This latter proposes that mood disorders are caused by impairments in neuronal communication and decreased neurogenesis, following chronic stress and/or disturbances in monoaminergic signaling. Antidepressants and mood stabilizers would then stimulate neurogenesis and restore the functionality of neuronal network[21].

This hypothesis finds its origins in the evidence of the role of neurogenesis in depression and the neurogenic effects of antidepressants. Also, the serotoninergic system has an important role in brain development, and SSRI treatments in juvenile mice produce several behavioral impairments[58–61]. This suggests that the serotoninergic system is involved in neuronal network formation, and that antidepressants might act on such processes. As explained above, mood disorders patients exhibit decreased hippocampal size, which can be explained by a loss of neuronal circuitry[48,49,62]. Correspondingly, chronic lithium and antidepressants administration stimulates the generation of new neurons in the rat hippocampus[55,56], and this is paralleled by behavioral improvements[63]. Furthermore, generation and maturation of new neurons require several weeks, which would then explain the time required to see the effects of an

antidepressant treatment. Thus the network hypothesis integrates those two processes: the time required for neurogenic processes would be the time required for the appearance of therapeutic effects[21,23,64].

1.2.3.1. Key role of BDNF in the network hypothesis

Neurotrophic factors are critical mediators of neurogenesis during development, as well as regulation of neuronal survival in the adult brain. Therefore, they are thought to be key mediators of neuronal plasticity-related process underlying mood disorders etiology, in the frame of the network hypothesis. In particular, the brain-derived neurotrophic factor (BDNF) has been widely investigated and is thought to play a prominent role in mood disorders.

BDNF is involved in a wide repertoire of neuronal processes such as neuronal growth, migration, differentiation, axonal and dendritic growth and survival, and synapse formation[25,65]. BDNF is produced in an activity-dependent manner and is thought to be implicated in the selection and the stabilization of active synapses[66].

In the frame of mood disorders, several clinical and post-mortem studies have associated BDNF with MDD, BD and suicide[67–71]. BDNF and its receptor, the tropomyosin receptor kinase B (TRKB), were observed to be downregulated in the brain of suicide victims, while protein levels of BDNF were lowered in the serum and brain of mood disorders patients[69,72–74]. The specific BDNF polymorphism val66met has been strongly associated with mood disorders and its replication in rodents resulted in depressive, anxious and aggressive behavior[75–79].

In line with the network hypothesis of depression, BDNF has been shown to be involved in the development and function of serotoninergic neurons. Indeed, BDNF and TRKB are strongly expressed in 5-HT neurons of the raphe nuclei and *in vitro* studies have demonstrated that BDNF particularly stimulates differentiation, survival, and axon sprouting of 5-HT neurons[80–86]. In addition, BDNF expression has been found to be increased in the rat hippocampus by chronic, but not acute, antidepressants [87–89]. More importantly, BDNF and TRKB are required for antidepressant effects[89–91]. Furthermore, direct BDNF injection in the hippocampus produces antidepressant-like effects in several depression tests, while heterozygote and conditional BDNF knock-out mice are resistant to antidepressants[90,92]. In parallel, several studies have shown that acute and chronic stress provoke decreased expression of BDNF in the hippocampus[93–95].

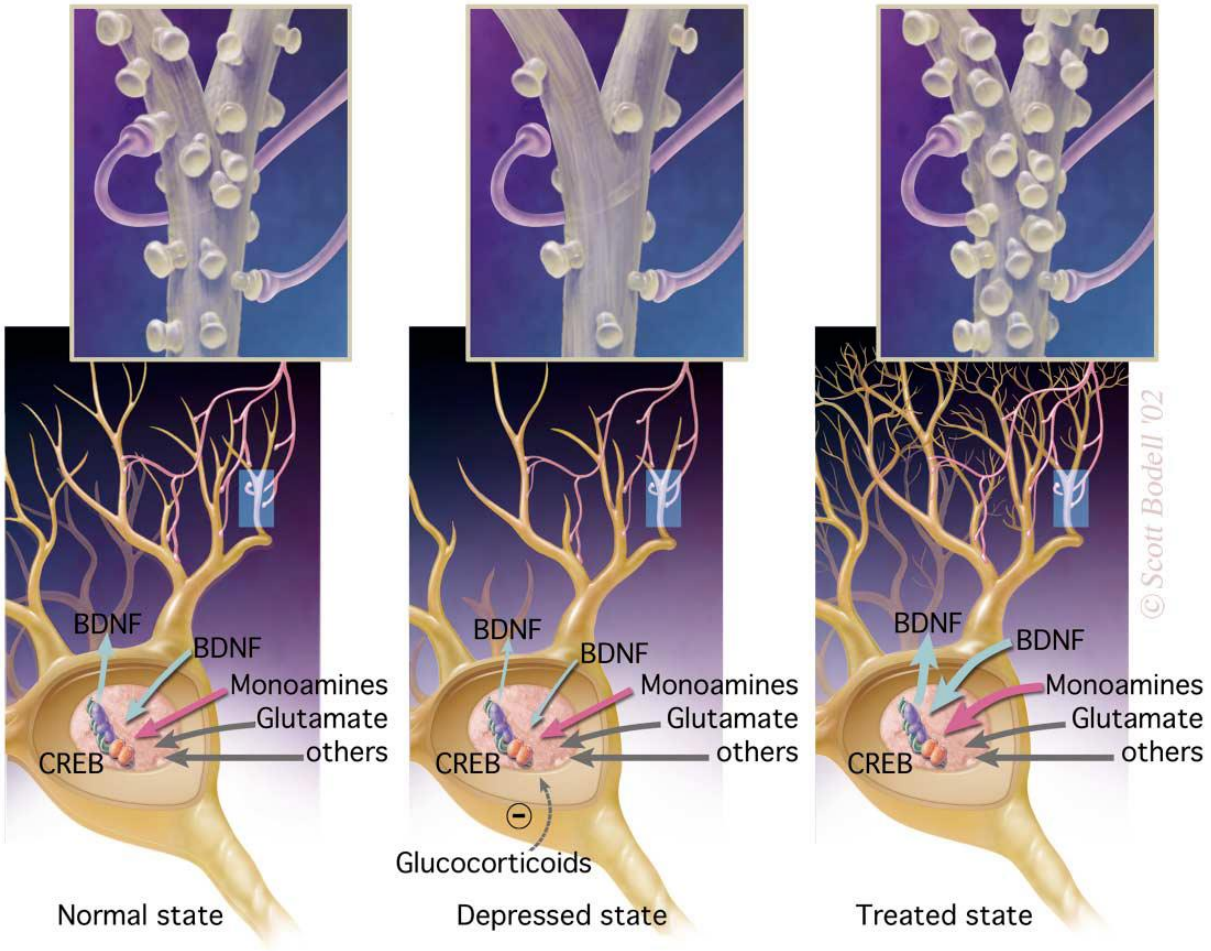

Figure 1.2 : The network hypothesis of depression. The left panel shows a normal hippocampal neuron receiving monoaminergic and glutamatergic innervation, among others. It also receives neurotrophic signaling such as BDNF. This leads to CREB-dependent BDNF expression and circuitry regulation. The middle panel shows a hippocampal neuron under chronic stress conditions. Constant increased glucocorticoid signaling leads to neuronal damages including reduction of dendritic arborization. BDNF signaling and expression are also reduced, and possibly mediate the effects on the dendrites. The right panel shows the effects of antidepressants. They increase monoaminergic and BDNF signaling, thus leading to CREB-dependent BDNF expression and restoration of neuronal connections. Taken from [23].

Therefore, the network hypothesis proposes that in mood disorders, neuronal circuitry is damaged following chronic stress. This will lead to decreased monoaminergic signaling, and diminished BDNF expression and function. Upon antidepressant treatment, monoaminergic and BDNF signaling are increased, leading to increased neuronal growth and arborization, thus restoring neuronal connectivity (**Fig. 1.2**).

Bdnf gene expression is highly complex as it contains at least 9 known alternative promoters[96]. *Bdnf* expression is regulated both by DNA accessibility through epigenetic mechanisms (which will be discussed in a later section) and the binding of transcription factors. CREB is a potent transcription factor that strongly regulates *Bdnf* expression, particularly its promoter IV, in an

activity-dependent manner[97,98]. The exon IV of *Bdnf* (*BdnfIV*) is of particular interest in mood disorders, as it is prominently expressed in brain structures associated with mood disorders (i.e. hippocampus, PFC). *In vitro* and *in vivo* studies have observed that *BdnfIV* accounts for approximately 50% to 75% of total *Bdnf* expression in the cortex[97], and it seems to have a critical role in synaptic plasticity in the same regions[99,100]. In addition, a recent study highlighted the role of *BdnfIV* in the regulation of numerous monoamine-related genes in the PFC and hippocampus[101]. Finally, antidepressants and mood stabilizers, as well as electroconvulsive therapy (ECS) were found to specifically increase *BdnfIV* expression[102–104].

Altogether, the numerous statements above strongly suggest a key role for BDNF in mood disorders. It is however important to know that BDNF function is not as clear as it seems. Indeed, numerous results in the literature might contradict the network hypothesis, or at least nuance it. For example, the effects of acute and chronic stress on BDNF are not as direct as they are presented in the hypothesis. Indeed, several studies have shown no effect of stress on BDNF expression, or even an increased expression of BDNF following stress[105–107]. However, the effects of stress on BDNF seem to be highly differential depending on the type of stress applied (acute vs. chronic, stressor used…)[105], brain region, time elapsed between stress application and BDNF measure, and BDNF promoter. Therefore, this rather suggests a very complex and dynamic regulation of BDNF by stress, and the underlying mechanisms of this regulation are still unclear. Also, it is important to notice that BDNF function seem to highly vary depending on the brain regions. Indeed, direct BDNF injection in the nucleus accumbens (Nac) and in the ventral tegmental area (VTA) produces pro-depressive effects[108,109].

All in all, while BDNF is undoubtedly highly involved in the etiology of mood disorders, its role and regulation tend to be far more complex than initially thought and still have to be widely investigated.

1.2.3.2. Involvement of the NR4A receptors family in the network hypothesis

The orphan nuclear receptors (NR4As) family is a subset of CREB-regulated genes of particular interest in the network hypothesis and for the present thesis. NR4AS are a family of transcription factors highly expressed in the brain, including regions such as the hippocampus and cortex[110,111]. NR4As are mainly involved in long-term memory mechanisms and learning

tasks[112,113], yet they have been associated with mood disorders. Indeed, mood disorders patients were found to exhibit lower levels of NR4A1 and NR4A2 (also known respectively as Nurr77 or NGFIB and Nurr1) mRNA and protein in the PFC[114]. In line with this, NR4A2 and NR4A3 (also known as Nor1) have been shown to be involved in neuronal growth, migration and survival, as well as in synaptic formation[115–118]. Rodent studies further highlighted the involvement of NR4As in mood disorders, as their expression is decreased following social isolation[119].

In line with the network hypothesis, NR4As were shown to be involved in the regulation of the HPA axis, particularly in CRH regulation[120,121]. Also, NR4As seem to be specifically involved in the differentiation and maturation of monoaminergic neurons, particularly dopaminergic neurons[122–124]. Finally, NR4A2 was found to regulate *Bdnf* expression *in vitro*[125]. However, the molecular pathways involved in such regulation, and more generally in the processes activated by NR4As are still widely unknown.

1.2.4. Epigenetic regulation of stress and mood disorders

The recent emergence of findings regarding epigenetic mechanisms has provided new insights on mood disorders and stress regulation. Indeed, many studies have highlighted the importance of epigenetic gene regulation (including modifications such as DNA methylation and chromatin remodelling) in stress response and particularly in depression. Furthermore, it has also allowed a better understanding of how stress could durably affect gene expression and lead to the development of depressive symptoms.

1.2.4.1. Overview of epigenetic mechanisms

Epigenetic mechanisms involve all changes in gene expression that are not due to the DNA sequence itself. They mainly consist of changes on DNA methylation and chromatin remodelling. Additional mechanisms such as small non-coding micro-RNA are also considered as epigenetic mechanisms[126,127]. DNA and histone modification will be briefly described here, as these two mechanisms were found to be critically involved in mood disorders.

DNA methylation is a mechanism by which a methyl group is added on the cytosine/guanine repeats (CpG) on the DNA sequence. When occurring at a gene promoter, DNA methylation will generally repress the transcription of such gene. It will also promote the binding of several

proteins, such as methyl-CpG-binding protein 2 (MeCP2), which will further repress gene transcription[126,128–130]. DNA methylation is achieved by DNA methyl-transferases (DNMTs) which will covalently bind a methyl group on CpG (**Fig. 1.3**). While DNMT3a and DNMT3b catalyse *de novo* methylation on previously unmethylated DNA, DNMT1 maintains methylation patterns during DNA replication[126,129].

Histone modifications affect DNA accessibility, as they act on chromatin conformation. DNA strands are densely folded around octamers of histone proteins whose N-terminal tails are exposed and can therefore be modified at distinct amino acid residues. These modifications include acetylation, ubiquitylation and SUMOylation at lysine residues, methylation at lysine and arginine residues, phosphorylation at serine and threonine residues and ADP-ribosylation at glutamate residues[130]. Histone acetylation on lysine residues is mediated by histone acetyl transferases (HAT) and is associated with transcriptional activation, as it will lead to chromatin unfolding, thus providing better accessibility to DNA sequence. Histone deacetylation is catalyzed by histone deacetylases (HDACs) (**Fig. 1.3**). Other histone modifications can either promote or repress gene expression, depending on the residue modified[131–133].

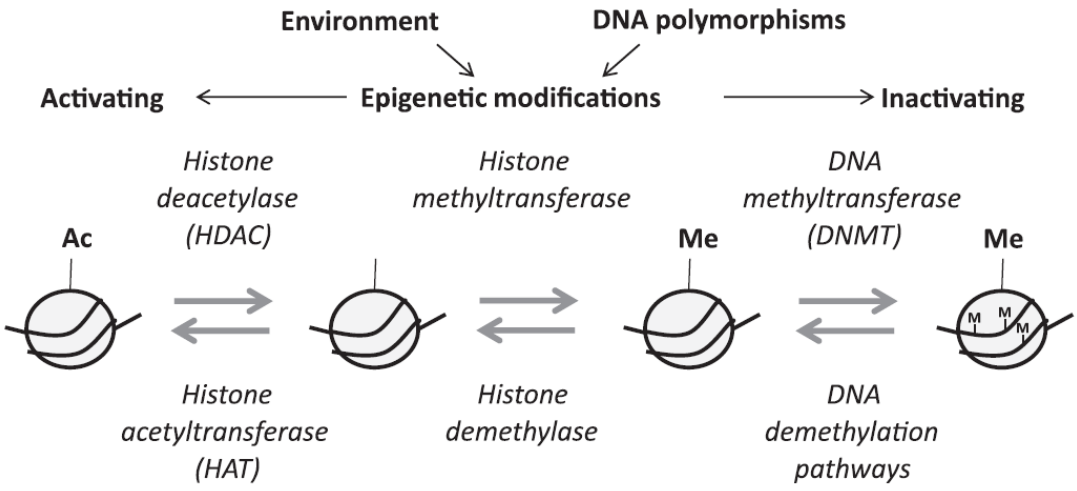

Figure 1.3 : Overview of the principal epigenetic mechanisms. Epigenetic modifications can be influenced by both environmental and/or genetic factors. Histone acetylation (Ac), regulated by histone acetyl transferases (HAT) and histone deacetylases (HDAC) generally increases transcriptional activity. On the opposite, histone methylation (Me) at some specific sites, regulated by histone methytransferases and histone demethylases, reduces transcriptional activity. Finally, direct DNA methylation of cytosines at CpG dinucleotides in DNA, catalyzed by DNA methyltransferases (DNMT) represses gene transcription. Taken from [139].

1.2.4.2. Involvement of epigenetic mechanisms in mood disorders

As already mentioned earlier, depression is thought to occur through complex interactions between genetic predispositions and environmental factors such as chronic or early-life stress. Epigenetic modifications might be underlying stress-induced long-lasting alterations in gene expression. Several pieces of evidence towards this hypothesis have been gathered over the last decades and mainly involve DNA methylation and histone acetylation.

1.2.4.2.1. DNA Methylation

There are several pieces of evidence of the involvement of DNA methylation modifications in the regulation of stress response. Most interestingly, post-mortem studies have shown increased methylation at the promoter of the glucocorticoid receptor (GR) gene in suicide victims with a history of childhood abuse[134]. This hypermethylation was correlated with decreased hippocampal GR expression, thus suggesting impaired feedback inhibition of the HPA axis, which is in line with the stress hypothesis of mood disorders mentioned above. In rodents, *GR* methylation has also been widely studied and found to be a critical regulator of stress response. It was indeed demonstrated that maternal behavior had a high influence on *GR* regulation[135]. In this study, they observed that rat mothers naturally showed different levels of maternal care, as measured by pup licking and grooming (LG) behavior. Interestingly, they showed that pups of mothers with high LG behavior displayed lower methylation at GR promoter than pups of low-LG mothers, indicating lower stress reactivity. On the opposite, pups of low LG mothers presented increased *GR* promoter methylation and lower hippocampal *GR* expression. This was correlated with increased behavioral and physiological stress reactivity. Of note, these epigenetic markings could be inherited, but they also could be reversed by acting on histone acetylation with the HDAC inhibitor trichostatin A (TSA).

Epigenetic effects of stress at the adult age have also been investigated in rodents. The chronic social defeat protocol is a behavioral paradigm that induces depressive-like symptoms, accompanied by increased levels of CRF[136]. This increase in CRF levels was correlated with decreased methylation on its gene promoter. Treatment with the antidepressant imipramine was able to restore normal CRF levels and normal methylation profile of its gene. Chronic stress also induced increased methylation on specific *Bdnf* promoters, therefore causing hippocampal damages[137–139]. Again, these effects could be reversed by imipramine treatment.

However, imipramine seemed to rather act on histone acetylation at specific *Bdnf* promoters, rather than on DNA methylation[138].

These various findings highlight the importance of DNA methylation as a critical modulator of gene expression in response to early-life and adult stress. While these long-lasting modifications can be inherited, it is also interesting to note that they can be reversed through the use of various treatments, such as antidepressants of HDAC inhibitors.

1.2.4.2.2. Histone acetylation

First evidence of the involvement of histone acetylation in MDD came through the study of the effects of ECS. Several studies have shown that ECS in rodents induced significant increases of histone H4 acetylation, prominently at *Bdnf* promoters, as well as histone H3 hyperacetylation in the hippocampus[103,140,141]. These first results suggested that chromatin remodelling might be an important mechanism underlying depression etiology.

This was further studied and confirmed by Tsankova et al. in 2006[138]. In this landmark study, it was found that chronic social defeat stress induced a repressive histone methylation at several promoters of *Bdnf*, correlated with decreased expression of this factor. Imipramine treatment was able to reverse these effects by inducing hyperacetylation of histones at *Bdnf* promoters. This was also paralleled by a downregulation of HDAC5. This study highlighted the dynamic chromatin remodelling occurring following chronic stress and upon antidepressant treatment.

These new insights were followed by the finding that HDAC inhibitors have strong antidepressant effects in rodents. Indeed, several molecules such as sodium butyrate, valproic acid or suberoylanilide hydroxamic acid (SAHA) were found to have a selective HDAC inhibitory activity that had strong antidepressant effects in several animal models of depression[138,142–144]. Of interest, some of these molecules were found to regulate the expression of a subset of genes, in a similar way as the antidepressant fluoxetine[142]. In line with this, imipramine was also found to induce histone H3 hyperacetylation, therefore further suggesting the involvement of this mechanism in antidepressant response[138].

In summary, epigenetic mechanisms provide new insights about the molecular mechanisms of mood disorders and antidepressant response. They also bring a new understanding of the possible pathways underlying the interaction between gene and environment, which is critical

for the development of these troubles. Furthermore, they give a solid hypothesis as to how stress can sustainably affect gene expression and thus lead to the development of such troubles.

1.2.5. Involvement of the agmatinergic system in psychiatric disorders

Besides the frame of the monoamine-stress-neurotrophic hypothesis of mood disorders, recent studies have highlighted that other more peculiar molecular pathways and physiological processes were affected in these troubles. The agmatinergic system is one of them and it has been particularly investigated in the present thesis.

1.2.5.1. Overview of the agmatinergic system

Agmatine is a ubiquitous and highly conserved compound, found in a wide variety of living organisms, including mammals[145]. It is a decarboxylation product of L-arginine, and its biosynthesis is catalyzed by arginine decarboxylase (ADC). This process competes with other arginine-dependent pathways: the urea cycle (occurring only in the liver), nitric oxide (NO) synthesis and creatine synthesis (**Fig. 1.4**) [146]. Agmatine is degraded by the enzyme agmatinase (Agm) into putrescine, which is the precursor of the higher polyamines spermine and spermidine. The latter have been associated with glutamate and gamma-aminobutyric acid (GABA) synthesis, thus placing agmatine as a potential mediator of polyamine and neurotransmitter production in the brain [146,147]. The importance of agmatine is also strengthened by its competition with the urea cycle and NO synthesis. Furthermore, agmatine has been found to specifically inhibit NO synthase [148,149]. It has been questioned whether agmatine degradation by Agm rather serves a purpose of polyamines synthesis or agmatine inactivation, the latter being apparently privileged[150,151].

Agmatine is distributed in many body tissues, including the brain, stomach, intestines and aorta[146,152]. The few studies on agmatine and Agm distribution in the brain, suggest a high activity in the hypothalamus, medulla oblongata, hippocampus, striatum and cortex[151,153,154]. Both agmatine and Agm seem to be present in several cell types, such as principal neurons, interneurons and glial cells[155–158]. Interestingly, they were also observed to be particularly present in mitochondria[154,159].

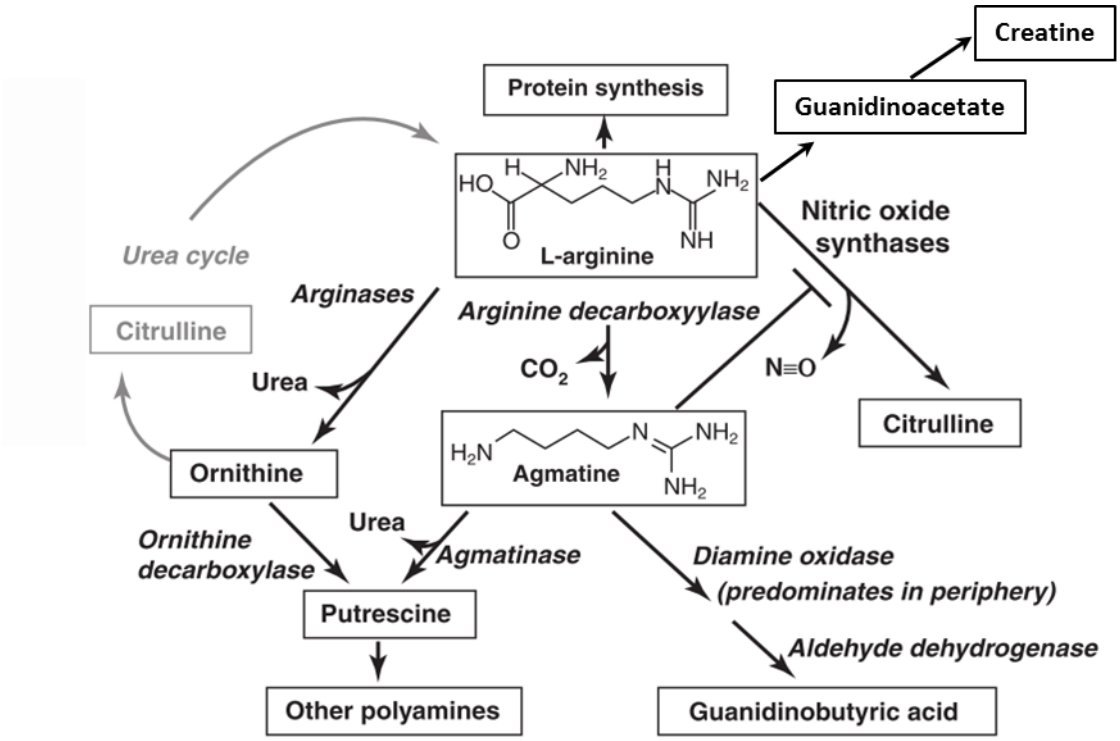

Figure 1.4 : Pathways involved in agmatine biosynthesis and degradation. Arginine is metabolized into agmatine, through the action of arginine decarboxylase. Agmatine is then degraded either by agmatinase into putrescine (leading to polyamine synthesis) or by diamine oxidase into guanidinobutyric acid. Arginine decarboxylation into agmatine is a pathway that competes with other arginine-related pathways such as the urea cycle (occurring only in the liver), nitric oxide synthesis and creatine synthesis. Agmatine also inhibits nitric oxide synthases. Adapted from [146].

While agmatine has been observed to have an overall regulatory and cytoprotective effect in peripheral organs[146,160] (such as in the heart[161], kidney[162–164] and stomach[152]), its physiological function in the brain is still not completely understood.

Several pieces of evidence have emerged and suggest an important role for agmatine in neuromodulation and in neurotransmission. There is actually a hypothesis that proposes agmatine as a potential new neurotransmitter. This proposition is based on several findings. First, agmatine concentration in the brain is similar to other classic neurotransmitters[145,165]. Second, agmatine is synthesized and stored in synaptic vesicles[166], in several types of neurons[155,156], and released from axons by Ca^{2+}-dependent depolarization[167,168]. Third, once released, agmatine can bind to a wide variety of receptors. Indeed, it binds with high affinity to all subclasses of α_2-adrenergic receptors and imidazoline receptors[169–172], and it specifically blocks ligand-gated ion channels, particularly glutamatergic NMDA receptors (NMDAR)[173]. Some studies suggest that agmatine can also bind nicotinic receptors[174] and serotoninergic 5HT-2A and 5HT-3 receptors[175]. Finally, agmatine can be either reuptaken, possibly via

voltage- or ligand-gated Ca^{2+} channels, or specifically degraded by Agm[173]. In addition, agmatine has also been observed to be synthesized and released in astrocytes, thus proposing these cells as potential agmatine reservoirs[157] (**Fig. 1.5**).

While this hypothesis is being debated, a wide variety of studies have helped unravelling the physiological effects of agmatine in the brain. Most notably, agmatine has been found to have an overall protective effect against neurotoxic and ischemic brain injuries, pain, epileptic seizures, and opioid addiction (for comprehensive reviews of these actions, see[146,153,160]). But agmatine has also been associated with several psychiatric disorders, such as schizophrenia, addiction and, of particular interest with the present work, MDD[153].

1.2.5.2. Evidence for the involvement of the agmatinergic system in MDD

There is substantial evidence that agmatine might contribute to modulate MDD, as observed by several clinical and preclinical studies. In humans, plasma levels of agmatine were found to

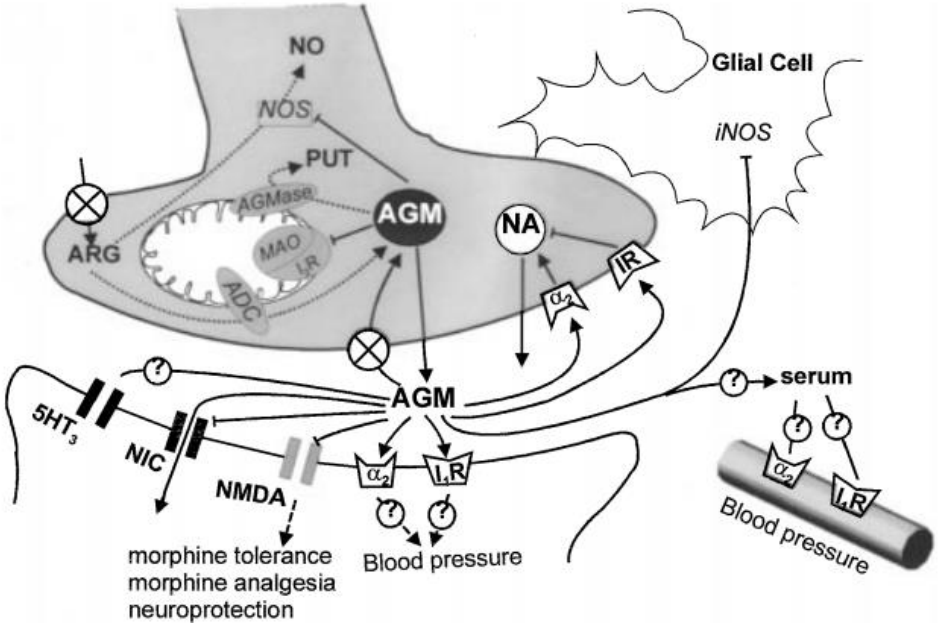

Figure 1.5 : Schematic representation of a putative agmatinergic synapse. L-arginin (ARG) is metabolized into agmatine (AGM) through the action of mitochondrial arginine decarboxylase (ADC). Agmatine is then stored in vesicles or degraded by mitochondrial agmatinase (AGMase) into putrescine. Inside the cell, AGM can inhibit monoamine oxidase (MAO), via imidazoline I_2 receptor (i_2R), and nitric oxide synthase (NOS). Agmatine can also be released into the synaptic cleft, where it can interact with a wide variety of pre- and post-synaptic receptors: activation of α_2 adrenergic (α_2) and imidazoline receptors(IR), and blockade of nicotinic cholinergic (NIC) and NMDA receptors. Agmatine might also activate $5HT_3$ receptors. Through its action on α_2-adrenergic and imidazoline receptors, agmatine might regulate noradrenaline (NA) release. Through the same receptors, it also regulates blood pressure in the periphery. After release, agmatine might possibly be reuptaken, through an as yet unknown transporter, or go into the post-synaptic neuron through nicotinic cholinergic receptors. Agmatine can also enter glial cells and regulate nitric oxide synthesis. Agmatine can also be synthetized and stored in astrocytes, which would then act as reservoirs (not shown here). Taken from[158].

be higher in depressed patients than in healthy control, which was normalized upon treatment with the antidepressant bupropion[176]. It is important to notice though, that to our knowledge no human studies on agmatine levels in the depressed brain had been performed yet. However, Agm levels have been measured in post-mortem tissues of MDD patients and revealed higher levels of this enzyme in the hippocampus, thus rather suggesting decreased agmatine levels in the brain[150]. This is further supported by several rodent studies that showed decreased agmatine levels in the hippocampus and PFC upon acute stress, alongside structural changes, such as reduced dendrite density[177,178]. In line with these findings, exogenously administered agmatine was found to have neuroprotective effects against both acute and chronic stress, as it prevented stress-induced neuronal damages, both *in vitro* and *in vivo*[177–181]. It also has a protective effect against the induction of inflammation-induced depressive symptoms through the administration of the pro-inflammatory cytokine tumor necrosis factor α (TNFα)[182]. Finally, acute and sub-chronic agmatine treatment were found to have behavioral antidepressant effects in rodents, in paradigms such as the forced-swim test (FST) and tail suspension (TS) test[183,184].

The molecular mechanisms underlying agmatine antidepressant effects have been investigated, but are still not well understood. It was observed that agmatine treatment has the ability to reverse monoamine reduction and Ca^{2+} overloading, in a similar way as classical antidepressants[185]. A certain amount of co-administration studies have shown that the antidepressant effects of agmatine were abolished when preventing its binding to imidazoline I_1 and I_2receptors, 5HT-2A and 5HT-3 receptors, α_2-adrenergic receptors, and NMDA receptors [186–188]. This suggests that the behavioral effects of agmatine are certainly due to its ability to bind to a wide variety of receptors, thus activating numerous signaling pathways. Indeed, several target receptors of agmatine have been shown to be involved in stress and mood regulation. For example, imidazoline receptors have the ability to regulate monoamines levels[189], and activation of I_2 receptors inhibits MAO-A and has antidepressant effects[189,190]. Furthermore, tricyclic antidepressants were found to regulate both imidazoline and α_2-adrenergic receptors binding sites[191–195], thus suggesting pleiotropic effects of agmatine treatment. Agmatine has not been considered yet for a potential antidepressant use in human. However, one pilot study has observed that chronic agmatine administration

produces antidepressant effects and seems to be well tolerated, while not producing harmful secondary effects[196], thus opening the door for a potential therapeutic use of agmatine.

Altogether, the different findings presented above strongly suggest an important role for agmatine in the brain, and have helped raise interest in this compound. Of note, two recent studies have highlighted the fact that chronic and sub-chronic agmatine treatment induced the phosphorylation of CREB, as well as activation of synaptic plasticity and cell survival pathways, accompanied by increased BDNF protein levels in mouse hippocampus[184,197]. These results are of particular interest considering the network hypothesis of MDD presented above, and contribute to the suggested role of agmatine as a potent neuromodulator.

1.3. The role of the transcription factor CREB and its coactivator CRTC1 in mood disorders

As mentioned several times above, the transcription factor CREB is a converging point of many pathways involved in mood disorders, and it is by far the most extensively studied transcription factor implicated in these diseases. This pleiotropic factor is involved in many processes in the central nervous system and a wide variety of studies have shown its involvement in mood disorders, thus highlighting the complexity of its functions in these troubles. The newly discovered CREB-regulated transcription coactivators (CRTCs), and particularly CRTC1, are a major interest for our group and have been associated with the pathological process leading to mood disorders. Thus, this section will specifically describe the involvement of CREB and CRTC1 in these psychiatric diseases.

1.3.1. CREB

CREB was first identified in 1987 by Montminy et al. [198] and since then, it is known to be a major pleiotropic transcription factor implicated in several crucial neuronal processes, such as neuron's survival and growth, neurogenesis, synaptogenesis, synaptoplasticity, and in long-term potentiation (LTP)[199,200]. CREB is present in three isoforms (CREBα, CREBΔ and CREBβ), all three of them having the capacity to induce CRE-dependent transcription in response to increased levels of cAMP[201]. CREB belongs to the basic leucine zipper (bZip) superfamily of transcription factors, and with two other proteins (cAMP-response element modulator (CREM), and activating transcription factor 1 ATF-1)) it forms the CREB family[199]. CREB binds

to the gene promoter CRE sequence as a dimer, through its bZip domain, but can also heterodimerize with ATF1 and CREM[199,202–204].

CREB is activated through its phosphorylation at serine 133 (Ser133), which promotes the association of CREB with CREB-binding protein (CBP), therefore leading to the assembly of a protein complex[205,206], which will then stimulate gene transcription partly through CBP's acetyltransferase activity.

Two principal pathways can trigger CREB phosphorylation and activation (**Fig. 1.6**):

(1) The adenylate cyclase/cAMP/PKA pathway, which is activated by noradrenaline (NA) and serotonin (5HT). The activation of G protein-coupled receptors (GPRCs), with a Gαs protein, by a monoamine neurotransmitter, activates the adenylate cyclase (AC), which in turn produces cAMP. The latter activates the protein-kinase A (PKA), which translocates to the nucleus, where it phosphorylates dimerized CREB[207,208].

(2) The Ca^{2+} pathway, which is activated by increased Ca^{2+} levels in the cytoplasm. This phenomenon can be achieved through different mechanisms: 1) GPCRs with a Gαq protein activate IP3, which opens Ca^{2+} channels present on the endoplasmic reticulum, leading to the increase of intracellular concentration of Ca^{2+}; 2) Voltage-dependent Ca^{2+} channels open in the presence of depolarization; 3) Glutamate receptors (NMDA receptors) open and let Ca^{2+} enter in the neuron. These different events all lead to cytoplasmic Ca^{2+} increase, an event that activates calcium-calmodulin-dependant kinases, which in turn phosphorylate CREB[208–211].

The implication of CREB in mood disorders is firstly supported by several genetic and clinical studies. Indeed, the chromosomal region 2q33-q35 containing the *CREB* gene has been observed as a susceptibility locus for recurrent MDD in women[212,213], and several *CREB* polymorphisms have been associated with MDD, BD, suicidal behavior, as well as aggressiveness[214–217]. Post-mortem CREB levels were found to be decreased in the temporal cortex of depressed patients, whereas an increase in CREB levels was found in patients taking antidepressants at the time of death[218,219]. Also, CREB levels and DNA binding activity were significantly decreased in the prefrontal cortex and the cingulate gyrus of BD patients[220].

In parallel, several mood disorders treatment have been showed to regulate CREB levels and activity. Antidepressants were found to provoke an increase in mRNA levels of CREB in the rat hippocampus. Some of them, such as fluoxetine, also produced an increase in CREB

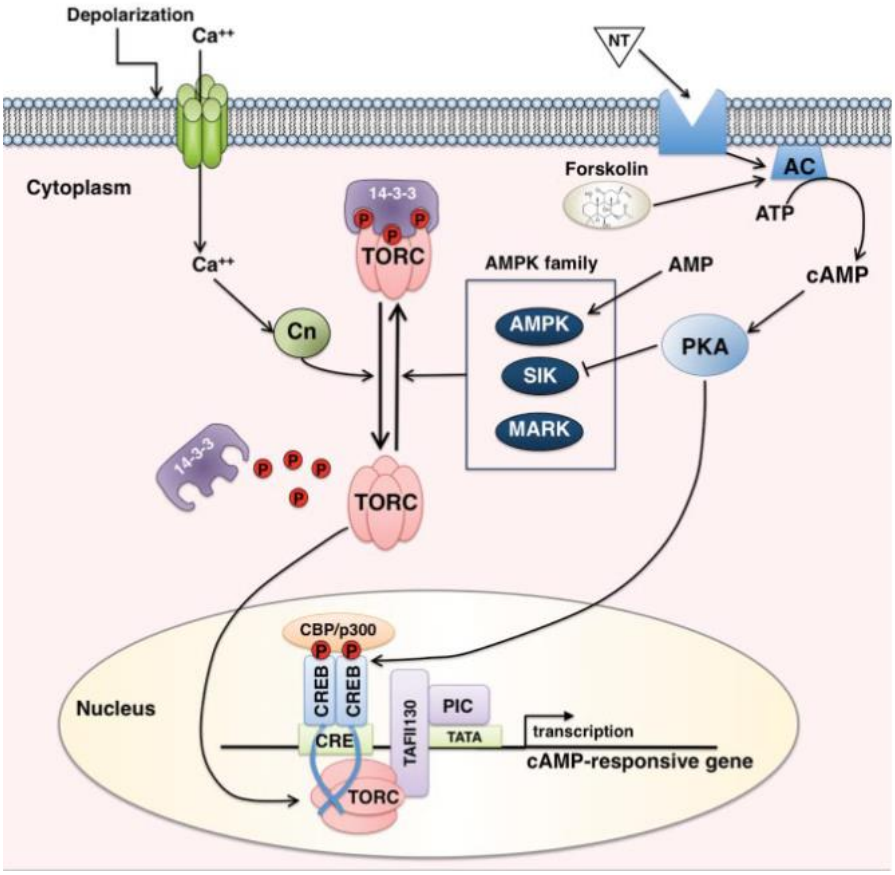

Figure 1.6 : The CREB-CRTC1 pathway. CREB can be activated by the cAMP pathway (right side). Upon binding of a neurotransmitters (NT) to its G-protein coupled receptor, adenylate cyclase (AC) is activated and catalyses cAMP production, which in turn activates the protein kinase A (PKA). The protein kinase A translocates then to the nucleus and phosphorylates dimerized CREB, thus leading to gene transcription. CREB can also be activated following increased intracellular Ca^{2+} concentration, which leads to activation of calcium-calmodulin-dependent kinases that will also phosphorylate and activate CREB (not shown here). CRTC1 (represented here as TORC) is present in its phosphorylated (inactivated) form in the cytoplasm, sequestrated by the scaffolding protein 14-3-3. CRTC1 is controlled by the opposing effects of the calcium-sensitive phosphatase calcineurin (Cn) and the AMP kinase family (AMPK, SIK, MARK). Upon neuronal excitation, increase in intracellular Ca^{2+} leads to the activation of calcineurin, which will catalyze CRTC1 dephosphorylation. Additionally, activation of PKA leads to the inhibition of AMP kinases, thus preventing CRTC1 phosphorylation. When activated, CRTC1 translocates to the nucleus and interacts with CREB in order to facilitate the recruitment of the transcription complex (including CREB binding protein (CBP) and the $TAF_{II}130$ polymerase subunit). Taken from[236].

phosphorylation, binding and protein levels[221]. Chronic lithium treatment increases the phosphorylation of the existing CREB, while not increasing its levels, and promotes its binding to DNA, leading to an increase of CREB-mediated gene transcription[222]. Human studies have correspondingly observed that lithium-treated patients present increased levels of phosphorylated CREB[223].

However, changes in CREB activity may vary depending on the type of treatment, the brain region, and the signaling pathway activated. Indeed, overexpression of CREB in the dentate

gyrus of rat produces antidepressant effect in several depression-related behavioral paradigms, whereas it has no effect when overexpressed in the CA1 region or in the prefrontal cortex[224]. Furthermore, an overexpression of CREB in the amygdala or in the NAc produces a pro-depressive behavior[225,226]. Interestingly, transgenic CREB-deficient mice exhibit antidepressant-like behaviors and exhibit a significant increase in hippocampal neurogenesis, but not in BDNF levels[227,228]. However, CREB-deficient mice still respond to antidepressants[228,229].

Altogether, these findings suggest that the implication of CREB in mood disorders, while being undeniable, is not clear and may depend on the brain region and the pathway involved.

1.3.2. CRTC1

The family of CRTCs, formerly known as transducers of regulated CREB activity (TORCs), was first discovered in 2003 by Conkright et al. [230]. These coactivators were found to promote CREB-regulated gene transcription, independently of CREB Ser133 phosphorylation. This happens via CRTCs binding as tetramer to the bZIP domain of CREB, thus favoring the interaction between CREB and the RNA polymerase II preinitiation complex component $TAF_{II}130$[230]. CRTCs can also directly interact with CBP and help its recruitment on CREB target genes promoter[231].

Three CRTCs isoforms have been characterized: CRTC1, CRTC2 and CRTC3, with CRTC1 being particularly highly expressed in cortical and hippocampal post-mitotic neurons[232,233]. Several studies have shown that CRTC1 and CRTC2 activate the transcription of several CREB-regulated genes when Ca^{2+} and cAMP are simultaneously present in the neuron, therefore acting as cAMP/Ca^{2+} coincidence detectors[232–236].

In resting conditions, CRTC1 is found in the cytoplasm in a phosphorylated form, bound to 14-3-3 proteins[235]. Synaptic activity inducing both Ca^{2+} and cAMP signals leads to the dephosphorylation of CRTC1 via two actions: cAMP inhibits salt-inducible kinase (SIK) 1/2 (promoting CRTC1 phosphorylation) and calcium signaling directly dephosphorylates CRTC1 via calcineurin. Dephosphorylated CRTC1 translocates to the nucleus and activates CREB-regulated gene transcription. (**Fig.1.6**)[232,233,235].

Several studies have demonstrated that CRTC1 is essential to many important neuronal processes, including dendritic growth, long-term synaptic plasticity and glucose

metabolism[232,233,237,238]. Several pieces of evidence also suggest that CRTC1 might play an important role in the etiopathogenesis of mood disorders. Indeed, CRTC1 has been demonstrated to be critical for activity-induced dendritic growth of cortical neurons during development[233]. Furthermore, BDNF expression occurs in a CRTC1-dependent manner in the adult rat hippocampus[232,237]. CRTC1 has also been shown to be required for the maintenance of L-LTP in the hippocampus[232,237] . In addition, the interaction of CRTC1 with CREB is required for the BDNF-dependent dendritic growth of cortical neurons[239]. The same study also demonstrated that the nuclear translocation of CRTC1 is induced by the activation of NMDAR by glutamate, which is also essential for BDNF effects on dendritic growth. Finally, CRTC1 has been shown to be a primary target of lithium, which enhances CREB-CRTC1 association, by helping CRTC1 oligomer formation, and preparing it for its association with CREB[240–242] . In parallel with its role in mood disorders, several recent studies have highlighted the importance of CRTC1-dependent gene transcription in spatial memory formation and its possible implication in Alzheimer's disease[243,244].

All these different findings suggest that CRTC1 might be a mediator of mood disorders etiology, possibly through its prominent role in CREB-regulated neuroplasticity gene transcription.

1.4. Mood disorders treatments

Although the etiology and the neurobiological bases of mood disorders are not clearly understood, there is a broad range of treatments available, which will be briefly summarized here.

1.4.1. Antidepressants

Regarding MDD, a wide variety of drug and non-drug treatments are currently available, with significant positive effects on patients, and are summarized in **Table 1.2**.

Non-drug treatments mainly include psychotherapy (however only effective for mild depression) and electro-convulsive therapy (efficient, but only used for the most severe cases)[24]. In the last decade, deep brain stimulation (DBS) has emerged as a potential new therapy and has been proven very effective[245]. Yet, for the vast majority of cases, classical antidepressant drugs remain the most frequent treatment[24].

Type of treatment	Mode of action	Examples
*Medication**		
Tricyclics	Inhibition of mixed noradrenaline and serotonin reuptake	Imipramine, desipramine
Selective serotonin reuptake inhibitors (SSRIs)	Inhibition of serotonin-selective reuptake	Fluoxetine, citalopram
Noradrenaline reuptake inhibitors (NRIs)	Inhibition of noradrenaline-selective reuptake	Atomoxetine, reboxetine
Serotonin and noradrenaline reuptake inhibitors (SNRIs)	Inhibition of mixed noradrenaline and serotonin reuptake	Venlafaxine, duloxetine
Monoamine oxidase inhibitors (MAOIs)	Inhibition of monoamine oxidase A (MAO_A). Inhibition of MAO_B does not have antidepressant effects	Tranylcypromine, phenelzine
Lithium	Lithium has many molecular actions (for example, inhibition of phosphatidylinositol phosphatases, adenylyl cyclases, glycogen synthase kinase 3β and G proteins) but which of its actions is responsible for its antimanic and antidepressant effects is unknown	
Atypical antidepressants	Unknown. Although these drugs have purported monoamine-based mechanisms (for example, bupropion inhibits dopamine reuptake, mirtazapine is an α_2-adrenergic receptor antagonist and tianeptine an activator of monoamine reuptake), these actions are not necessarily the mechanisms that underlie the drugs' therapeutic benefit	Bupropion, mirtazapine, tianeptine
Non-medication		
Electroconvulsive therapy (ECT)	General brain stimulation	
Magnetic stimulation	General brain stimulation? A magnetic field is thought to affect the brain by inducing electric currents and neuronal depolarization	
Vagal nerve stimulation (VNS)	Unknown	
Psychotherapies	Exact mechanism is uncertain, but is thought to involve learning new ways of coping with problems	Cognitive-behavioural therapy, interpersonal therapy
Deep brain stimulation	In severely ill patients, stimulation of a region of the cingulate cortex found to function abnormally in brain imaging scans reportedly has antidepressant effects[84]	

Table 1.2 : Table of currently available antidepressant drug and non-drug treatments. Taken from[24].

As previously explained, all the currently available antidepressants focus on the monoamine metabolism. First generation antidepressants, monoamine oxidase inhibitor and tricyclic antidepressants, provided template for the development of second generation antidepressants: selective serotonin reuptake inhibitors (SSRIs), noradrenaline reuptake inhibitors (NRIs), and serotonin and noradrenaline reuptake inhibitors (SNRIs), which are more specific, but still acting on the same system[24,246]. Although all these drugs have a clear efficiency, they however need weeks of treatment before producing a response. Moreover, they are associated with a lot of side effects and only less than 50% of depressed patients reach total remission. In fact, treatment-resistant depression (TRD), a condition in which the patient does not show any response to one or more treatment trials, is estimated to occur in 12 to 20% of depressed patients[247].

Although most antidepressant drugs are effective (except for treatment-resistant depression), and can be combined with non-drug therapies (e.g.; combination of antidepressant treatment and psychotherapy have been proven very effective[248]), intensive study of neurobiological bases of depression is needed in order to develop alternative drugs, acting beyond the monoaminergic system.

1.4.2. Mood stabilizers

Mood stabilizing agents are the most frequent drugs used to treat BD, and lithium is by far the most used of these agents[249]. Indeed, it efficiently treats both manic and depressive episodes, reduces the recurrence of such episodes and also decreases suicidal behaviors[249–251]. As mentioned above, lithium has neuroprotective properties, which probably underlie its effects, but its mode of action is still unknown. Lithium freely enters in cells through sodium channels and inhibits several enzymes in the cytoplasm, mainly the inositol monophosphatase (IMPase) and the glycogen synthase kinase 3 (GSK-3). These enzymes have critical roles in pathways such as the phosphoinositol (PI) pathway and the Wnt signaling pathway[249,252–255]. The PI pathway is involved in processes such as cell division and neuronal excitability, and is therefore thought to play a crucial role in the mood stabilizing effects of lithium[256–258]. However, lithium inhibitory action on GSK-3 is also of interest, as this inhibition leads to increase in CREB activity[222,253,259]. Of note, GSK-3 inhibition is also a signaling pathway elicited by BDNF[260,261]. Lithium effects could therefore also rely on this process.

Although its mechanisms of action are still extensively investigated, lithium remains a highly frequent drug for the treatment of BD; yet it is associated with many side effects.

Apart from lithium, other mood stabilizing agents are currently used and valproate (VPA) is one of them. It mainly acts on manic behavior, it has been indeed shown that it dampens the high-frequency Na^+ channel firing occurring in manic phases, and it also enhances the release of GABA[262–264]. VPA has another interesting characteristic, as it can act as a HDAC inhibitor, which further strengthens the role of epigenetic modifications in mood disorders (developed in a previous section)[265,266].

As for MDD, combination of treatments are often used, especially when it comes to treat both manic and depressive symptoms. Combination of antipsychotic and antidepressant drugs have been proven effective, and can be further combined with psychotherapy. Similarly as for

MDD, BD treatments also require weeks before producing a visible therapeutic effect, and they are also associated with non-negligible side effects, thus increasing the need for investigating new therapeutic opportunities[267].

1.4.3. New focus on NMDA receptor inhibitors

In the last decade, NMDAR antagonist have received increased interest as potential new antidepressant drugs, because of their newly discovered rapid antidepressant effects. This new field of investigation started when it was observed that a single sub-anesthetic dose of the NMDAR antagonist ketamine had rapid (72h) and long-lasting (up to 2 weeks) antidepressant effects, even in patients exhibiting treatment-resistant depression[268,269]. Several studies could replicate these effects, however with a certain variability of response[270–273]. Nevertheless, the effect of ketamine was repeatedly assessed and proven effective, also in BD patients[269,274]. Yet the psychomimetic properties of this agent and its abuse potential renders it impossible to be used as a long-term antidepressant. But this allowed to discover a new mechanism potentially involved in mood disorders and provided new lines of investigation for the development of new treatments[275,276]. Therefore, the molecular mechanisms underlying NMDAR antagonists effects have been heavily investigated.

The antidepressant effects of ketamine and other NMDAR blockers could be reproduced in rodents, in several paradigms. Most studies focused on the FST, a paradigm in which ketamine is highly effective. It showed rapid (30min) effects that could last up to 1 week after a single injection[276–282]. Yet, the amplitude and duration of the effect were different depending on the mouse or rat strain, and the protocol used[276]. Ketamine also had antidepressant effects in paradigms such as the TS[277,278,283–285], novelty-suppressed feeding[286–289], learned helplessness (LH)[284,286,290,291] and sucrose preference test[292–294]. It was also effective at reversing depressive-like behavior and molecular alterations of animals which underwent a chronic stress procedure[289,292,295–298].

The molecular cascades activated by ketamine were also investigated and have been partly unraveled. Ketamine inhibition of NMDAR leads to a suppression of tonic glutamate input to GABAergic neurons, a mechanism that results in a general disinhibition of glutamate signaling in the PFC[276,299]. This will lead to an upregulation of glutamatergic AMPA receptor (AMPAR)

and an overall increased neuronal activity[299]. AMPAR have been shown to be critical for ketamine effects and the NMDA/AMPA ration is a critical mediator of such effects[293].

Ketamine and other NMDAR antagonists have also been demonstrated to rapidly (30 min) activate the mammalian target of rapamycin (mTOR) pathway, which is involved in several neuroplasticity pathways, such as LTP[286]. Through this pathway, ketamine rapidly activates the expression of immediate-early genes involved in glutamate-mediated synaptic plasticity. This mTOR activation also induces the dephosphorylation and inhibition of the eukaryotic elongation factor (eEF2)[287,289]. This inhibition leads to an overall increase in protein translation, including increased BDNF translation. It has been recently demonstrated that rapid antidepressant effect of ketamine and MK-801 (another NMDAR antagonist) depend on BDNF translation, induced by eEF2 dephoshorylation[289] (**Fig. 1.7**). An even more recent study has also determined, through optogenetic experiments, that the rapid and sustainable effects of ketamine were dependent on the infralimbic PFC activity[300]. Furthermore, ketamine treatment significantly increased the number and function of spine synapses in this region[300]. In line with the involvement of epigenetic mechanisms in mood disorders, it has also been very recently observed that ketamine produces increased histone acetylation in cultured neurons, alongside repression of HDAC5 activity[301]. Although these various new findings still have to be confirmed, compounds acting similarly as NMDAR antagonists might constitute an interesting new line of investigations.

In summary, while many treatments are available for mood disorders, there are still 50% of patients that are not responding, therefore urging the need for alternate therapies. However, new promising agents, such as the NMDAR antagonists, suggest that the development of such

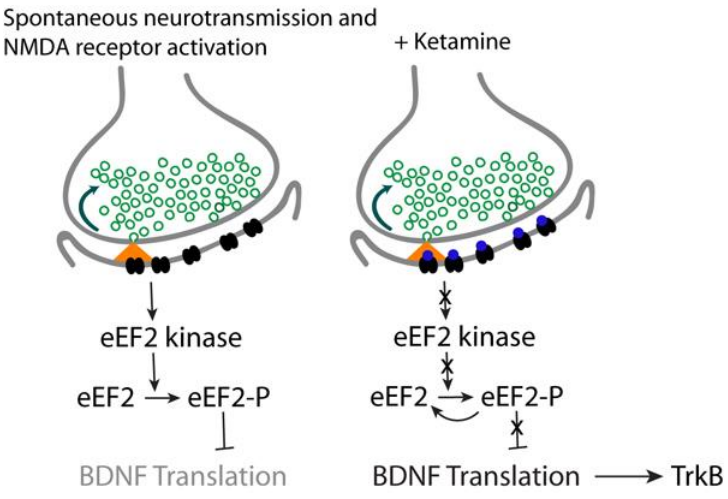

Figure 1.7 : Mechanisms underlying ketamine effects on BDNF translation. Upon normal NMDAR activity, the eukaryotic elongation factor 2 (eEF2) kinase phosphorylates and activates eEF2, which prevents protein translation. Upon NMDAR blockade by ketamine, inhibition of eEF2 kinase occurs and leads to dephosphorylation and inactivation of eEF2. This will enhance protein translation, including BDNF translation. BDNF can therefore bind and activates its receptor TrKB, thus activating synaptic plasticity-related pathways. Taken from[289].

therapies might be possible in the future. But it also highlights once again the complexity of the mechanisms involved in mood disorders, and the need to understand their etiology, in order to better treat them.

1.5. Animal models of mood disorders

As demonstrated above, mood disorders are complex and heterogenic diseases, resulting from several environmental and physiological factors. They are characterized by a wide range of highly variable physical and psychological symptoms, as underlined by the variety of systems that are observed to be disturbed in these troubles. Hence, animal models of mood disorders are difficult to develop. Indeed, face validity in animal models of mood disorders is restrained to some symptoms of the disease that can be modulated in animals, whereas others cannot (such as psychological troubles)[302]. An animal model presenting all the aspects of the disease is therefore impossible to obtain, and the ones available only reflect a small subset of the complexity of mood disorders. Yet, many different animal models of mood disorders have been developed over the years and are commonly used in today's research[302–305]. This section will summarize these different models.

1.5.1. Rodent models of mood disorders

An ideal animal model of mood disorders (or any other condition) should present three criteria:

(1) Construct validity, in which the etiology of the disease is similar as observed in humans.

(2) Face validity, in which the animal presents similar symptoms and endophenotypes as in humans.

(3) Predictive validity, in which the animal model responds to the same treatments as humans[305].

No animal model combining these three features exists for mood disorders[306]. As explained above, many symptoms and aspects of these troubles cannot be modeled in animals, therefore complete face validity cannot be obtained. However, construct and predictive validity can be modeled in some animal models and can be tested in a wide variety of behavioral paradigms (**Table 1.3**).

Model	Main Features
Forced swim test	Antidepressants acutely increase the time an animal struggles in a chamber of water; lack of struggling thought to represent a state of despair.
Tail suspension test	Antidepressants acutely increase the time an animal struggles when suspended by its tail; lack of struggling thought of represent a state of despair.
Learned helplessness	Animals exposed to inescapable footshock take a longer time to escape, or fail to escape entirely, when subsequently exposed to escapable foot shock; antidepressants acutely decrease escape latency and failures.
Chronic mild stress	Animals exposed repeatedly to several unpredictable stresses (cold, disruption of light-dark cycle, footshock, restraint, etc.) show reduced sucrose preference and sexual behavior; however, these endpoints have been difficult to replicate, particularly in mice.
Social stress	Animals exposed to various types of social stress (proximity to dominant males, odors of natural predators) show behavioral abnormalities; however, such abnormalities have been difficult to replicate, particularly in mice.
Early life stress	Animals separated from their mothers at a young age show some persisting behavioral and HPA axis abnormalities as adults, some of which can be reversed by antidepressant treatments.
Olfacotry bulbectomy	Chemical or surgical lesions of the olfactory bulb cause behavioral abnormalities, some of which can be reversed by antidepressant treatments.
Fear conditioning	Animals show fear-like responses when exposed to previously neutral cues (e.g., tone) or context (cage) that has been associated with an aversive stimulus (e.g., shock).

Table 1.3 : Table of the most current animal models of depression and their main features. Taken from[23].

Depressive-like behaviors can be observed in animals following acute or chronic stress[306]. Therefore, most behavioral paradigms currently used rely on the application of one or several stressors. Some of the most commonly used tests rely on the application of one acute stress. These include the FST, TS and LH paradigms:

- FST: in this test, the animal is put in the water (which is a stress for rodents) for a short period of time, with no possibility to escape. The animal will start to present an immobile floating behavior, which is interpreted as a reflector of the stressed state of the animal, as well as a resignation facing the inescapability of the situation[307–310].
- TS: in this paradigm, the animal is briefly suspended by the tail, which is a highly stressful event. During this time, the animal will seek to escape by actively moving. However, it will soon start to give up and stay completely immobile. As in the FST, this immobility time is measured and considered as "depressive-like behavior"[311,312].
- LH: in this test, the animal is first subjected to short electric shocks, with no possibility to escape. In a second phase, the electric shocks are repeated, but this time the animal has the possibility to escape to another compartment. The latency to escape is

measured and if this latency time is high, it is interpreted as a depressive-like behavior, as the animal does not seek to escape the stressful situation[313,314].

These three paradigms allow to measure what is called the "behavioral despair" of the animals. It is described as the resignation of the animal facing the stressful event and a lack of motivation to seek an escape. This interpretation has however been extensively debated as it could also be explained by a learning process, in which the animal might have learned that there is no escape[315]. Furthermore, these paradigms present poor construct validity, as they do not reflect the development of depression as observed in humans (as it generally occurs following chronic and not acute stress). Nevertheless, these tests are widely used, mainly because of their high predictive validity. Indeed, the behavioral despair measured in these paradigms can be efficiently reversed by nearly all available antidepressant and mood stabilizing drugs, when administered acutely. Therefore, these tests, especially the FST, are commonly used as screening procedures for potential new antidepressant agents[302,306].

Another subset of depression-related behavioral tests involves the application of a chronic stress protocol. These tests have a good construct validity as they better reflect the development of depression as observed in humans. In these paradigms, one or several stressors are applied chronically (several weeks) and this leads to the establishment of a chronic stressed state in the animal, which will also present depressive-like symptoms such as anhedonia and social impairments[316–318]. There are several existing chronic stress protocols that can be applied. The chronic unpredictable mild stress (CUMS) procedure is based on the application of several mild stressors (circadian perturbation, swimming session, injections…) in an unpredictable schedule. This protocol is known to induce anhedonia in rodents, which is a core symptom of depression, thus providing good construct and face validity to this model[316]. Anhedonia can be measured in rodent by the sucrose preference test. In this test, the animal can freely access either water or sweetened water. The animals would naturally prefer sweetened water, yet when they present anhedonia this preference is strongly diminished[318–320].

Anhedonia also occurs in rodents following a chronic social defeat stress protocol. Here the experimental animal is put daily in the presence of an aggressive dominant mouse. This procedure mimics the social stress that can occur in humans and that leads to depression. The consequence of this protocol is the development of an anhedonic behavior, but also of social

withdrawal as well as a metabolic syndrome (weight gain and leptin resistance), which is also a clinical feature of MDD[321–323].

Chronic corticosterone administrations, as well as long term isolation can also be used as stressors and induce depressive-like behavior in rodents[302,324,325]. Maternal separation is also a stress procedure that can be used to study the effects of early-life stress, as it induces long term and heritable behavioral impairments that can be related to MDD[45,326]. As already mentioned in a previous section, chronic stress procedures also induce durable chemical and morphological changes in the brain, such as neuronal death and cellular damages. These chronic stress models also have good predictive validity as their effects can be reversed by chronic, but not acute, antidepressant treatment, which is similar to what is observed in humans.

Recently, the open-space forced swim test (OSFS) procedure was developed[327,328]. This model is based on repeated swimming sessions, similar as the FST, for several weeks. During the first days of the test, the animals will progressively develop a stressed state, as reflected by progressive increased immobility time in each swimming session. The swimming sessions then continue, while in parallel a chronic antidepressant treatment is administered. Floating time is measured in each session and progressively decreases as the treatment starts to be effective. This procedure is interesting as it allows to chronically stress the animals in a first phase, and then assess the effects of antidepressant in a second phase. Furthermore, it is only sensitive to chronic antidepressant treatment.

In the frame of BD, there is currently no existing animal model that combines features of both depressive and manic phase, as well as a cyclic alternation of these two phases[306,329]. Therefore, the models focus on either one of the two phases. Regarding depressive episodes, the same paradigms as detailed above are used. On the opposite, there are few models of mania available. It can however be modeled in animals, either pharmacologically of genetically. The most used model includes a treatment with psychostimulants, such as amphetamines or cocaine. These can produce a wide spectrum of mania-like behaviors, such as hyperactivity, heightened alertness, insomnia, and changes in sleep patterns, and these behaviors can be reversed by mood stabilizers[330–332]. There are also several genetically-engineered mice exhibiting manic-like behaviors[333]. One mutant model is based on a *Clock* gene downregulation[334]. These mice exhibit several symptoms of mania as they are

hyperactive, need less sleep, and show an increased inclination for stimulants and reward, and chronic administration of lithium reduces these behaviors. Another transgenic model relies on the overexpression of GSK-3, which induces hypophagia, hyperlocomotion, and reduces immobility in the FST[335].

In summary, although mood disorders are difficult to model in animals, several paradigms allow to reproduce some aspects of these troubles in rodents. However, there is not a single "test for depression" or "test for BD". Instead, to be qualified as "depressive" or "bipolar" an animal should rather present the phenotypes of interest in different types of behavioral assays that include several aspects of the diseases: stress, anhedonia, social behavior, activity, treatment response. One should also be very careful with the high risk of anthropomorphism that comes along with behavioral observation. Furthermore, molecular changes in the different pathways presented above should also be investigated and correlated to the behaviors observed.

1.5.2. *Crtc1*$^{-/-}$ mice

As described above, CRTC1 is a key modulator of many important neuronal processes and might be an important mediator of mood disorder etiology. To further investigate the role of CRTC1 in the brain, we and others have generated simultaneously a line of complete *Crtc1*$^{-/-}$ mice by using the same genetrap strategy[238,336]. Altarejos et al. first observed that CRTC1-deficient mice exhibited a hyperphagic, obese and infertile phenotype. Therefore, the CREB-CRTC1 pathway was thought to mediate the effects of hormones and nutrients on the energy balance and fertility[238]. However, these findings were partly contradicted by our laboratory, that found no major infertility in *Crtc1*$^{-/-}$mice[336]. Therefore, it was concluded that the CREB-CRTC1 pathway is involved in the hypothalamic control of energy balance, but its effect on mice fertility is less clear.

Our group then undertook an extensive behavioral and molecular characterization of *Crtc1*$^{-/-}$ mice and found that they exhibited many endophenotypes related to mood disorders[337]. Indeed, in addition to their mild obese phenotype (which starts to appear only after the age of 14 weeks), these mice also presented several social impairments: male *Crtc1*$^{-/-}$ mice presented increased aggressiveness, as well as decreased social interaction and sexual motivation. Mutant mice also exhibited increased behavioral despair in the FST, as well as

anhedonia in the sucrose preference test. Their emotional response was found to be exacerbated in the fear-conditioning paradigm, and *Crtc1*$^{-/-}$ mice also presented higher anxiety and neophobia[337].

Interestingly, *Crtc1*$^{-/-}$ mice presented a blunted response to the antidepressant fluoxetine. Indeed, in the OSFS paradigms, mutant animals exhibited increased immobility time, which could not be reduced by fluoxetine. They also showed no response to this treatment in the TS test. Interestingly however, fluoxetine could successfully reverse their aggressiveness, as well as their anxiety in the novelty-induced hypophagia (NIH) paradigm[337].

At the molecular level, *Crtc1*$^{-/-}$ mice presented several interesting alterations, as a reduced dopamine and serotonin turnover was observed in the prefrontal cortex. Furthermore, downregulation of several neuroplasticity-related genes in significant regions involved in mood disorders could be seen: total *Bdnf* and its exon IV were both reduced significantly in the hippocampus and the PFC. The same observation was done for BDNF receptor, *TrkB*. Moreover, the three *Nr4as* were also significantly reduced in the hippocampus and the PFC. Other neuroplasticity-related genes were also analyzed, such as *c-fos*, *FosB*, *Crem-Icer*, *Somatostatin* and *Cartpt*, which were all found to be downregulated in the PFC and hippocampus of mutant mice[337].

Altogether, these results suggest that *Crtc1*$^{-/-}$ mice can be considered as a potent new animal model for mood disorders, as these animals encompass several aspects of both MDD and BD: increased behavioral despair, social impairments, anxiety, exacerbated aggressiveness and metabolic troubles. Furthermore, most of the key genes known to be involved in mood disorders are strongly downregulated in these mice. Moreover, *Crtc1*$^{-/-}$ mice could also be considered as potential model for treatment-resistant depression, as they are insensitive to fluoxetine.

This highly interesting mouse line also further highlights the importance of CRTC1 in mood disorders and its potential role in the etiology of these diseases. It also suggests its involvement in antidepressant response.

The main aim of this thesis was therefore to further investigate the involvement of CRTC1 in the etiology of mood disorders, by determining which systems are affected by CRTC1 deletion and contribute to their behavioral and molecular phenotype.

2. ARTICLES

Note: Manuscripts of the papers presented in this section are provided in the Appendix section, p.121

2.1. "The HDAC inhibitor SAHA improves depressive-like behavior of CRTC1-deficient mice: possible relevance for treatment-resistant depression"

Elsa M. Meylan[a,b], Olivier Halfon[b], Pierre J. Magistretti[c,d,a], Jean-René Cardinaux[a,b]

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Journal: *Neuropharmacology*

Issue: Volume 107, August 2016, p. 111-121. Published online in March 2016

2.1.1. Summary

Following our previously published paper showing the blunted response to the antidepressant fluoxetine displayed by *Crtc1*$^{-/-}$ mice[337], we further investigated the involvement of CRTC1 in behavioral and molecular antidepressant response. In this study, we showed that *Crtc1*$^{-/-}$ mice similarly responded to the tricyclic antidepressant desipramine: their immobility in the OSFS could not be reduced by the treatment, while it decreased their anxiety in the NIH paradigm. Supporting the blunted response to this tricyclic antidepressant, we found that desipramine significantly increased the expression of *Bdnf*, *Bdnf IV* and *Nr4a1-3* in the hippocampus and prefrontal cortex of wild-type (WT) mice, but failed to do so in *Crtc1*$^{-/-}$ mice. As already described, epigenetic regulation of neuroplasticity gene expression has been associated with

depression and antidepressant response, and histone deacetylase (HDAC) inhibitors have been shown to have antidepressant-like properties. We therefore tested the effects of the HDAC inhibitor SAHA on WT and *Crtc1*$^{-/-}$ mice. We showed that unlike conventional antidepressants, chronic systemic administration of the HDAC inhibitor SAHA could partially rescue the depressive-like behavior of *Crtc1*$^{-/-}$ mice, as it significantly decreased their immobility in the OSFS. This behavioral effect was accompanied by an increased expression of *Bdnf*, but not *Nr4as*, in the prefrontal cortex of *Crtc1*$^{-/-}$ mice, suggesting that this epigenetic intervention was able to restore the expression of a subset of genes, by acting downstream of CRTC1.

2.1.2. Contribution

I contributed to the experimental design and planning of this study. I performed all the experiments as well as the analysis of the results (behavioral recordings, calculations and statistical analyses). Finally, I wrote the entirety of the article.

2.2. "Involvement of the Agmatinergic System in the Depressive-like Phenotype of the *Crtc1* Knockout Mouse Model of Depression"

Elsa M Meylan[1,2], Lionel Breuillaud[1,2], Tamara Seredenina[3], Pierre J Magistretti[4,5,1], Olivier Halfon[2], Ruth Luthi-Carter[3,6], and Jean-René Cardinaux*[,1,2].

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Journal: *Translational Psychiatry*

Issue: Volume 6, July 2016, e852.

2.2.1. Summary

In order to complete the analysis of the molecular phenotype of *Crtc1*$^{-/-}$ mice, a microarray gene expression profiling was performed and revealed an upregulation of agmatinase in the cortex of *Crtc1*$^{-/-}$ mice. Knowing the involvement of the agmatinergic system in mood disorders, we further investigated the possible dysregulation of this system in *Crtc1*$^{-/-}$ mice. Quantitative polymerase chain reaction and Western blot analyses confirmed *Agmat* upregulation in both male and female *Crtc1*$^{-/-}$ prefrontal cortex (PFC) and hippocampus. Agmat staining and confocal immunofluorescence microscopy further revealed an increased number of Agmat-expressing cells, notably parvalbumin- and somatostatin- interneurons. We therefore hypothesized that the dysregulated agmatinergic system of *Crtc1*$^{-/-}$ mice might contribute to their depressive-like phenotype. Indeed, acute agmatine treatment improved the depressive-like behavior of *Crtc1*$^{-/-}$ mice in the forced swim test, suggesting that exogenous agmatine has a rapid antidepressant effect through the compensation of agmatine deficit due to upregulated *Agmat*. Through western blot analyses, we observed that in WT mice PFC, agmatine rapidly increased BDNF levels and decreased eEF2 phosphorylation, indicating that agmatine might be a fast–acting antidepressant with NMDA receptor antagonist properties. Interestingly, the induction of BDNF translation was only observed in female mice, thus suggesting sex-specific molecular effects of agmatine and BDNF regulation. Also, the effects of agmatine on BDNF and eEF2 were not present in *Crtc1*$^{-/-}$ mice, which implies that the behavioral antidepressant effects of agmatine do not solely rely on BDNF.

2.2.2. Contribution

I contributed to most of the experimental design and planning of this study. I performed all the experiments and result's processing (behavioral recordings, calculations and statistical analyses), except for the microarray profiling, which was performed by Dr. L. Breuillaud, Dr. T. Seredenina and Prof. R. Luthi-Carter. Finally, I wrote the entirety of the article, except for the parts related to the microarray, which were written by Dr. J.R. Cardinaux.

3. MATERIAL AND METHODS

3.1. Mice production and housing

3.1.1. Animals

Mice were bred and housed under a 12 light-dark cycle with *ad libitum* access to tap water and standard rodent chow diet (3436 Kliba Nafag, Provimini Kliba AG, Kaiseraugst, Switzerland). *Crtc1*$^{-/-}$ mice were generated as previously described[336,337]. For the present experiments, WT and *Crtc1*$^{-/-}$ mice result from a crossing between heterozygous *Crtc1*$^{+/-}$ mice, previously backcrossed for seven generations with C57BL/6N mice (Charles River Laboratories, Saint-Germain-sur-l'Arbresle, France). Animals were weaned at the age of 21 days and group housed. At the age of 5 weeks, animals were isolated in order to avoid excessive aggressiveness from *Crtc1*$^{-/-}$ mice. Treatments, behavioral and molecular procedures began when the animals reached the age of 8 weeks.

3.1.2. Genotyping

Genotyping was performed shortly after weaning. Genomic DNA from ear biopsy was extracted using a 20 min incubation at 95°C with solution A (25mM NaOH, 0.2mM EDTA) and addition of solution B (40mM TrisHCl pH 5.0). Samples were genotyped by PCR amplification of WT and *Crtc1*$^{-/-}$ alleles. The following primers were used: (a) forward 5'-GGCAGTACATAGCTTCTCTGGTGA-3', (b) reverse 5'-TGCAGGGCAGAGTCAGAGTTGGT-3' and (c) reverse 5'-GACAGTATCGGCCTCAGGAAGAT CG-3'. WT allele was amplified using primers (a) and (b), and *Crtc1*$^{-/-}$ allele using primers (a) and (c). The following PCR program was used: 3min at 94°C, 35 cycles of 15s at 94°C, 45s at 58°C, 90s at 72°C, and finally 7min at 72°C. Amplified DNS fragments were resolved in a 1% agarose gel.

3.2. Drugs and treatment

3.2.1. Lithium treatment

At the age of 8 weeks, male WT and *Crtc1*$^{-/-}$ mice received a 4-week chronic lithium treatment in the food, as previously described[338]. This treatment protocol was chosen because of its ability to reach and maintain blood lithium levels within the human therapeutic range (0.4-1.2mM). Lithium-enriched chow (Rodent diet, grain-based, $LiCO_4$ 2.4g/kg) and its equivalent control (Rodent diet, grain-based, Fat (4.5%)) were purchased from Custom Animal Diets, LLC

(Bangor, PA, USA). Mice fed with lithium-enriched food received an additional bottle of saline (0.9% NaCl) to prevent electrolyte imbalances resulting from lithium administration. To assess the effects of the treatment, and to monitor possible harmful effects, mice body weight was regularly measured, as well as food and liquids consumptions for the whole treatment duration.

3.3. Behavioral experiments

3.3.1. Lithium experiment protocol

Chronic lithium treatment began when mice reached the age of 8 weeks. WT and *Crtc1*$^{-/-}$ mice were randomly split in two subgroups: one was fed with lithium-enriched chow, and the other one was fed with control chow. Behavioral experiments started 2 weeks after the beginning of the treatment on day D70 (70 days of age) while continually treated until the end of the experiment. All behavioral tests were conducted in the dark phase of the inverted 12-hr light-dark cycle according to standard procedures. Behavioral experiments were carried out in the following order and at the following days: forced-swim (D70-D71), tail suspension (D73), social interaction (D77), and resident-intruder (D79). Mice were then sacrificed on day D81.

3.3.2. Forced-swim test

A two-day test (day 1 and day 2) was performed. Mice were put during 5min in a glass beaker (26cm tall x 18cm diameter) filled to a depth of 22cm with tap water (26 ± 0.5°C), in a room with a light intensity of ~35 lux. This depth was sufficient to ensure that mice could not escape or touch the floor of the container. Sessions were videotaped from above and manually analyzed with Ethovision 3.1 software (Noldus Information Technology, Wageningen, the Netherlands) for floating and climbing behavior. Mice were judged immobile when they remained floating passively in the water, except for minor movements to keep their heads above the water.

3.3.3. Tail suspension test

Mice were individually suspended by their tail on a horizontal metal rod at ~35cm height using medical adhesive tape placed approximately 2cm from the tip of the tail. Each assay was videotaped during 5min and immobility time was manually scored with Ethovision. Mice were judged to be immobile when they hung passively without moving.

3.3.4. Social interaction test

The experiment was conducted in a room with a low light intensity of ~25 lux, to avoid any stressful condition. A social interaction box was built with two different compartments (A and B, 38x29x21cm each), separated by a wooden wall with a small door allowing the mouse to freely access to the two zones. Each zone contains a small compartment (10x10x21cm) made of plastic walls with small holes, to allow the mice to smell and see the content of the plastic container, without permitting a direct interaction (**Figure 3.1**).

The test was separated in two phases: in the first phase (habituation, 5 min), the experimental mouse was allowed to explore the new environment during 5min, in order to assess whether the mouse has already a zone preference before beginning the test. Immediately after the habituation, the second phase started (test, 15min). An unfamiliar adult male mouse (CD-1 mice, Charles River Laboratories) was put in one of the plastic containers with a box on the top, to prevent it from climbing the wall, and an inanimate object was put in the other plastic container. Location of the stranger mouse and the inanimate object was randomly changed between each experimental mouse tested. Between each experimental mouse and before beginning the experiment, the social interaction chamber was washed with water and 70% ethanol.

A zone of ~6cm was defined around the periphery of each plastic container (interaction zones A and B). During the 15 min of the test, time spent and distance moved in each of the four zones (compartment A, compartment B, interaction zone A and interaction zone B) were

Figure 3.1 : Social Interaction Test set-up. A plastic box was speratаed by a wooden wall with a hole, creating two different zones (A and B, 38 x 29 x 21 cm each). Each zone contained a smaller plastic compartment (10 x 10 x 21 cm) with holes on the walls. Virtual zones of ~6cm (interaction zones A and B) were created around both of the smaller plastic compartments.

measured with Ethovision. A preference ration was then calculated as follows:

$$Preference = \frac{time\ or\ distance\ in\ mouse\ zone\ (test)}{total\ time\ or\ distance\ (test)} - \frac{time\ or\ distance\ in\ mouse\ zone\ (habituation)}{total\ time\ or\ distance\ (habituation)}$$

3.3.5. Resident-intruder test

Cages were changed for all the experimental mice (residents) 8 days prior to conducting the test and no more changes were done afterwards. On test day, an unfamiliar adult WT male mouse of the same genetic background (C57BL/6N, Charles River Laboratories) was placed in the resident male cage. The intruder mice were group-housed and matched with resident mice for weight (intruder mice always had ~2-3 g less than resident mice). The two mice were allowed to interact for 15min. Each intruder mouse was only used once. The interaction between the two mice was videotaped and scored manually with Ethovision for attack latency, frequency and duration.

3.3.6. Stress procedure

For the experiment on the effects of stress on CRTC1, male C57BL/6N mice of 3 and 6 weeks old were purchased (Charles River Laboratories) and group-housed. When mice reached the age of 5 weeks (adolescents) and 8 weeks (adults), they were submitted to a stress procedure. Mice underwent the first four days of the OSFS as previously described[337], i.e. a 15 min forced-swimming session during 4 consecutive days. 24h after the last swimming session, animals were sacrificed.

3.4. Molecular experiments

3.4.1. Animal sacrifices and brain samples

For the lithium experiments, animals were sacrificed two days after the last behavioral tests (at 81 days of age). Mice were sacrificed through cervical dislocation, decapitated and the brain was rapidly placed in a stainless steel adult mouse brain slicer matrix with 1 mm coronal section slice intervals. Six coronal slices were made from the second frontal slice channel of the matrix. Brain slices were placed on microscope slides and immediately frozen in dry ice, and then stored at -80°C. Medial PFC and dorsal HIP were collected with a micropunch (ø 1 mm, Stoeltling, Wood Dale, IL, USA) in corresponding brain slices. PFC and hippocampus samples were kept at -80°C.

For the experiment on the effects of stress on CRTC1, adolescent and adult mice were sacrificed 24h after the last swimming session. For this experiment, the samples were obtained as follows: mice were sacrificed through cervical dislocation and decapitated. The brain was rapidly placed in a stainless steel adult mouse brain slicer matrix with 1 mm coronal section slice intervals. A first cut included the PFC from which the olfactory bulbs and associated structures were removed. Total hippocampi were unrolled from the cortex. All the structures were quickly frozen in dry ice and stored at -80°C until further processing. Half of each sample was used for RNA extraction and the other half for protein extraction.

For the ELISA experiments, male WT and *Crtc1*$^{-/-}$ mice were sacrificed at 8 weeks of age. Brain micropunches were obtained with a similar procedure as for the lithium experiment. Micropunches of the hippocampus and striatum were collected on the corresponding slices and stored at -80°C.

For the qPCR experiment, male WT and *Crtc1*$^{-/-}$ mice were sacrificed at 8 weeks of age. PFC and hippocampus samples were obtained using the same procedure as for the stress experiment.

3.4.2. RNA extraction and RT-qPCR

RNA was extracted and purified from the PFC and the hippocampus using the RNeasy Plus Minikit (Qiagen) according to manufacturers' instructions. RNA concentrations were measured with the help of a NanoDrop Lite (Thermo Scientific, Wilmington, DE, USA). cDNA was obtained performing a 50µl reverse transcription reaction, using 100ng of RNA with Taqman RT Reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). A 10µl-mix containing 0.87µl of cDNA, 0.5µl of primers (F+R, 5µM each), 4.96µl of the SYBR Green PCR Master Mix (Applied Biosystems) and 3.67µl of water was added in each well of a 96-well plate, where the amplification was conducted using an ABI PRISM 7500 real-time PCR system (Applied Biosystems). The following RT-qPCR program was used: 2min at 50°C, 10min at 95°C, 45 cycles of 15s at 95°C, and 1min at 60°C. PCR reactions were run followed by a dissociation reaction to determine specificity of the amplified product. Relative gene expression was then quantified with the ΔΔCT method[339], using the housekeeping β-actin gene for normalization. All primers used for the various experiments, at a concentration of 250nM, are presented in **Table 3.1**.

3.4.3. Cytokines ELISA

Proteins were extracted from hippocampus and striatum. Samples were manually homogenized with a microtube pestle in RIPA buffer [50 mM Hepes (pH 7.6), 150 mM NaCl, 1 mM EDTA (pH 7.5), 2.5 mM EGTA (pH 8.0), 10% Glycerol, 1% NP-40, 1% Deoxycholate, 0.1% SDS, with a protease inhibitor cocktail (Sigma, St-Louis, MO, USA), and extracted for 20 min at 4°C. Protein quantification was done with the Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were then measured for protein levels of the cytokines TNFα, IL-1β, IL-6 and IFN-γ using corresponding enzyme-liked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA), according to manufacturer's instructions. Cytokine concentrations were normalized over total protein levels.

Gene	Forward Sequence	Reverse Sequence
ß-Actin	5'-GCTTCTTTGCAGCTCCTTCGT-3'	5'-ATATCGTCATCCATGGCGAAC-3'
Bdnf total	5'-AAAACCATAAGGACGCGGACTT-3'	5'-GAGGCTCCAAAGGCACTTGA-3'
Bdnf I *Bdnf II* *Bdnf III* *Bdnf IV* *Bdnf V*	5'-AAGTCACACCAAGTGGTGGGC-3' 5'-AAGCCGGTGTCGCCCTTA-3' 5'-TTGGAGGGCTCCTGCTTCTC-3' 5'-GTAAGAGTCTAGAACCTTGGGGACC-3' 5'-TGGGGCAGACGAGAAAGCG-3'	5'-GGATGGTCATCACTCTTCTCACCT-3'
Cart	5'-TTCCTGCAATTCTTTCCTCTTGA-3'	5'-GGGAATATGGGAACCGAAGGT-3'
Crtc1	5'-CAGGACTTGGGCCTGGAA-3'	5'-AGACAGACAAGACCCTTTCTAAGCA-3'
Dnmt1	5'-CCATTGGCCTGGAGATTAAG-'3	5'-GGCTCTGGGTGAGAGCACTA-'3
Dnmt3a	5'-GAGGGAACTGAGACCCAC-'3	5'-CTGGAAGGTGAGTCTTGGCA-'3
Dnmt3b	5'-GCCCATGCAATGATCTCTCT-'3	5'-CCAGAAGAATGGACGGTTGT-'3
Gad1	5'-CAGAAAACGCCCCCAGAAC-3'	5'-CGGGAGACCAAGTTTCATTTCC-3'
Gad2	5'-AAAATCCCTGGCTTCATTGAG-3'	5'-TTAGATCGGTATGCCAGGCG-3'
GR	5'-CGGTTTCAGAAGTGCCTAGC-3'	5'-TTGCCTGGAACCTGGAATAG-3'
IDO	5'-GCCCTGGGTTGGAGATCATAC-3'	5'-CATGCAGGGTAGAGTCATTCTC-3'
Npy	5'-TGCTTACTCTCTTTTCCCTTCC-'3	5'-CATCAGACCTGGTGCTTCA-'3
Nr4a1	5'-CACAGGTCACCCTCGATTTTT-'3	5'-ACCATCCAACGATCTCTCTCATC-'3
Nr4a2	5'-TCCGGCTTTTGGTCCTTCG-'3	5'-ATGCCGCCCGTGAACTTTT-'3
Nr4a3	5'-TGGCTCGACTCCATTAAAGAC-3'	5'-TGCATAGCTCCTCCACTCTCT-3'
Tet1	5'-GAGCCTGTTCCTCGATGTGG-'3	5'-CAAACCCACCTGAGGCTGTT-'3

Table 3.1 : Table of primers used.

3.5. Statistical analyses

Statistical analyses were performed using the Statistica 8.0 Software (StatSoft Inc., Tulsa, OK, USA). All data are presented as mean ± SEM. P-values of $p < 0.05$ were considered as statistically significant. A Shapiro-Wilk test was first performed to assess data normality. All results were found to follow normal distribution; therefore parametric tests could be used.

For the behavioral and molecular data of the lithium experiment, two-way ANOVAs (with genotype and treatment as independent variables) were performed, followed by a Fisher LSD post-hoc test. For the assessment of physiological effects of lithium treatment on mice, a two-way ANOVA with repeated measures was performed, as the weight, and the food and liquids consumptions were repeatedly measured throughout the entirety of the protocol. A Fisher LSD post-hoc test was carried out afterwards.

For the stress experiment, qPCR data were analyzed with a Student t-test, as only two groups were compared each time (stress vs. control).

For the qPCR and ELISA experiments, a Student t-test was performed, as only two groups were compared each time (WT vs. *Crtc1*$^{-/-}$).

4. RESULTS

4.1. Effects of lithium on *Crtc1*$^{-/-}$ mice

Note: All the data presented in this section were obtained and analyzed by a master student, Sara Dias, under my direct supervision. Figures were reworked by myself.

As presented in the introduction, *Crtc1*$^{-/-}$ mice present a certain amount of behavioral and molecular features of depressive but also bipolar disorders. As demonstrated by Breuillaud et al.[337], and in section 2.1, *Crtc1*$^{-/-}$ mice have a blunted response to classical antidepressants. We were therefore interested in studying the behavioral and molecular response of *Crtc1*$^{-/-}$ mice to a chronic lithium treatment. Male WT and *Crtc1*$^{-/-}$ mice were thus exposed to an oral

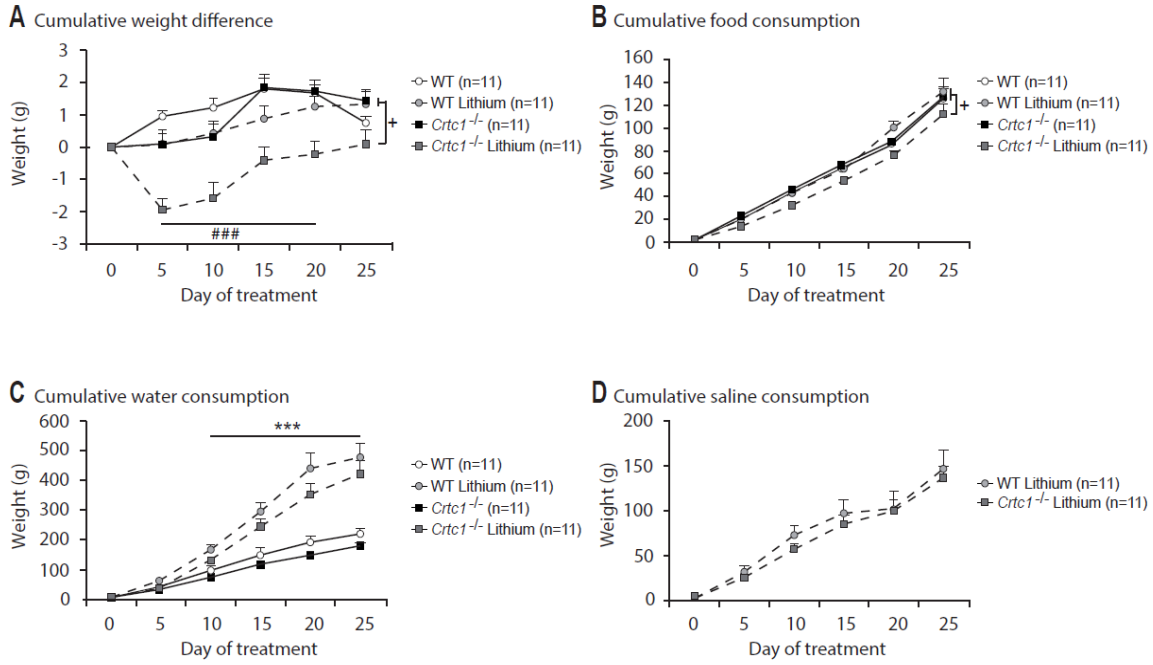

Figure 4.1: Physiological effects of lithium. (A) Cumulative weight difference. All groups of mice, except for lithium-treated *Crtc1*$^{-/-}$ mice, progressively gained weight throughout the procedure. Control WT mice displayed a slight weight loss on the last five days of treatment. Lithium-treated *Crtc1*$^{-/-}$ mice showed a significant weight loss during the five first days of treatment, but slowly regained weight afterwards, until reaching back to baseline after 20 days ($^{+++}p<0.001$, vs. themselves on day 0). At the end of the procedure, they had gained significantly less weight than control Crtc1$^{-/-}$ mice ($^{+}p<0.05$, vs. *Crtc1*$^{-/-}$ mice). (B) Cumulative food consumption. All groups of mice ate a similar amount of food throughout the procedure, except for lithium-treated *Crtc1*$^{-/-}$ mice which ate slightly less food. At the end of the treatment, they had eaten less food than control *Crtc1*$^{-/-}$ mice ($^{+}p<0.05$, vs. *Crtc1*$^{-/-}$ mice). (C) Cumulative water consumption. Control WT and *Crtc1*$^{-/-}$ mice drank a similar amount of water throughout the procedure. Both lithium-treated WT and *Crtc1*$^{-/-}$ mice drank significantly more water, starting from day 10 (*$p<0.001$, vs. WT mice) until the end of the treatment. (D) Cumulative saline consumption of lithium-treated WT and *Crtc1*$^{-/-}$ mice. Both groups drank a similar amount of saline solution during the entirety of the treatment.**

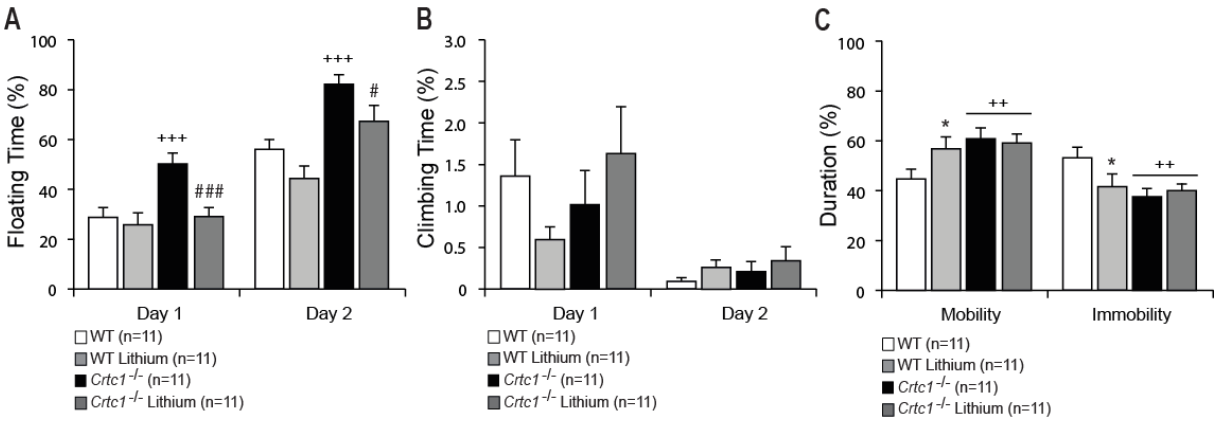

Figure 4.2: Effects of lithium on the behavioral despair of WT and *Crtc1*$^{-/-}$ mice. (A) Floating behavior in the FST. On both days of test, untreated *Crtc1*$^{-/-}$ mice displayed a significant increased immobility time ($^{+++}p<0.001$, vs. WT mice). Lithium significantly decreased the immobility time of *Crtc1*$^{-/-}$ mice ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, vs. *Crtc1*$^{-/-}$ mice), Lithium had no effect on the immobility time of WT mice. (B) Climbing behavior in the FST. No difference in the climbing time could be observed between the different groups. (C) Mobility and immobility in the TS. Both groups of *Crtc1*$^{-/-}$ mice displayed an increased mobility and a corresponding decreased immobility compared to WT mice ($^{++}p<0.01$, vs. WT mice). Lithium had no effect on the mobile and immobile behavior of *Crtc1*$^{-/-}$ mice, while it significantly increased the mobility (and thus decreased the immobility) of WT mice ($^{*}p<0.05$, vs. WT mice).

2-week lithium treatment, followed by several behavioral tests, under continuous treatment (see 3.3.1).

4.1.1. Physiological effects of lithium

Through the entirety of the treatment, mice weight was regularly measured, as well as food, water and saline consumption (**Figure 4.1**). Measure of mice cumulative weight difference (**Figure 4.1 A**) revealed that both WT groups and untreated *Crtc1*$^{-/-}$ mice presented a normal progressive weight gain, apart from control WT group which showed a slight weight decrease in the last five days of the treatment. Lithium-treated *Crtc1*$^{-/-}$ mice significantly lost weight during the five first days of the treatment (-1.94 g, $^{\#\#\#}p<0.001$). After that, mice progressively regained weight until the end of the treatment. However, they still presented a decreased weight as compared to control *Crtc1*$^{-/-}$ mice at the end of the experiment (-1.34g, $^{+}p<0.05$). Cumulative food consumption (**Figure 4.1 B**) was significantly lower for *Crtc1*$^{-/-}$ mice throughout the experiment ($^{+}p<0.05$), which correlated with their weight loss. Measure of cumulative water consumption (**Figure 4.1 C**) showed that both lithium-treated groups consumed significantly more water, after day 10 and until the end of the procedure, than the control groups ($^{***}p<0.001$). Saline consumption (**Figure 4.1 D**) did not differ between the two lithium-treated groups.

4.1.2. Behavioral effects of lithium

4.1.2.1. Behavioral despair

The behavioral effects of lithium were first assessed in the FST during two consecutive days. On day 1, untreated *Crtc1*$^{-/-}$ mice presented an increased immobility time compared to untreated WT mice (+23.7%, $^{+++}p<0.001$) (**Figure 4.2 A**). Lithium significantly decreased the immobility time of *Crtc1*$^{-/-}$ mice (-21.9%, $^{\#\#\#}p<0.001$). No effect of lithium could be seen on the behavioral despair of WT mice. On day 2, untreated *Crtc1*$^{-/-}$ mice spent again more time immobile than WT mice (+32.1%, $^{+++}p<0.001$) and this immobility was once more reduced by lithium (-14.8%, $^{\#}p<0.05$). No effect of lithium could be observed on WT mice on day 2. Regarding climbing behavior (**Figure 4.2 B**), no effect of genotype or treatment could be observed on both days of test.

WT and *Crtc1*$^{-/-}$ mice were next tested in the TS test (**Figure 4.2 C**). Lithium treatment significantly decreased the immobility time of WT mice (-11.7%, $^{*}p<0.05$), with a corresponding increase in mobility time. Both groups of *Crtc1*$^{-/-}$ mice presented a lower immobility time (and a corresponding higher mobility time) (-13.8%, $^{++}p<0.01$) than WT mice. Altogether, these results showed that lithium was able to reduce the behavioral despair of *Crtc1*$^{-/-}$ mice in the FST, but not in the TS. Surprisingly, in the latter paradigm, *Crtc1*$^{-/-}$ mice presented a decreased behavioral despair as compared to WT mice.

4.1.2.2. Social behavior

The effects of lithium were then assessed in the social interaction test. A social preference ratio was calculated (see 3.3.4) in order to measure the preference of the experimental mice

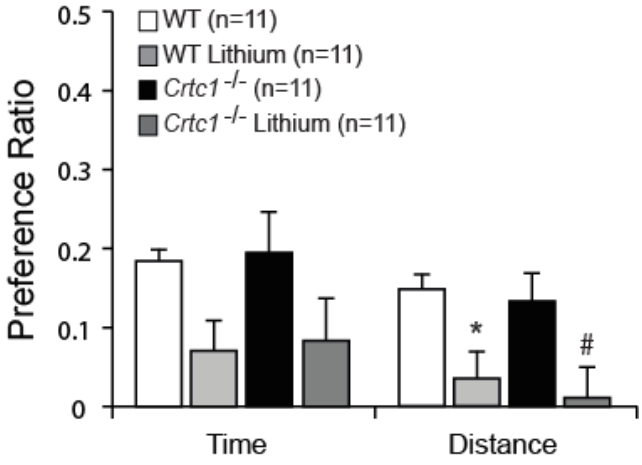

Figure 4.3: Effects of lithium in the social interaction test. The preference ratio is presented for both the time spent and the distance travelled in the mouse zone. No significant effect of genotype or treatment could be seen on the time parameter. Lithium has however a strong tendency to decrease the time spent in the mouse zone. Both lithium-treated WT and *Crtc1*$^{-/-}$ mice displayed a decreased preference ratio in the distance moved in the mouse zone ($^{*}p<0.05$, vs. WT mice, $^{\#}p<0.05$, vs. *Crtc1*$^{-/-}$ mice).

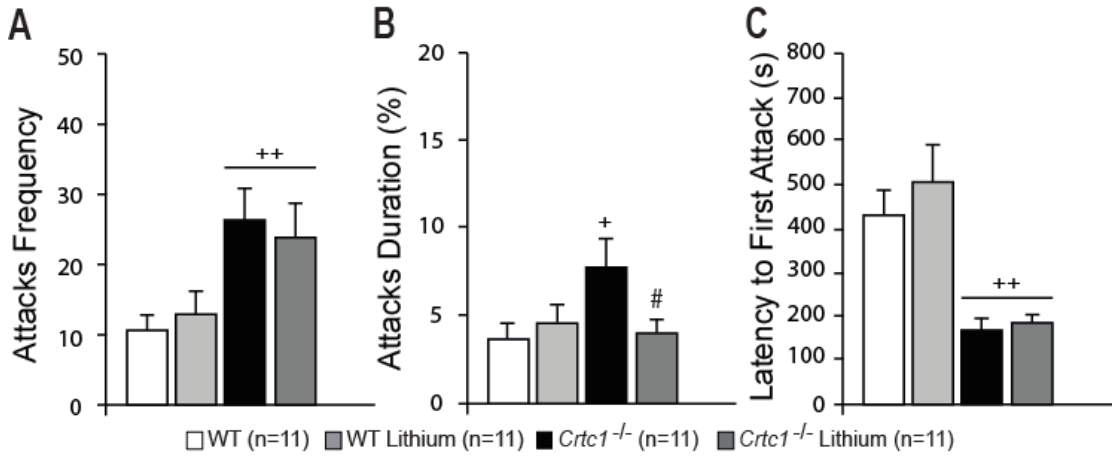

Figure 4.4: Effects of lithium on the aggressive behavior of *Crtc1*$^{-/-}$ mice. (A) Frequency of attacks. Both groups of *Crtc1*$^{-/-}$ mice attacked more frequently the intruder than the WT mice ($^{++}p<0.01$, vs. WT mice). Lithium had no effect on the frequency of attacks. (B) Duration of the attacks. Untreated *Crtc1*$^{-/-}$ mice attacked the intruder for a longer time than WT mice ($^{+}p<0.05$, vs. WT mice). Lithium significantly decreased the attacks duration of *Crtc1*$^{-/-}$ mice ($^{\#}p<0.05$, vs. *Crtc1*$^{-/-}$ mice) but had no effects on WT mice. (C) Latency to the first attack. Both groups of *Crtc1*$^{-/-}$ mice attacked the intruder significantly earlier than WT mice ($^{++}p<0.01$, vs. WT mice). Lithium had no effect on this parameter.

for the unknown mouse without any influence of some initial zone preferences in the habituation phase (data not shown). This ratio is presented in **Figure 4.3**. No significant effect of lithium could be seen on the time spent in the mouse zone. However, a strong tendency to a decreased time spent in this zone could be observed in the two lithium-treated groups. Regarding the distance travelled in the mouse zone, lithium significantly decreased this parameter in WT ($^{*}p<0.05$) and *Crtc1*$^{-/-}$ mice ($^{\#}p<0.05$) compared to their respective control groups. These results suggest that lithium might have a negative effect on the social behavior of both WT and *Crtc1*$^{-/-}$ mice.

4.1.2.3. Aggressive behavior

The effects of lithium on aggressive behavior were finally assessed in the resident intruder test (**Figure 4.4**). Both control and lithium-treated *Crtc1*$^{-/-}$ mice presented an increased frequency of attack, as compared to WT mice (+13.4 attacks, $^{++}p<0.01$), with no effects of lithium on this parameter (**Figure 4.4 A**). Regarding attack duration, untreated *Crtc1*$^{-/-}$ mice attacked the intruder for a significant longer time than WT mice (+4.1%, $^{+}p<0.05$). Lithium significantly reduced the attack duration of *Crtc1*$^{-/-}$ mice (-4.3%, $^{\#}p<0.05$), while showing no effect on the attacks duration of WT mice (**Figure 4.4 B**). Finally, both groups of *Crtc1*$^{-/-}$ mice presented a shorter latency to attack the intruder than WT mice (-99 s, $^{++}p<0.01$), and lithium

had no effect on this parameter (**Figure 4.4 C**). These results suggest that lithium is able to reduce the aggressiveness of *Crtc1*$^{-/-}$ mice by reducing the duration of the attacks, but has no effects on the frequency of the attacks or the latency to the first attack.

4.1.3. Molecular effects of lithium

After the behavioral assessments presented above, mice were sacrificed and gene expression analysis was performed in the hippocampus and PFC. The effects of lithium were assessed on several CREB-regulated genes that are presented below.

4.1.3.1. Crtc1

Crtc1 gene expression was measured in control and lithium-treated WT mice (**Figure 4.5**). No difference of *Crtc1* levels could be observed, therefore suggesting that lithium did not interfere with *Crtc1* gene regulation.

4.1.3.2. Bdnf and BdnfIV

The expression of *Bdnf* and its exon *BdnfIV* were next measured (**Figure 4.6**). In the hippocampus, no significant effect of genotype or treatment could be seen (**Figure 4.6 A**). However, lithium had a strong tendency to decrease *Bdnf* expression in WT mice, while having no effect on *BdnfIV*. Also, both groups of *Crtc1*$^{-/-}$ mice presented a non-significant decrease of *Bdnf* and *BdnfIV* expression compared to WT mice. In the PFC, control and lithium-treated *Crtc1*$^{-/-}$ mice presented significant lower levels of *Bdnf* and *BdnfIV* compared to WT mice (respectively -44%, $^{++}p<0.01$; -38%, $^{+}p<0.05$) (**Figure 4.6 B**). In WT mice, lithium significantly reduced the expression of *Bdnf* (-32%, $^{*}p<0.05$), but had no effect on *BdnfIV* expression. These results suggest that lithium decreased the expression of *Bdnf* in WT mice, but did not affect *Bdnf* expression in *Crtc1*$^{-/-}$ mice.

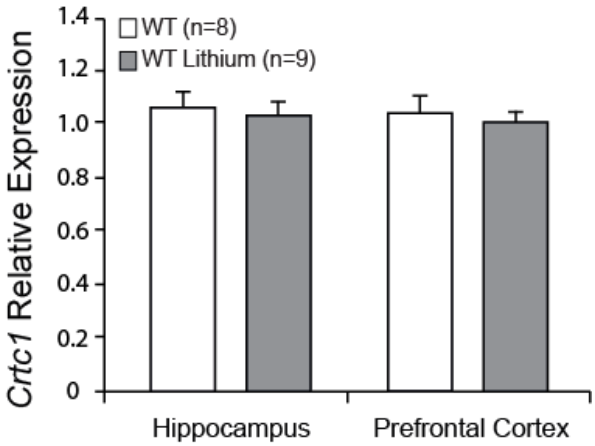

Figure 4.5: Effects of lithium on *Crtc1* expression. The expression of the Crtc1 gene was not influenced by lithium treatment in the hippocampus and PFC of WT mice.

4.1.3.3. Npy and Cart

The metabolism-related genes neuropeptide Y (NPY) and the cocaine and amphetamine-

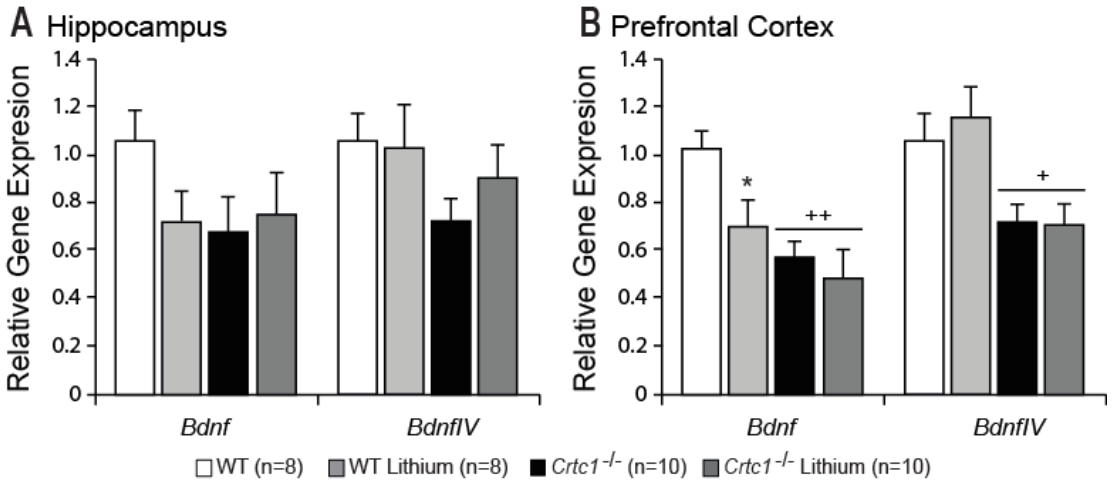

Figure 4.6: Effects of lithium on *Bdnf* and *BdnfIV* expression. (A) Gene expression of *Bdnf* and *BdnfIV* in the hippocampus. No significant effect of genotype or treatment could be seen in this structure. Lithium however had a tendency to decrease *Bdnf* expression in WT mice, and both groups of *Crtc1*$^{-/-}$ mice presented apparent lower levels of *Bdnf* and *BdnfIV*. (B) Gene expression of *Bdnf* and *BdnfIV* in the PFC. Both groups of *Crtc1*$^{-/-}$ mice displayed lower levels of *Bdnf* and *BdnfIV* mRNA ($^{+}p<0.05$, $^{++}p<0.01$, vs. WT mice) with no effect of lithium. In WT mice, lithium significantly decreased *Bdnf* expression in the PFC ($^{*}p<0.05$, vs. WT mice) but had no effect on *BdnfIV* expression.

regulated transcript (Cart) were then assessed, because of their involvement in mood disorders and their regulation by CREB$^{340–342}$ (**Figure 4.7**). *Npy* was observed downregulated in control *Crtc1*$^{-/-}$ mice in both the hippocampus and PFC (respectively -41%, $^{++}p<0.01$; -42%,

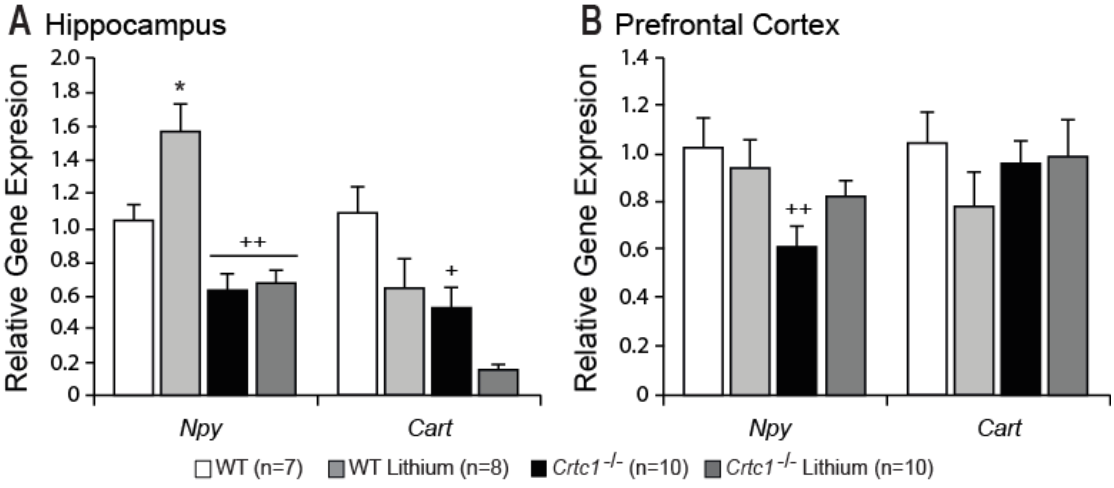

Figure 4.7: Effects of lithium on *Npy* and *Cart* expression. (A) Gene expression of *Npy* and *Cart* in the hippocampus. Both groups of *Crtc1*$^{-/-}$ mice presented a decreased expression of *Npy* ($^{++}p<0.05$, vs. WT mice) with no effect of lithium. Lithium significantly increased *Npy* expression of WT mice ($^{*}p<0.05$, vs. WT mice). *Cart* was downregulated in untreated *Crtc1*$^{-/-}$ mice ($^{+}p<0.05$, vs. WT mice). Lithium had a non-significant tendency to decrease *Cart* expression in both WT and *Crtc1*$^{-/-}$ mice. (B) Gene expression of *Npy* and *Cart* in the PFC. Untreated *Crtc1*$^{-/-}$ mice displayed decreased *Npy* expression ($^{++}p<0.01$, vs. WT mice). Lithium had no effect on *Npy* expression in WT mice, but showed a non-significant tendency to increased *Npy* mRNA in *Crtc1*$^{-/-}$ mice. No difference of *Cart* expression could be seen between the different groups.

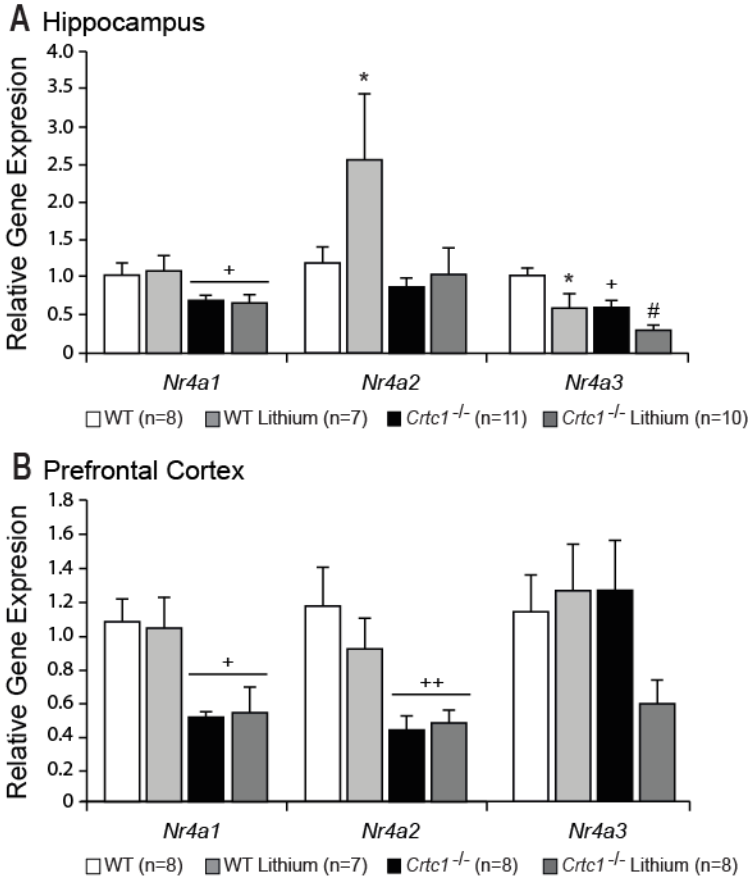

Figure 4.8: Effects of lithium on *Nr4a1-3* expression. (A) Gene expression of *Nr4a1-3* in the hippocampus. Both untreated and lithium-treated *Crtc1*$^{-/-}$ mice displayed lower levels of *Nr4a1* expression (^{+}p<0.05, vs. WT mice). Lithium had no effect on *Nr4a1* expression, but significantly increased *Nr4a2* levels in WT mice (*p<0.05, vs. WT mice). *Nr4a3* was downregulated in *Crtc1*$^{-/-}$ mice (^{+}p<0.05, vs. WT mice), and lithium significantly decreased *Nr4a3* levels in both WT (*p<0.05, vs. WT mice) and *Crtc1*$^{-/-}$ mice ($^{\#}p$<0.05, vs. *Crtc1*$^{-/-}$ mice). (B) Gene expression of *Nr4a1-3* in the PFC. Both groups of *Crtc1*$^{-/-}$ mice presented lower levels of *Nr4a1* and *Nr4a2* expression (^{+}p<0.05, ^{++}p<0.01, vs. WT mice). Lithium had no effect on any *Nr4a* mRNA levels in this structure.

^{++}p<0.01). Lithium significantly increased *Npy* expression in the hippocampus of WT mice (+51%, *p<0.05) but had no effect in the PFC (**Figure 4.7 A and B**). Lithium did not significantly affect *Npy* expression in *Crtc1*$^{-/-}$ mice, although it had a slight non-significant tendency to increase *Npy* in the PFC.

Regarding *Cart* expression, control *Crtc1*$^{-/-}$ mice presented lower *Cart* levels in the hippocampus, compared to WT mice (-52%, ^{+}p<0.05) (**Figure 4.7 A**). Lithium had no significant effects on *Cart*, but showed a strong tendency to decrease its expression in both genotypes. In the PFC, no genotype or treatment effects could be observed (**Figure 4.7 B**).

These data show that both *Npy* and *Cart* are downregulated in *Crtc1*$^{-/-}$ mice. Lithium increased *Npy* expression in the hippocampus of WT mice but was apparently ineffective in *Crtc1*$^{-/-}$ mice. On the opposite, lithium seemed to downregulate *Cart* expression in both WT and *Crtc1*$^{-/-}$ mice.

4.1.3.4. Nr4a1-3

Finally, the effects of lithium on *Nr4a1-3* were measured (**Figure 4.8**). In the hippocampus, lithium had no effect on *Nr4a1* expression, and both control and lithium-treated *Crtc1*$^{-/-}$ mice

presented lower *Nr4a1* levels (-36%, $^{+}p<0.05$) (**Figure 4.8 A**). Lithium strongly increased *Nr4a2* expression in WT mice (+116%, $*p<0.05$) but had no effect on *Crtc1*$^{-/-}$ mice. Lithium also significantly decreased the expression of *Nr4a3* in both WT (-42%, $*p<0.05$) and *Crtc1*$^{-/-}$ mice (-30%, $*p<0.05$). Untreated *Crtc1*$^{-/-}$ mice also presented lower basal *Nr4a3* levels (-41%, $^{+}p<0.05$). In the PFC, lithium did not have any effects on the expression of the three *Nr4as* (**Figure 4.8 B**). Both groups of *Crtc1*$^{-/-}$ mice exhibited lower levels of *Nr4a1* (-53%, $^{+}p<0.05$) and *Nr4a2* (-64%, $^{++}p<0.01$) as compared to control WT mice. These results confirmed the previously observed *Nr4as* downregulations observed in *Crtc1*$^{-/-}$ mice. Lithium also seemed to have differential effects on *Nr4a2* and *Nr4a3* in both genotypes.

4.2. Effects of stress on *Crtc1* and *Bdnf* expression in adult and adolescent mice

To further investigate the role of CRTC1 in mood disorders etiology, we were interested in studying the regulation of *Crtc1* gene in response to stress. We also investigated the effect of

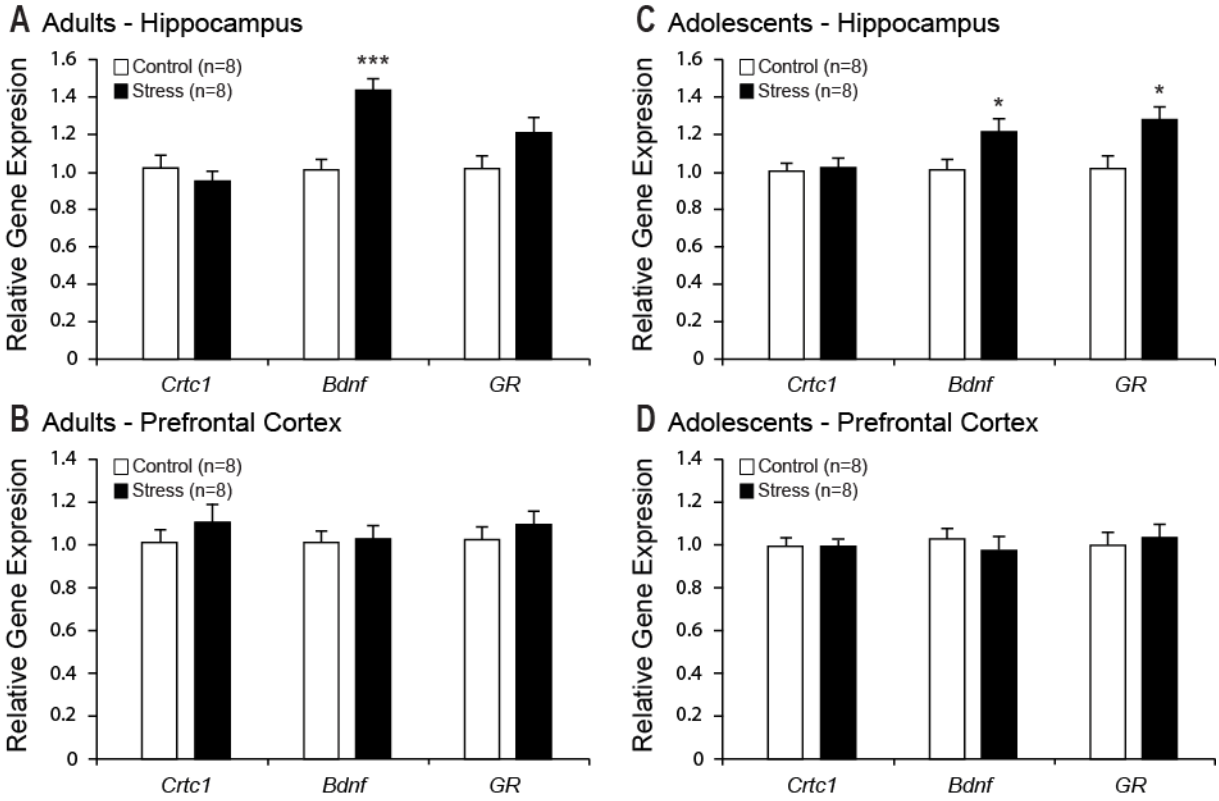

Figure 4.9: Effects of stress on *Crtc1*, *Bdnf* and *GR* expression in adult and adolescent mice. (A) Hippocampus of adult mice. No effect of stress could be seen on *Crtc1* and *GR* expression, while it induced a significant increase in *Bdnf* expression (*$p<0.001$, vs. control mice). (B) PFC of adult mice. Stress had no effect on the expression of *Crtc1*, *Bdnf* or *GR*. (C) Hippocampus of adolescent mice. Stress had no effect on *Crtc1* mRNA levels, but significantly upregulated the expression of *Bdnf* and *GR* ($*p<0.05$, vs. control mice). (D) PFC of adolescent mice. Stress had no influence on *Crtc1*, *Bdnf* and *GR* gene expression.**

stress on *Bdnf* and its different exons, as well as the regulation of the glucocorticoid receptor gene (*GR*). We were also interested in knowing if the regulation of these various genes might be different depending on the brain development. In this pilot study, male adult and adolescent WT mice underwent 4 consecutive days of swimming sessions, corresponding to the first part of the OSFS protocol. Gene expression analysis was performed afterwards in the hippocampus and prefrontal cortex.

4.2.1.1. Effects of stress on Crtc1, Bdnf and GR expression

We first assessed the effects of the stress procedure on the expression of *Crtc1*, *Bdnf* and *GR* (**Figure 4.9**). A significant increase of *Bdnf* expression was observed in the hippocampus of adult stressed mice (+43%, ***p<0.001), while *Crtc1* and *GR* levels were unchanged (**Figure 4.9 A**). Stress did not affect *Crtc1*, *Bdnf* or *GR* expression in the PFC of adult mice. In juvenile animals, stress increased the expression of *Bdnf* (+21%, *p<0.05) and *GR* (+27%, *p<0.05) in the hippocampus, but had no effect on *Crtc1* levels (**Figure 4.9 C**). In the PFC, *Crtc1*, *Bdnf* or *GR* expression was unchanged (**Figure 4.9 D**). These results suggest that the stress protocol

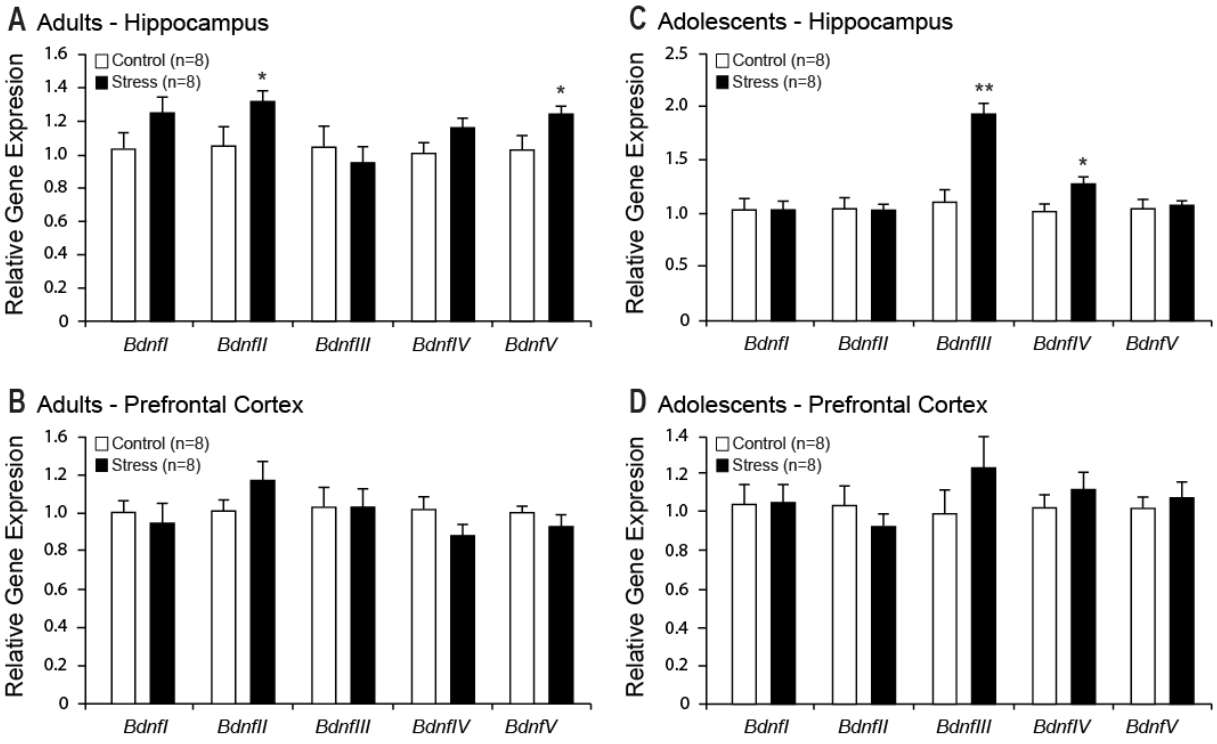

Figure 4.10: Effects of stress on the *BdnfI-V* exons. (A) Hippocampus of adult mice. Stress significantly upregulated the exons *II* and *V* of *Bdnf* (*p<0.05, vs. WT mice) and had no effect on the other exons. (B) PFC of adult mice. Stress didn't affect any *Bdnf* exon. (C) Hippocampus of adolescent mice. Stress significantly upregulated exons *III* and *IV* of Bdnf (p<0.01, *p<0.05, vs. WT mice) and had no effect on the other exons. (D) PFC of adolescent mice. No *Bdnf* exon was affected by stress in this structure.**

used had no influence on *Crtc1* gene regulation, but induced an increase in *Bdnf* expression in both adult and adolescent mice. Stress also induced the expression of *GR*, but only in juvenile animals.

4.2.1.2. Effects of stress on BdnfI-V exons

The effects of stress in the different *Bdnf* exons I to V were measured to better understand the stress-induced increase in total *Bdnf* (**Figure 4.10**). In adult mice, stress upregulated *BdnfII* and *BdnfV* in the hippocampus by stress (respectively +31%, **p*<0.05; +27%, **p*<0.05), while the expression of the other exons was unchanged (**Figure 4.10 A**). In the PFC, stress had no significant effect on the different *Bdnf* exons could be observed (**Figure 4.10 B**). In adolescent mice, *BdnfIII* and *BdnfIV* were upregulated by stress in the hippocampus (respectively +84%, ***p*<0.01; +25%, **p*<0.05), with no other changes observed (**Figure 4.10 C**). In the PFCstress did not have any effect on the various *Bdnf* exons (**Figure 4.10 D**). These data suggest that the stress-induced total *Bdnf* upregulation is due to a differential regulation of its various exons, depending on the development stage of the animal.

4.3. Future investigation lines in *Crtc1*$^{-/-}$ mice

Because of the pleiotropy of CREB, and possibly of CRTC1, and because of the numerous behavioral alterations observed in *Crtc1*$^{-/-}$ mice as well as the fact that these latter are complete knock-out animals, we hypothesize that many physiological processes might be altered in these mice. Furthermore, each of these processes might participate in the establishment of their mood disorder-like phenotype, as these diseases are multifactorial. We therefore performed several pilot studies, investigating different physiological systems of these mice, in order to determine several investigation lines for future studies in these mice.

4.3.1. Epigenetic system of *Crtc1*$^{-/-}$ mice

Because of the involvement of epigenetic mechanisms in stress response and in mood disorders, we were interested in studying possible alterations of the expression of epigenetic enzymes in *Crtc1*$^{-/-}$ mice. As observed in our article presented in section 2.1, we observed no

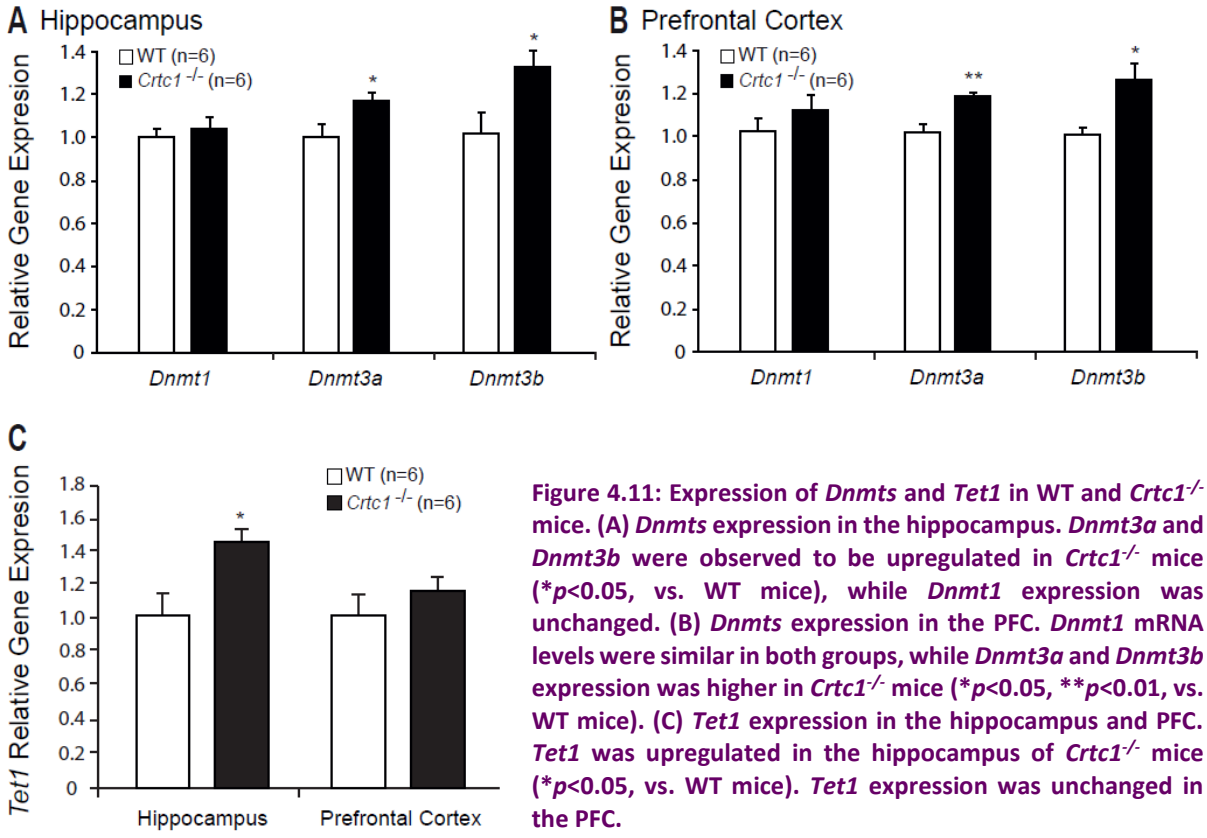

Figure 4.11: Expression of *Dnmts* and *Tet1* in WT and *Crtc1*$^{-/-}$ mice. (A) *Dnmts* expression in the hippocampus. *Dnmt3a* and *Dnmt3b* were observed to be upregulated in *Crtc1*$^{-/-}$ mice (p*<0.05, vs. WT mice), while *Dnmt1* expression was unchanged. (B) *Dnmts* expression in the PFC. *Dnmt1* mRNA levels were similar in both groups, while *Dnmt3a* and *Dnmt3b* expression was higher in *Crtc1*$^{-/-}$ mice (**p*<0.05, ***p*<0.01, vs. WT mice). (C) *Tet1* expression in the hippocampus and PFC. *Tet1* was upregulated in the hippocampus of *Crtc1*$^{-/-}$ mice (**p*<0.05, vs. WT mice). *Tet1* expression was unchanged in the PFC.**

major difference in *Hdacs* expression in *Crtc1*$^{-/-}$ mice. In this study we performed gene expression analysis of the different DNA methyltransferases (*Dnmts*) and of the tet-methylcytosine-dioxygenase (*Tet1*) (**Figure 4.11**).

The expression of *Dnmt1*, *Dnmt3a* and *Dnmt3b* was assessed in the hippocampus and PFC of WT and *Crtc1*$^{-/-}$ mice. In the hippocampus, we observed no difference in expression levels of *Dnmt1*, but *Dnmt3a* and *Dnmt3b* were upregulated in *Crtc1*$^{-/-}$ mice (respectively +16%, **p*<0.05; +31%, **p*<0.05) (**Figure 4.11 A**). Similar observations were made in the PFC, where *Dnmt1* expression was unchanged, while *Dnmt3a* and *Dnmt3b* expression were increased in *Crtc1*$^{-/-}$ mice (respectively +17%, ***p*<0.001; +24%, **p*<0.05) (**Figure 4.11 B**). Regarding *Tet1* expression, *Crtc1*$^{-/-}$ mice displayed higher levels of *Tet1* mRNA than WT mice in the hippocampus (+43%, **p*<0.05). *Tet1* expression in the PFC was similar in both groups (**Figure 4.11 C**). These results suggest a possible alteration of epigenetic regulation in *Crtc1*$^{-/-}$ mice, particularly concerning DNA methylation.

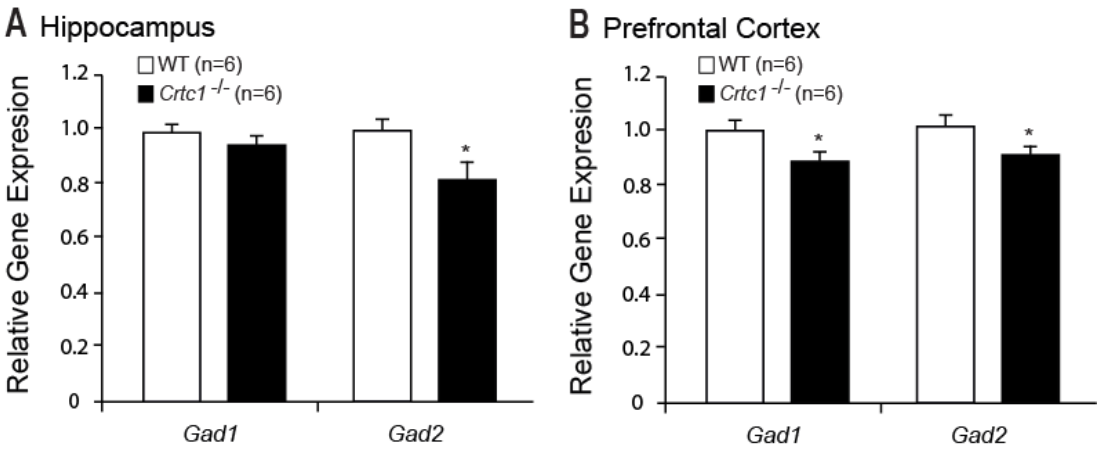

Figure 4.12: Expression of *Gad1* and *Gad2* in WT and *Crtc1*$^{-/-}$ mice. (A) *Gad1* and *Gad2* expression in the hippocampus. *Gad1* expression was similar in both groups of mice, while *Gad2* was downregulated in *Crtc1*$^{-/-}$ mice (p*<0.05, vs. WT mice). (B) *Gad1* and *Gad2* expression in the PFC. Both *Gad1* and *Gad2* were downregulated in the PFC of *Crtc1*$^{-/-}$ mice (*py0.05, vs. WT mice).**

4.3.2. GABAergic system

Dysregulation of the two isoforms of the glutamate decarboxylase (GAD), GAD67 and GAD65, which catalyze the decarboxylation of glutamate into GABA, have been associated with several psychiatric disorders. Dysregulation of GAD67 has been particularly associated with BD[343,344]. To determine whether *Crtc1*$^{-/-}$ mice might have similar alterations of the GABAergic system, we measured the expression of *Gad1* and *Gad2* (respectively encoding for GAD67 and GAD65) in the hippocampus and PFC of WT and *Crtc1*$^{-/-}$ mice (**Figure 4.12**).

In the hippocampus, we observed no difference of *Gad1* expression, whereas *Gad2* was downregulated in *Crtc1*$^{-/-}$ mice (-18%, **p*<0.05) (**Figure 4.12 A**). In the PFC, both *Gad1* and *Gad2* were downregulated in *Crtc1*$^{-/-}$ mice (respectively -12%, **p*<0.05, -10%, **p*<0.05) (**Figure 4.11 B**). This suggests that *Crtc1*$^{-/-}$ mice might present impairments of their GABAergic system.

4.3.3. Inflammatory system

Several recent findings have highlighted the role of the immune inflammatory system in mood disorders. Several pro-inflammatory cytokines have been found to be involved in MDD etiology, but also in synaptic plasticity regulation[345,346]. The tryptophan-degrading enzyme indoleamine-2,3- dioxygenase (IDO) has been found to be a key enzyme for the development of depressive symptoms induced by pro-inflammatory cytokines[347]. Here, we measured the levels of several cytokines, as well as *IDO* gene expression in WT and *Crtc1*$^{-/-}$ mice, in order to investigate whether these latter might present alterations of their inflammatory system.

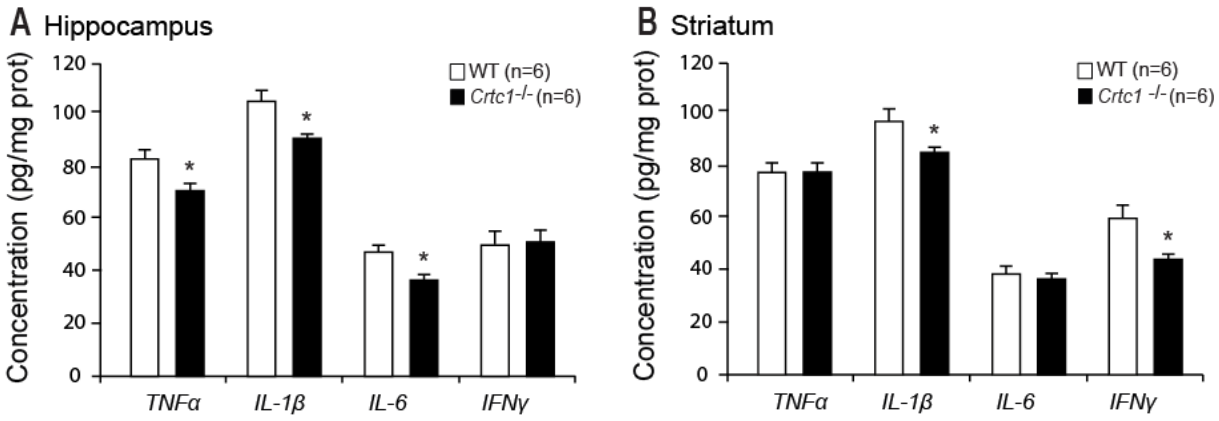

Figure 4.13: Cytokines measurements in WT and *Crtc1*$^{-/-}$ mice. (A) Cytokines concentrations in the hippocampus. *Crtc1*$^{-/-}$ mice displayed decreased levels of the cytokines TNFα, IL-1β and IL-6 in this structure (p*<0.05, vs. WT mice). IFNγ levels were unchanged. (B) Cytokines concentration in the striatum. Similar levels of TNFα and IL-6 were observed, while *Crtc1*$^{-/-}$ mice presented lower concentrations of IL-1β and IFNγ than WT mice (**p*<0.05, vs. WT).**

4.3.3.1. Cytokines measurements

Hippocampus and striatum concentration of the pro-inflammatory cytokines TNFα, interleukine-1β (IL-1β), interleukine-6 (IL-6) and interferon γ (IFNγ) were measured by ELISA assay in male WT and *Crtc1*$^{-/-}$ mice (**Figure 4.13**). In the hippocampus, *Crtc1*$^{-/-}$ mice presented lower levels of TNFα (-15%, **p*<0.05), IL-1β (-14%, **p*<0.05) and IL-6 (-22%, **p*<0.05) (**Figure 4.13 A**). In the striatum, *Crtc1*$^{-/-}$ mice displayed lower levels of IL-1β (-12%, **p*<0.05) and TNFγ (-26%, **p*<0.05) than WT mice (**Figure 4.13 B**). No difference in levels of TNFα and Il-6 were observed.

4.3.3.2. IDO gene expression

Gene expression of *IDO* was assessed in the hippocampus and PFC of male WT and *Crtc1*$^{-/-}$ mice (**Figure 4.14**). *IDO* was upregulated in the hippocampus of *Crtc1*$^{-/-}$ mice (+32%, **p*<0.05). No difference of *IDO* expression between the two genotypes was observed in the PFC.

Altogether, these preliminary data suggest a possible alteration of the inflammatory system of *Crtc1*$^{-/-}$ mice, as they display lower levels of several cytokines, as well as an increased expression of *IDO*.

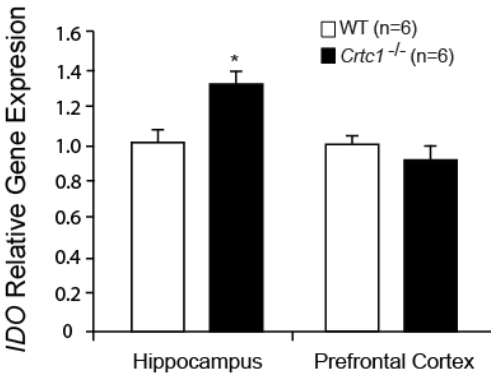

Figure 4.14: Expression of *IDO* in WT and *Crtc1*$^{-/-}$ mice. In the hippocampus, *Crtc1*$^{-/-}$ mice displayed an upregulation of *IDO* mRNA (p*<0.05, vs. WT mice). *IDO* levels in the PFC were similar in both genotypes.**

5. DISCUSSION

The principal aim of the present thesis was to provide a further and deeper characterization of the role of CRTC1 in the pathophysiology of mood disorders. More specifically, the importance of CRTC1 in CREB-related gene regulation was particularly investigated in order to unravel the importance of such regulation in the etiology of mood disorders. Targeted characterization of behavioral and molecular response of *Crtc1*$^{-/-}$ mice to several drug treatments was used and has allowed us to identify several etiologic and therapeutic mechanisms in which CRTC1 plays a critical role.

5.1. The effects of desipramine and SAHA on *Crtc1*$^{-/-}$ mice

As previously demonstrated by our group, *Crtc1*$^{-/-}$ mice present a blunted response to the classical antidepressant fluoxetine[337]. While the behavioral and molecular phenotype characterization of *Crtc1*$^{-/-}$ mice highlighted a role for CRTC1 in mood disorder etiology, this particular feature also suggested its involvement in the therapeutic response to antidepressants. We further investigated this finding by first assessing the effect of another classical antidepressive drug, the tricyclic antidepressant desipramine (article presented in section 2.1).

As for fluoxetine, *Crtc1*$^{-/-}$ mice were resistant to the effects of desipramine in the OSFS protocol, but responded to this antidepressant in the NIH paradigm. These results suggest that for both serotoninergic- and adrenergic-targeting drugs, CRTC1 is required for a proper behavioral response to their antidepressant effects. On the other hand, CRTC1 is not required for the anxiolytic effects of these two drugs in the NIH test, or it could suggest that these effects are not solely mediated by CRTC1-dependant pathways.

Gene expression analysis of *Bdnf* and *Nr4as* allowed us to better characterize the molecular mechanisms underlying the behavioral response of *Crtc1*$^{-/-}$ mice to desipramine. Indeed, this treatment induced an increased expression of *Bdnf,* its exon *IV*, *Nr4a1* and *Nr4a2* in WT mice, but failed to do so in *Crtc1*$^{-/-}$ mice. These interesting results imply that CRTC1 is required for the desipramine-induced expression of these genes, and that such induction might be regulating the behavioral response. In the light of these results, we hypothesized that the restoration of the expression of the above-cited genes might potentially rescue the phenotype of *Crtc1*$^{-/-}$ mice.

We therefore decided to assess the effects of the HDAC inhibitor SAHA in *Crtc1*$^{-/-}$ mice. This treatment was chosen because of the strong involvement of epigenetic mechanisms in mood disorders[348], and because HDAC inhibitors have been shown to induce *Bdnf* and *Nr4as* expression[116,143,349], as well as to regulate several patterns of genes similarly as antidepressants[142,350]. Behavioral experiments showed that *Crtc1*$^{-/-}$ mice responded to SAHA in the OSFS protocol, as their immobility was progressively decreased by the treatment. However, they never reached back the level of WT mice, thus suggesting only a partial effect of SAHA. In the NIH paradigm however, SAHA seemed inefficient on mutant animals, yet WT animals presented an abnormally high anxious behavior in this test, thus these results should be interpreted cautiously. Gene expression analysis revealed that SAHA was able to restore normal *Bdnf* and *BdnfIV* expression in the PFC of *Crtc1*$^{-/-}$ mice, but was unable to restore *Nr4a*s expression in these animals.

The partial antidepressant effect of SAHA on *Crtc1*$^{-/-}$ mice might be explained by its ability to restore normal *Bdnf* levels in the PFC. Indeed, *Bdnf* expression and levels, particularly in the PFC, have been negatively correlated with immobility time in the FST[351,352], thus suggesting a key role in behavioral despair regulation. We could therefore hypothesize that SAHA was able to reduce *Crtc1*$^{-/-}$ mice immobility in the OSFS through its restoration of normal *Bdnf* levels in these animals. However, SAHA could not restore normal *Nr4a1-3* levels in mutant animals, while it strongly upregulated all *Nr4as* in WT mice. This corroborates previous findings showing that HDAC inhibitors could increase *Nr4a1-3* expression, via CREB-CBP, a crucial mechanism for memory consolidation. The present results suggest that CRTC1 is also required for HDAC-induced *Nr4a1-3* upregulation (which makes sense, as CRTC1 helps recruiting CBP). This would be in line with the recent findings about the involvement of CRTC1 in cognitive behaviors and in spatial memory[244,353].

The inability of SAHA to induce *Nr4a1-3* expression in *Crtc1*$^{-/-}$ mice might be a cause of their incomplete behavioral response to this drug in the OSFS. Indeed, as previously explained, *Nr4as* have been tightly associated with mood disorders and *Bdnf* regulation[114,125]. There has been however very few studies about the possible regulation of behavioral despair by *Nr4as*. One study shows a negative correlation between floating behavior and *Nr4a3* levels in the amygdala, suggesting a possible regulation of this behavior by these genes[354]. Therefore, the lack of *Nr4a1-3* induction by SAHA in *Crtc1*$^{-/-}$ mice might be a possible reason for their

incomplete behavioral response. Another possibility could be an abolished *Nr4as*-induced *Bdnf* expression. Indeed, *Nr4a2* is known to regulate *Bdnf* expression, and it can also specifically bind its promoter *IV*, a mechanism involved in neuroprotection[355]. One could therefore imagine that in the absence of SAHA-induced *Nr4as* upregulation, an additional *Bdnf* increase is abolished, thus preventing total behavioral response. Therefore, reversal of behavioral despair would be the results of several CRTC1-dependent and independent pathways converging to an overall increase of *Bdnf* expression.

It is nevertheless quite obvious that *Bdnf* and *Nr4as* are not the only genes involved in behavioral response to antidepressants. It is indeed more plausible that several different pathways and molecules regulate this complex behavior. Furthermore, differential regulation of such pathways could occur depending on the brain structure. Indeed, we mainly focused here on the hippocampus and PFC as these are the main structures involved in mood disorders, but it would be interesting to study other regions. As explained above, *Nr4a3* regulation in the amygdala contributes to behavioral despair. Moreover, BDNF and CREB have pro-depressive effects when upregulated in the NAc. Regarding this statement, a very recent study has demonstrated that BDNF antidepressant effects greatly depended on TrkB levels in different cell types in the NAc, and that BDNF-TrkB effects were not always mediated by CREB[356]. The blunted or incomplete response of *Crtc1*$^{-/-}$ mice to the various treatments presented above could also be caused by many other features than a dysregulation of *Bdnf* or *Nr4as*.

One of them could be the fact that these animals are complete knock-out animals. Indeed, CREB, BDNF and CRTC1 are all involved in processes such as neuronal growth, and CREB has been known for a long time to be involved in brain development, neuronal differentiation, as well as neurogenesis in adults[357–359]. Hence, complete CRTC1 deletion could have a great impact on brain development and thus, some endophenotypes of *Crtc1*$^{-/-}$ mice could be due not only to a direct effect of the lack of CRTC1 (i.e. genes dysregulation), but also to the results of an altered development. Consequently, it is then possible that some behavioral and molecular features of these mice are only partially reversible, and even irreversible.

In the SAHA experiment, we also observed a peculiar result concerning *Bdnf* expression in *Crtc1*$^{-/-}$ mice. Indeed, we observed an upregulation of *Bdnf* in the hippocampus of mutant mice, instead of the usual downregulation displayed by these animals. Our hypothesis is that

the daily injections applied during several weeks have provoked a considerable stress on these animals, and thus has led to an abnormal stress-induced *Bdnf* regulation. We investigated this hypothesis in a pilot experiment that will be discussed in a future section (see 5.4).

To summarize, our findings have shown that CRTC1 is necessary for a proper behavioral and molecular response to classical antidepressants, probably because of its requirement for the induction of *Bdnf* and *Nr4as* expression. By acting on the epigenome of these mice, we were able to partially restore normal behavioral despair in these mice, paralleled by *Bdnf* levels restoration in the PFC. Complete gene expression rescue was however not possible and suggests the involvement of other mechanisms. To further characterize the importance of CRTC1 in the response to antidepressants, a region-specific rescue of *Crtc1* should be done. Indeed, by restoring *Crtc1* expression in *Crtc1*$^{-/-}$ mice in a specific area, we would be able to determine in which region(s) CRTC1 is mainly required for a normal behavior as well as for proper response to antidepressants. Furthermore, this experiment could also be performed at several developmental stages, and thus would help to determine the importance of CRTC1 expression in brain development, and its consequences in adulthood.

5.2. The agmatinergic system of *Crtc1*$^{-/-}$ mice

Our second article, presented in section 2.2, aimed at characterizing the involvement of the agmatinergic system in the depressive-like phenotype of *Crtc1*$^{-/-}$ mice. Genome-wide microarray analysis performed in the motor cortex of female mice revealed a considerable increase of *Agm* mRNA in *Crtc1*$^{-/-}$ animals. This upregulation was confirmed at both gene and protein levels specifically in the PFC and was also observed in the hippocampus, in both male and female mutant mice. Characterization and counting of Agm-expressing cells showed that mutant mice exhibited an increased number of such cells, and that these were mainly, but not only, interneurons. Agm was mainly co-localized with parvalbumin (PV)- and somatostatin (Sst)- interneurons, with very few colocalization with calretinin (CR)-interneurons.

The fact that *Crtc1*$^{-/-}$ mice presented increased levels of Agm led us to hypothesize that they might have decreased levels of agmatine, as well as an overall dysregulated agmatinergic system. This is in line with the current knowledge that agmatine has strong neuroprotective and antidepressant effects. We therefore assumed that this agmatine impairment might be contributing to their depressive-like phenotype, and that by acting on this system, we might

be able to restore a normal phenotype. Male and female WT and *Crtc1*$^{-/-}$ mice were thus acutely treated with agmatine and tested in the FST. We found that agmatine was able to reverse the increased behavioral despair displayed by *Crtc1*$^{-/-}$ mice in this paradigm. These results strongly suggest that the impaired agmatinergic system of CRTC1-deficient animals contributes to their phenotype. In a final experiment, we were interested in investigating the molecular mechanisms underlying the fast-acting antidepressant effects of agmatine. Because of its rapid effect (30 min) and its ability to block NMDAR, we hypothesized that it might act in a similar way as ketamine. We therefore measured peEF2 and BDNF protein levels, and indeed observed that agmatine induced a dephosphorylation of eEF2 in the PFC of WT mice, but not in *Crtc1*$^{-/-}$ mice. Interestingly, agmatine also induced an increase in BDNF translation in the PFC, but only in female WT mice. It had no effect on BDNF levels in male WT mice and in mutant animals. These latter also displayed lower basal levels of both BDNF and peEF2.

This study highlighted for the first time a link between the CREB-CRTC1 pathway and the agmatinergic system. As *Crtc1*$^{-/-}$ mice present a strong increase in Agm levels and Agm-expressing cells, it suggests that CREB-CRTC1 might be able to impact on agmatine turnover. It is possible that Agm gene expression is directly regulated by this pathway, as well as possibly the other enzymes involved in agmatine regulation. But it could also be the result of an indirect effect of CRTC1 deletion. Indeed, we have shown that Agm-expressing cells were present in higher number in *Crtc1*$^{-/-}$ mice, and that these cells were mainly interneurons. It could thus be possible that the CREB-CRTC1 pathway is involved in the generation and differentiation of such neurons, and hence, that the lack of CRTC1 might impair the development of these cells, therefore being present in an increased number. The higher levels of Agm observed in these mice would then be a consequence of a dysregulation of GABAergic interneurons development.

This would be possible because, as explained above, *Crtc1*$^{-/-}$ mice are complete knock-out animals and thus, CRTC1 deficiency during brain development might affect the overall neuronal circuitry. Furthermore, there has been evidence of the involvement of CREB, but also BDNF in the development of GABAergic interneurons in several regions of the brain[360–363]. To investigate a possible impairment of this system, a complete characterization of the various GABAergic interneuron subpopulations should be performed in *Crtc1*$^{-/-}$ mice. This has led us

to perform a pilot study in that direction, which will be discussed in a later section (see section 5.5.2).

We hypothesized that the increased Agm levels observed in *Crtc1*$^{-/-}$ mice might lead to decreased agmatine levels. This hypothesis is also supported by the fact that acute agmatine injections could rescue their depressive-like phenotype, by compensating this agmatine decrease. However, precise agmatine measurements in the brain should be performed in male and female *Crtc1*$^{-/-}$ mice, in order to confirm this hypothesis. In addition, measurement of levels of the other enzymes involved in agmatine metabolism (e.g. ADC) should be performed, in order to fully characterize the agmatinergic system of *Crtc1*$^{-/-}$ mice.

Investigation of the molecular mechanisms underlying the antidepressant effects of agmatine revealed that it might be a fast-acting antidepressant, through NMDAR inhibition. Indeed, we have showed that agmatine was able to induce the activation of the mTOR pathway, as highlighted by dephosphorylation of eEF2. This was paralleled by an increase in BDNF translation, and this precise mechanism has been shown to be critical for ketamine and other NMDAR antagonists antidepressant effect[276,289]. In addition, these effects were only seen in the PFC, which is in line with a recent study showing that ketamine's effects were mediated by the infralimbic PFC[300].

However, the effects of agmatine on BDNF and eEF2 were only present in WT animal, and the effects on BDNF were only observed in females, thus suggesting other mechanisms mediating the behavioral response to agmatine. Furthermore, *Crtc1*$^{-/-}$ mice interestingly displayed basal lower levels of phospho-eEF2, which suggests a dysregulation of this pathway in these animals. Yet it is also possible that agmatine fails to induce visible BDNF increase in *Crtc1*$^{-/-}$ mice, since these animals present decreased basal BDNF mRNA and protein levels. Decreased eEF2 phosphorylation might then be a possible compensatory mechanism in an attempt to maximize protein translation. Also, the differential regulation of BDNF in WT male and female mice is interesting, but its reasons remain unclear. Yet, it has already been observed that BDNF has different effects in male and female mice, and *Bdnf* heterozygous mice have different phenotype depending on the gender[364,365]. Moreover, there has been compelling evidence that BDNF, as well as other neuroplasticity mediators, can be regulated by female hormones, such as estradiol and progesterone, and by the estrus cycle[366–372].

Nevertheless, activation of eEF2-dependent translation by agmatine is apparently not necessary for its behavioral effect, as the latter was present in both genotypes and both genders. This implies that other pathways influencing behavior can be activated by agmatine. This is in line with the fact that antidepressant effects of agmatine require the activation of several of its targets, as explained in the introduction[183,186–188].

While we have provided evidence that the agmatinergic system is involved in the depressive-like phenotype of *Crtc1*$^{-/-}$ mice, it is however not yet possible to precisely determine what this contribution is. As explained before, agmatine can act on many systems; therefore, dysregulation of agmatinergic signaling could have a severe general impact. Indeed, agmatine also activates imidazoline and α_2-adrenergic receptors, which have been both involved in mood disorders. Imidazoline receptor I_2 ligands can inhibit monoamine oxidase A (MAO-A), thus regulating monoamines levels, and also produce antidepressant effects on rats in the FST[189,190]. The tricyclic antidepressant imipramine has also been shown to regulate imidazoline binding sites upon chronic treatment[191,192], and this class of antidepressants also regulates α_2-adrenergic receptors[193,195]. These examples provide evidence that a dysregulation of agmatinergic pathways may impact several mood-associated processes, which could all contribute to the depressive-like phenotype of *Crtc1*$^{-/-}$ mice. Moreover, dysregulation of agmatine levels might equally affect arginine availability. This would then impact other arginine-dependent pathways, such as NO synthesis and urea cycle, therefore causing important physiological effects. All in all, these observations strengthen the fact that agmatine dysregulation contributes to the phenotype of *Crtc1*$^{-/-}$ mice; however, the specific pathways involved remain unknown yet.

Finally, although we have demonstrated a link between the CREB-CRTC1 pathway and the agmatinergic system, it is however not known how these two systems are connected. Indeed, CRTC1 deficiency leads to agmatinergic dysregulation, but the mechanisms underlying this direct or indirect consequence are not known. A direct mechanism would be the regulation of the expression of Agm, and possibly other enzymes of the agmatinergic system (e.g. ADC) by CREB and CRTC1. Therefore, it should be investigated whether the transcription of these genes is directly regulated by CREB and CRTC1, either by bioinformatics analysis or *in vitro* assay. A more indirect mechanism could be a developmental effect of CRTC1 deletion that would have affected for instance their GABAergic circuitry (and possibly other systems), which would then

lead to impaired agmatinergic signaling. These various hypotheses should thus be investigated in order to better understand how CREB-CRTC1-regulated transcription could affect the agmatinergic system.

5.3. The effects of lithium on *Crtc1*$^{-/-}$ mice

We have demonstrated that *Crtc1*$^{-/-}$ mice present a blunted response to classical antidepressants, thus suggesting the involvement of CRTC1 in such response. Since mutant animals present a behavioral phenotype that can be related to both MDD and BD, we were then logically interested in studying the response of *Crtc1*$^{-/-}$ mice to a mood stabilizing treatment. Consequently, we decided to treat *Crtc1*$^{-/-}$ mice with lithium, as this agent is the most used and most efficient treatment for BD, and targets several signaling pathways (see 1.4.2). Of particular interest for this study, lithium has also been shown to directly act on CRTC1-CREB association[240–242]. In order to investigate the importance of this effect in lithium action, we chronically treated male WT and *Crtc1*$^{-/-}$ mice, and then assessed the effects of this treatment on their behavioral and molecular phenotype.

5.3.1. Physiological effects of lithium

WT and *Crtc1*$^{-/-}$ mice were chronically treated with lithium-enriched food, a protocol that we chose because of its proved ability to reach and maintain blood lithium levels within the human therapeutic range (0.4-1.2 mM)[338]. Yet, this study also stated that prolonged lithium treatment could cause electrolyte imbalance, hence the proposition to provide a bottle of saline solution to treated mice. We thus also chose to monitor the physiological effects of lithium through the entirety of the experiment, in order to investigate possible harmful effects of the treatment.

Weight and food consumption measurements showed us that both WT groups and control *Crtc1*$^{-/-}$ mice presented a normal weight evolution, and consumed similar amount of food. However, lithium-treated *Crtc1*$^{-/-}$ mice displayed a weight decrease in the first five days of the treatment, which was paralleled by a decrease in food consumption. This decrease cannot be explained by a possible aversive taste of the food, as treated WT mice presented a normal food intake. But we had previously demonstrated that *Crtc1*$^{-/-}$ mice seem to present neophobia (for example in the Open-field test)[337]. Therefore, the decrease in food consumption observed in mutant mice treated with lithium would rather be due to the

presence of a novel type of food. This explanation is supported by the fact that after these first days, *Crtc1*$^{-/-}$ mice started to consume food in a normal amount. This initial difference of food consumption probably had no effect on the experiments afterwards, as weight gain and food intake evolved normally after the first days, over a period of 4 weeks.

Water consumption monitoring showed that both WT and *Crtc1*$^{-/-}$ mice treated with lithium progressively drank a much higher amount of water than control groups, thus resulting in polyuria. This feature is one of the core symptoms of a condition known as diabetes insipidus, one of the principal side effects of lithium[373]. Lithium enters cells of the renal collecting duct through epithelium Na^+ channels (ENaC). It then accumulates in the cytoplasm, until reaching sufficient concentration to inhibit its target molecules, including GSK-3. This latter controls the regulation of aquaporin channels, regulating water reabsorption. Upon GSK-3 inhibition by lithium, aquaporins are downregulated, thus decreasing water reabsorption. This leads to polyuria and a consequent increased water consumption. Furthermore, ENaC subunits are also downregulated, which then results in decreased Na^+ reabsorption (natriuresis), hence the need of the additional saline bottle, to counteract this Na^+ loss. Saline consumption measurement showed that both treated WT and *Crtc1*$^{-/-}$ mice drank similar amount of saline solution throughout the treatment, thus probably compensating for Na^+ loss.

In summary, the only harmful effect of lithium treatment that we could observe was the development of diabetes insipidus. This suggests that treated mice might present electrolytes imbalance, even if the saline bottle helped compensating this. Although treated animals seemed healthy until the end of the experiment, one must keep in mind that these animals presented a condition that could interfere with the behavioral and molecular assessments. There have been few studies about the effects of diabetes insipidus on rodent behavior, but the ones existing suggest a possible decrease in cognitive function[374–377]. Therefore, behavioral analysis should be interpreted carefully, as a deleterious effect of this condition cannot be excluded.

5.3.2. Behavioral effects of lithium

We investigated the effects of lithium on several aspects of the behavioral phenotype of *Crtc1*$^{-/-}$ mice. The first behavior that we measured was the behavioral despair, in the FST and TS paradigms. In the FST, we observed again the characteristic behavioral despair of *Crtc1*$^{-/-}$

mice, as reflected by their increased immobility levels on both days of test. Interestingly, lithium significantly reduced the floating behavior of *Crtc1*$^{-/-}$ mice on both days. This results shows that lithium is effective in knock-out animals in this test, thus suggesting that lithium effects on behavioral despair do not require the CREB-CRTC1 association. Surprisingly, we did not observe any antidepressant effect of lithium on WT mice, although a slight tendency to decrease immobility could be seen on day 2. The reasons of this lack of effect are unclear, as lithium has been shown to be effective in WT mice[378]. Yet, behavioral effects of lithium seem to be highly dependent on its plasmatic concentration[379]. Plasmatic lithium levels measurements in parallel to behavioral assessment should be performed to investigate this hypothesis.

In the TS paradigm, both groups of *Crtc1*$^{-/-}$ mice interestingly presented lower levels of immobility than WT mice, with no effect of lithium on this parameter, while in WT mice, it significantly decreased their immobility time. Since mutant animals presented increased behavioral despair in the FST, one could expect the same in the TS. However, in our previous study, we already observed no difference in immobility levels in the TS, rather even a slight tendency to decreased behavioral despair[337]. Here, both treated and untreated *Crtc1*$^{-/-}$ mice displayed a significant decreased immobility. Interestingly, CREB knock-out mice similarly behave in the TS, which is explained by a possible overactivation of stress response, inducing overactivation of escaping behavior[229]. However, these CREB mutant mice present a general antidepressant phenotype in several other paradigms, which is not the case of *Crtc1*$^{-/-}$ mice. Of particular interest for these results, *Shank3* mutant mice, which are a model of manic-like behavior, display a similar behavior in the TS, with no effect of lithium[380]. This is in line with the fact that *Crtc1*$^{-/-}$ mice present phenotypes of both MDD and BD. Thus, the behavior displayed by *Crtc1*$^{-/-}$ mice in the TS might be the manifestation of manic-like behavior. The fact that lithium was ineffective at normalizing this behavior suggests that CRTC1 is required for lithium effect on this test. Moreover, results from both the FST and TS paradigms suggest that restoration of normal mobility in these two tests do not occur through the same mechanisms, as initially thought. This hypothesis should be further investigated, as it would help to better understand the mechanisms involved in these behavioral despair paradigms.

We next investigated the effects of lithium on social behavior. While *Crtc1*$^{-/-}$ mice have been shown to display social withdrawal[337], we did not observe any impairment of social behavior

in mutant mice, probably because the paradigm used here was different. Interestingly, lithium seemed to decrease the preference for the new mouse in both genotypes. There have been few studies on the effects of lithium on social behavior in rodents. One study however suggests rather an increase of social behavior induced by lithium[381], but again, a different paradigm was used in this study. Apathy has been known to be a side effect of lithium, which might explain this lack of social interest. However, this explanation is contradictory with the effects on mobility observed in the FST and TS. It is not impossible that the effects of lithium on social behavior might be due to diabetes insipidus, as we observed its manifestation in lithium-treated mice. As explained above, this condition can cause impairments in cognitive function. Its effects on social behavior have not been investigated, to our knowledge, but it might be an interesting hypothesis to follow up.

Finally, the effects of lithium on aggressive behavior were assessed, in the resident-intruder test. The enhanced aggressiveness of *Crtc1*$^{-/-}$ male mice was replicated here, as untreated mutant animals attacked the intruder sooner, more often, and for a longer time than WT mice. While lithium had no effect on attacks frequency and latency, it significantly decreased the attacks duration of *Crtc1*$^{-/-}$ mice. No effect of lithium could be seen in WT mice, but duration was already very low in these animals (<5%). It is thus possible that lithium effects were not visible, or that WT mice had reached a minimum threshold. The results of this test show that lithium can reduce the aggressiveness of *Crtc1*$^{-/-}$ mice, and therefore these effects are CRTC1-independent.

In summary, behavioral assessment of the effects of lithium showed that the depressive-like behavior of *Crtc1*$^{-/-}$ mice could be successfully reversed in the FST. These mice displayed an interesting manic-like behavior in the TS, which could not be rescued by lithium. The treatment had however a negative effect on the social behavior of WT and *Crtc1*$^{-/-}$ mice, nevertheless it partly reversed their aggressive behavior. Altogether, this suggests that the effects of lithium on behavioral despair and aggressiveness do not require CREB-CRTC1 association, or that they are not exclusively mediated by this pathway. On the opposite, mood stabilizing effect of lithium in the TS seems to require CRTC1 presence.

5.3.3. Molecular effects of lithium

After the different behavioral assessments discussed above, mice were sacrificed and the expression of several CREB-regulated genes was analyzed in the hippocampus and PFC, in order to investigate the molecular effects of lithium on WT and *Crtc1*$^{-/-}$ mice.

We first measured *Crtc1* expression in WT mice and observed no effect of lithium on its regulation. This is in line with previous study showing that lithium did not act on *Crtc1* mRNA expression, but rather on its oligomeric formation[242].

We then went on measuring *Bdnf* and *BdnfIV* expression, as lithium has been shown to induce *Bdnf* expression and to particularly activate its promoter *IV*[104,382]. Lithium had no effect in *Crtc1*$^{-/-}$ mice, but it significantly decreased *Bdnf* expression in WT mice. The absence of lithium effect in mutant mice is possibly due to their basal lower levels of *Bdnf*, which might not be further decreased by lithium. As explained above, lithium was previously shown to upregulate *Bdnf*, but our results show the opposite. Another study has however shown no effect of lithium on *Bdnf* expression[383]. The lithium-induced decrease in *Bdnf* levels could be explained by several hypotheses. Since molecular measurements were performed after the behavioral procedures, it is possible that the animals exhibited a certain amount of stress which would be evidenced in lithium-treated animals, showing *Bdnf* downregulation. Comparison with control animals which would not undergo the behavioral procedures should be done in order to investigate this hypothesis. It is also possible that the diabetes insipidus developed by lithium-treated mice interfered with *Bdnf* regulation. The effects of this condition on neurotrophic genes have not been investigated, yet diabetes insipidus might have some effects on general metabolism, which could then influence *Bdnf* expression, as this gene is also involved in metabolism and can be regulated by leptin, for example[384]. Beside the unexpected effect of lithium on *Bdnf* expression that merits further investigation, these results also suggest that the behavioral effects of lithium, particularly in the FST and TS, are not mediated by *Bdnf* or *BdnfIV*.

We then measured *Npy* gene expression, as this orexigenic CREB-regulated neuropeptide has been shown to be involved in mood disorders and upregulated by lithium[340,341]. *Crtc1*$^{-/-}$ mice presented basal lower *Npy* levels in the PFC and hippocampus, and this feature would merit further investigation in the frame of the obese phenotype displayed by mutant animals. Lithium also upregulated *Npy* in the hippocampus, which is in line with previous findings[341].

However, this increase only occurred in WT animals. This suggests that CREB-CRTC1 association is required for the effects of lithium on *Npy* expression.

Regarding *Cart* expression, lithium showed a non-significant tendency to downregulate this transcript in both WT and *Crtc1*$^{-/-}$ mice. Lithium effects on *Cart* have not been previously investigated, and this apparent downregulation should be confirmed and further investigated.

Finally, the effects of lithium on the *Nr4a* family were investigated. As previously shown, *Crtc1*$^{-/-}$ mice displayed downregulation of *Nr4a1-3* in the hippocampus and PFC[337]. Lithium significantly upregulated *Nr4a2* in the hippocampus of WT mice, but was ineffective in *Crtc1*$^{-/-}$ mice. In the same structure, it also induced a downregulation of *Nr4a3* in both genotypes. To our knowledge, the effects of lithium on *Nr4a1-3* have not been previously investigated. In our study, lithium increased *Nr4a2* and decreased *Nr4a3* in the hippocampus. Although these effects need to be replicated and confirmed, they interestingly suggest that the *Nr4as* might be involved in lithium effects.

In summary, this experiment with lithium has allowed us to see that the behavioral despair and the aggressiveness of *Crtc1*$^{-/-}$ mice can be rescued by lithium to a certain extent. Therefore, lithium mood stabilizing effect might not necessarily require CREB-CRTC1 association, or at least do not solely rely on it. Moreover, these effects are apparently not mediated by the action of lithium on *Bdnf*. We have also observed that *Crtc1*$^{-/-}$ mice might present a manic-like behavior in the TS, which could not be restored by lithium. At a molecular level, we saw that CRTC1 was required for the lithium-induced upregulation of *Npy* and *Nr4a2*. This also suggests the involvement of these genes in the therapeutic effects of lithium. We did however obtain some peculiar results, particularly the effects of lithium on social behavior and on *Bdnf* expression. Two major factors that could contribute to this would be either the stress generated by the various behavioral procedures and/or the diabetes insipidus caused by the treatment. Control experiments with unstressed mice and other administration routes (e.g. intraperitoneal on direct intracerebral injections would allow a better control of the dosage and could reduce the side effects) could be performed to investigate these issues.

Altogether, these results suggest that the mood stabilizing effects of lithium partly rely on CRTC1, although other alternative pathways contribute to its effects. This also highlights the fact that *Crtc1*$^{-/-}$ mice might be considered as an animal model of BD, because they present depressive-like, as well as manic-like symptoms. Finally, it would be interesting to assess the

effects of another mood stabilizer, such as valproate, on *Crtc1*$^{-/-}$ mice. Indeed, valproate has different mechanisms of action than lithium, and of interest, has an HDAC inhibitory activity. Knowing that we have demonstrated that this type of molecules can be effective in *Crtc1*$^{-/-}$ mice, it would be an interesting experiment to perform.

5.4. Stress regulation of *Crtc1* and *Bdnf*

During the various experiments presented above, we observed several times contradictory *Bdnf* regulation (especially in the SAHA and lithium experiments). We hypothesized that it might be due to the stress generated by the various behavioral experiments or by chronic injections. We therefore sought to investigate the effects of stress on *Bdnf* expression, but also on *Crtc1* gene regulation, in order to assess its possible involvement in stress response (which has never been studied before). These measurements were performed in adult and adolescent mice, as stress response might differ depending on brain development. In a pilot experiment, we thus assessed *Bdnf*, *Crtc1* and also *GR* gene expression in the hippocampus and the PFC of adult and juvenile mice that had undergone four days of swimming sessions (corresponding to the first phase of the OSFS protocol).

Crtc1 expression was unchanged by stress, neither in the hippocampus nor in the PFC, thus suggesting that changes in its levels are not involved in stress response. *Bdnf* however, was found to be upregulated by stress in the hippocampus of both adult and adolescent mice. This might be in line with the abnormally high *Bdnf* levels observed in the hippocampus of *Crtc1*$^{-/-}$ mice in the SAHA experiment. This would then result from an abnormal *Bdnf* regulation in response to stress (probably caused by the daily injections). *Bdnf* expression measurements following stress in *Crtc1*$^{-/-}$ mice should be done to investigate this hypothesis. In addition to *Bdnf*, *GR* expression was also upregulated in the hippocampus, but only in adolescent mice. This suggests an age-dependent differential response to stress.

As *Bdnf* expression was found to be upregulated by stress, we measured the expression of the various *Bdnf* exons separately. One study has indeed shown that the different exons of *Bdnf* were differentially regulated by stress, depending on the brain region and stressor applied[105]. We could indeed observe a differential *Bdnf* regulation in the hippocampus: *BdnfII* and *BdnfV* were upregulated by stress in adult mice, while *BdnfIII* and *BdnfIV* were upregulated in adolescent mice. Therefore, both adult and juvenile mice present increased total *Bdnf* mRNA;

but this increase is mediated by different promoters depending on the age. Yet, the contribution of each exon to final *Bdnf* levels and function are unknown.

The fact that *GR* expression was upregulated in juvenile mice suggests a lower stress reactivity due to an increased feedback inhibition of the HPA axis. This increase of *GR* expression could be therefore a protective mechanism against stress. The *Bdnf* increase observed in the same animals might even be related to the *GR* increase, as there is evidence that *Bdnf* expression can be regulated by glucocorticoids[385–391]. This hypothesis however needs further investigations.

In the literature, we can find many different results regarding *Bdnf* regulation by stress. While the general consensus is that acute and chronic stress downregulate *Bdnf*[88,352], there are many examples that show no effect of stress[351,392–395], or an upregulating effect[396]. This discrepancy can be explained by a probable highly dynamic regulation of *Bdnf* by stress. Therefore, one can observe very different results depending on the type of stress applied (acute vs. chronic), the duration of the stress, and the time elapsed between the stress and the measure. To better characterize *Bdnf* regulation by stress, a complete and extensive experiment should be done, where acute and chronic could be compared, and *Bdnf* would be measured at different time-points after the stress, and in different brain regions. This would help to obtain a better overview of the complex interaction of stress and *Bdnf*.

We did not observe any change in *Crtc1* regulation after the stress, yet it is still possible that CRTC1 mediates the effects of stress on *Bdnf* expression. Therefore, *Bdnf* levels after stress exposure should also be measured in *Crtc1*$^{-/-}$ mice, in order to see whether CRTC1 deficiency abolishes or enhances the effects of stress on *Bdnf*.

Altogether, these preliminary results show that stress can induce *Bdnf* expression, which could explain some of our results. We also demonstrate here that *Bdnf* regulation by stress is dynamic, and differentially regulated depending on the stage of brain development. This study therefore merits deeper investigations, as it would bring a better characterization of stress effects and its impact on *Bdnf* expression.

5.5. Future investigations in *Crtc1*$^{-/-}$ mice

To conclude this thesis, we have performed several preliminary studies, investigating different physiological systems that could be altered in *Crtc1*$^{-/-}$ mice. These results provide possible

investigation lines that should be further explored, in order to complete the characterization of *Crtc1*$^{-/-}$ mice.

5.5.1. Epigenetic system

As detailed in the introduction, epigenetic gene regulation plays a major role in mood disorders (see 1.2.4). In our article presented in section 2.1, we showed that we were able to restore the expression of some genes in *Crtc1*$^{-/-}$ mice, by acting on epigenetic mechanisms. We were also interested in investigating whether these animals might present an overall altered epigenome, as this might contribute to their mood disorders-like phenotype. Furthermore, CRTC1 helps recruiting CBP, which has an intrinsic HAT activity. We could thus hypothesize that CRTC1 deficiency would lead to a decreased CBP-mediated HAT activity, that would lead to impaired histone acetylation. The fact that the depressive-behavior of *Crtc1*$^{-/-}$ mice could be partially reversed by the HDAC inhibitor SAHA corroborates this hypothesis. Nevertheless, we investigated other mediators of epigenetic regulation, namely *Dnmts* and *Tet1*.

Our results suggest that *Crtc1*$^{-/-}$ mice might present epigenetic alterations. Indeed, while the expression of most *Hdacs* is unchanged, *Dnmt3a* and *Dnmt3b* expression was increased in the hippocampus and PFC of these animals, which might suggest overall increased DNA methylation. TET1 is involved in the process of DNA demethylation, and has been associated with neurogenesis and learning process[397,398]. The upregulation of *Tet1* that we observed in *Crtc1*$^{-/-}$ mice might be a possible compensatory mechanism for the increased *Dnmts* levels also seen in these mice. On the other hand, the upregulation of *Dnmts* could also be triggered by the increase of TET1, and therefore deeper investigations are needed to sort out this complex issue. Nevertheless, these preliminary results suggest an impaired epigenetic gene regulation, which is very likely to contribute to the molecular, and thus behavioral, phenotype of *Crtc1*$^{-/-}$ mice.

Further investigation of the epigenome of these mice will however be required to confirm or infirm these different hypotheses. It would also be interesting to look at the methylation profile of specific genes that are observed to be downregulated in *Crtc1*$^{-/-}$ mice. Nonetheless, these results unravel a new role for CRTC1 in epigenetic regulation.

5.5.2. GABAergic system

As previously suggested, *Crtc1*$^{-/-}$ mice might present impairments in GABAergic interneurons circuitry (see 5.2). This working hypothesis is plausible, as *Crtc1*$^{-/-}$ mice present decreased levels of *Bdnf*, and particularly *BdnfIV*. The latter has been involved in the development of GABAergic interneurons, as mice lacking *BdnfIV* present a deficiency in PV-interneurons[100]. *Bdnf* heterozygous mice also present dysregulation of interneuronal circuitry, mainly PV- and calretinin (CR)- interneurons[399], and BDNF has also been shown to participate in the development of calbindin (CB-) and CR-interneurons[400]. In addition, several types of interneurons were found reduced in the PFC of BD patients[343,344,401,402]. To conclude, *Crtc1*$^{-/-}$ mice also present a decreased expression of *Sst* gene, thus suggesting impairment of this interneuron subtype.

To our surprise, characterization of Agm-expressing cells has revealed an increased number of such cells, which were mainly interneurons. This would then rather suggest an increased number of interneurons. To unravel this issue, a characterization of interneuronal circuitry should be performed in *Crtc1*$^{-/-}$ mice. Here, we measured the expression levels of *Gad1* and *Gad2* genes, respectively coding for GAD67 and GAD65, the two isoforms of GAD, which catalyze GABA synthesis. Of interest, both isoforms have been associated with mood disorders, and CREB has been shown to mediate GAD65 expression[343,344,361]. In our pilot study, we could indeed observe a downregulation of both *Gad1* and *Gad2*, in the hippocampus and PFC of *Crtc1*$^{-/-}$ mice. This confirms that mutant mice might present impaired GABA metabolism, and thus an overall impairment of GABAergic signaling, which could contribute to their phenotype. However, a complete and quantitative morphological and electrophysiological study of this system is required, in order to investigate its potential contribution to the phenotype of *Crtc1*$^{-/-}$ mice.

5.5.3. Inflammatory system

There have been several pieces of evidence over the past decade of the involvement of immune system activation in mood disorders, and particularly in MDD. Several pro-inflammatory cytokines, mainly TNFα, have been shown to induce depressive-like behavior in various study, and to be increased in the brain of depressed patients[403–410]. Correspondingly, chronic stress induces increased levels of such cytokines in several brain regions[411,412].

Moreover, injections of lipopolysaccharide (LPS), which elicits activation of the immune system and release of pro-inflammatory cytokines, is sufficient to elicit depressive-like behavior[413–416]. The enzyme IDO has been shown to be critically involved in inflammation-induced depression[347,417]. This enzyme can be activated by TNFα, IFNγ, IL-1β and LPS; among others. Its activation leads to the metabolism of tryptophan into tryptophan catabolites (TRYCATs), thus depleting available tryptophan levels, therefore leading to decreased 5HT synthesis, which would then provoke depressive symptoms.

In this pilot experiment, we sought to investigate the potential imbalance of inflammatory mediators, as it might participate to the depressive-like phenotype of *Crtc1*$^{-/-}$ mice. We first measured the levels of various pro-inflammatory cytokines in the hippocampus and striatum of *Crtc1*$^{-/-}$ mice, as these regions seem to be involved in inflammation-mediated depression. We could observe decreased levels of TNFα, IL-1β and IL-6 in the hippocampus, and IL-1β and IFNγ in the striatum. These results indeed suggest imbalance of cytokines, yet they are contradictory with the evidence that cytokines induce depressive behaviors. However, there are some studies that have also suggested a protective role of TNFα and IFNγ, particularly in glial cells[346,418–420]. It is then possible that these cytokine depletions would lead to more vulnerable neurons or glial cells, which would be in line with the phenotype of *Crtc1*$^{-/-}$ mice. Yet, the involvement of cytokines in depression is very complex and is still not well understood. Furthermore, cytokines might have both harmful and protective effects on neurons, but the trend to move towards one of these effects or the other might rely on a complex chemical balance. In any case, our results still show a dysregulation of the immune system in *Crtc1*$^{-/-}$ mice that would merit further investigation.

Finally, measurements of *IDO* showed that *Crtc1*$^{-/-}$ mice presented increased expression of this gene in the hippocampus. This would suggest an increased activity of this enzyme, that would lead to serotonin depletion, and this might be a possible explanation for the decreased 5HT levels observed in these mice[337].

Altogether, the present preliminary results show that the immune system of *Crtc1*$^{-/-}$ mice might be altered and this alteration could participate in their phenotype. This also suggests the involvement of CRTC1 in a normal immune system regulation. To what extent CRTC1 participates in this regulation, and to what extent does their immune system participate in their phenotype still have to be unraveled and should be a focus for future studies.

5.6. General Discussion and Perspectives

The aim of the present thesis was to provide a deeper characterization of *Crtc1*$^{-/-}$ mice phenotype. More precisely, we wanted to investigate the impact of CRTC1 deficiency on several molecular mechanisms involved in mood disorders etiology. As these troubles are complex and heterogeneous, we sought to determine the contribution of such mechanisms to various behavioral and molecular features exhibited by knock-out animals. Through pharmacological approaches, we also attempted to rescue the behavioral and molecular phenotype of *Crtc1*$^{-/-}$ mice by acting on several systems.

The most striking result emerging from the numerous data presented here, is that CRTC1 can be definitely considered as a key mediator of a wide variety of cellular and physiological processes. This is mainly highlighted by the fact that our experiments have shown that *Crtc1*$^{-/-}$ mice are impaired in many systems, including the monoaminergic system, the agmatinergic system, the epigenome, the GABAergic metabolism and the inflammatory system. However, it is not yet possible to determine which of these impairments are direct or indirect consequences of CRTC1 deletion, as well as the possible interactions between these systems. Regardless of the mechanisms involved, these results definitely highlight the pleiotropic role and importance of CRTC1.

We had previously demonstrated that CRTC1 is importantly involved in mood disorders etiology[337]. This study, as well as the one presented in section 2.1, provide also strong evidence that CRTC1's function is critical for the therapeutic effects of classical antidepressants. Our results show that CRTC1 is indeed necessary for the induction of several genes by antidepressants, but also by lithium, including *Bdnf* and *Nr4as*. This suggests that the behavioral effects of antidepressants rely on CRTC1-mediated gene induction. To corroborate this hypothesis, we showed that *Bdnf* expression can be restored by counteracting CRTC1 deletion and acting on epigenetic gene regulation, alongside a partial rescue of the depressive-like behavior.

On the opposite, CRTC1 apparently does not mediate the antidepressant effects of lithium, but might interestingly be involved in its anti-manic effects. Of interest, CRTC1 is not involved in the anxiolytic and anti-aggressive effects of antidepressants and lithium. While CRTC1 deletion leads to the development of anxious and aggressive behaviors, these can be restored independently of its presence.

Although CRTC1 deficiency leads to the perturbation of many systems, we do not know yet to what extent these various impairments contribute to the phenotype of *Crtc1*$^{-/-}$ mice. We have demonstrated that by acting on the altered agmatinergic system of these animals, we were able to restore a normal behavioral phenotype. These results provide evidence that this system might be one of the various causes of their conditions. Furthermore, they also strengthen the findings that agmatine is an important neuromodulator potentially involved in mood disorders. Our data also suggest that agmatine might be a fast-acting antidepressant with ketamine-like properties, which is particularly interesting regarding the development of new therapeutic strategies.

Even if our various results allowed us to unravel some mechanisms underlying the phenotype of *Crtc1*$^{-/-}$ mice, as well as the role of CRTC1 in mood disorders, there are still many unresolved issues. Indeed, the pilot experiments that we performed reveal the wide scope of the molecular alterations resulting from CRTC1 deletion. Our data provide evidence that many systems might be impaired in knock-out animals. These include the epigenetic system, GABAergic circuitry and immune system, all of which could equally contribute to the phenotype of these animals. Furthermore, our various *Bdnf* measurements, as well as our study on stress and *Bdnf* also suggest impaired stress response.

This underlines again the broad complexity underlying mood disorders. It also suggests a pleiotropic regulatory role for CRTC1, and highlights it as a potent modulator involved in many important processes in the central nervous system. Thus, all the aforementioned systems impaired in mutant animals would merit a complete and further characterization, with the aim of characterizing the reach of CRTC1's regulatory function.

As already suggested above, a region-specific rescue of *Crtc1* expression in *Crtc1*$^{-/-}$ mice, followed by behavioral and molecular measurements, would be an important and interesting experiment to perform. It would indeed allow to better define the region(s) in which CRTC1 plays a critical role for the development of depressive-like behavior and/or for antidepressant response. In addition, this experiment could be done at several time points of brain development, in order to characterize the possible developmental effect of a complete CRTC1 deletion. As discussed before, this might contribute to their adult phenotype. To further investigate this issue, a characterization of CRTC1 expression and activity throughout brain

development could also be done. Correspondingly, punctual CRTC1 deletion or overexpression in WT animals, at different time points could be an interesting thing to do.

As CRTC1 deficiency produces depressive-like behavior, we could expect that higher CRTC1 levels would have opposite effects and produce an antidepressant effect. Viral-mediated *Crtc1* overexpression in different regions, at the adult age or during development would address this question, and the effects that it would induce would be of great interest.

Another important assessment that should be performed in *Crtc1*$^{-/-}$ mice would be to investigate the neurogenesis displayed by these animals. Indeed, as CRTC1 has been shown to be involved in CREB- and BDNF-regulated dendritic growth, and knowing the involvement of these two factors in neurogenesis[239,421], we could expect that this process would be altered in mutant animals. Thus, measures of neurogenesis, neuronal growth and survival, as well as dendritic arborization in the hippocampus of these mice would be another step towards the understanding of the phenotype of these animals.

To conclude, while some aspects of *Crtc1*$^{-/-}$ mice phenotype are now better understood, these animals still hold many challenging issues that need to be investigated. Although the use of this mouse line can help resolving a certain amount of questions regarding CRTC1 role in mood disorders, translational studies should also be performed. CRTC1 expression and activity in human samples, or genomic studies in mood disorders patients could also provide interesting outcomes. One study in psychiatric patients has already revealed an association between a *Crtc1* polymorphism and body weight gain[422]. Further research for CRTC1 mutations and their possible association with mood disorders should therefore be performed. This would lead to a more targeted preclinical research, and the results emerging from both animal and human studies might eventually lead to a better understanding of CRTC1 function and importance.

6. CONCLUSIONS

For several years, our group has been working on CRTC1, and the generation of *Crtc1*$^{-/-}$ mice has allowed to highlight the importance of this coactivator in the brain, and its involvement in mood disorders. One of the main features of these animals is their display of behavioral and molecular phenotypes that can be related to MDD but also to BD. *Crtc1*$^{-/-}$ mice could therefore be considered as a new animal model of mood disorders. Here, we aimed at better characterizing this mouse line, as well as providing new hypothesis regarding CRTC1 involvement in mood disorders etiology. There are several main points revealed by our results.

First, the *Crtc1*$^{-/-}$ mouse model is a highly valuable animal model of mood disorders. Indeed, it allows to investigate plenty of endophenotypes related to MDD and BD, both at the behavioral and molecular levels. Furthermore, we have demonstrated the resistance of these animals to classical antidepressant treatment. This fact renders these animals highly interesting for research focusing on treatment-resistant depression.

Second, the phenotype observed in *Crtc1*$^{-/-}$ mice seems to result from a combination of many impaired processes. This remarkably mimics the occurrence of mood disorders in human, as they often result from complex interaction between genetic factors, molecular and physiological alterations, and environmental stressors. This highlights again the intricacy and heterogenic nature of these diseases, and thus the difficulty to completely understand their etiology.

Third, CRTC1 is a major regulatory factor, which impacts, directly or not, many important processes of the central nervous system. We have demonstrated its importance in mood disorders and in antidepressant response, but due to its pleiotropic role, it might be involved in other diseases. Its implication in Alzheimer's disease has been recently highlighted[244], thus it could also be related to other memory- and learning- related troubles. Due to its role in CREB-regulated gene transcription, it might also be a mediator of neuroplasticity-related processes. Therefore, dysfunction of CRTC1 could be involved in neuropsychiatric diseases associated with altered neuronal plasticity and activity (mood disorders but also schizophrenia or affective disorders).

Finally, in the light of these various statements, clinical investigation of CRTC1 would be a major step towards the understanding of its function and its involvement in neuropsychiatric

disorders. The search for possible polymorphisms or mutations of CRTC1 in human affected with mood disorders, or other diseases, would help to better target the mechanisms that should be deeper investigated *in vitro* and *in vivo*. Thus, the knowledge gathered from both preclinical and clinical investigations might contribute to a better understanding of the involvement of CRTC1 in mood disorders, which eventually might unravel new perspectives for therapeutic research.

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8. APPENDIX

Content:

- Article: “The HDAC inhibitor SAHA improves depressive-like behavior of CRTC1-deficient mice: possible relevance for treatment-resistant depression"

- Article: “Involvement of the Agmatinergic System in the Depressive-like Phenotype of the *Crtc1* Knockout Mouse Model of Depression”

Neuropharmacology 107 (2016) 111–121

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

The HDAC inhibitor SAHA improves depressive-like behavior of CRTC1-deficient mice: Possible relevance for treatment-resistant depression

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ARTICLE INFO

Article history:
Received 30 July 2015
Received in revised form
4 March 2016
Accepted 6 March 2016
Available online 9 March 2016

Keywords:
Mood disorders
Antidepressants
Animal model of depression
CREB coactivator
BDNF
HDAC inhibitor
SAHA
Epigenetics

Chemical compounds studied in this article:
Desipramine hydrochloride (PubChem CID: 65327)
Vorinostat (PubChem CID: 5311)

ABSTRACT

Major depression is a highly complex disabling psychiatric disorder affecting millions of people worldwide. Despite the availability of several classes of antidepressants, a substantial percentage of patients are unresponsive to these medications. A better understanding of the neurobiology of depression and the mechanisms underlying antidepressant response is thus critically needed. We previously reported that mice lacking CREB-regulated transcription coactivator 1 (CRTC1) exhibit a depressive-like phenotype and a blunted antidepressant response to the selective serotonin reuptake inhibitor fluoxetine. In this study, we similarly show that *Crtc1*$^{-/-}$ mice are resistant to the antidepressant effect of chronic desipramine in a behavioral despair paradigm. Supporting the blunted response to this tricyclic antidepressant, we found that desipramine does not significantly increase the expression of *Bdnf* and *Nr4a1-3* in the hippocampus and prefrontal cortex of *Crtc1*$^{-/-}$ mice. Epigenetic regulation of neuroplasticity gene expression has been associated with depression and antidepressant response, and histone deacetylase (HDAC) inhibitors have been shown to have antidepressant-like properties. Here, we show that unlike conventional antidepressants, chronic systemic administration of the HDAC inhibitor SAHA partially rescues the depressive-like behavior of *Crtc1*$^{-/-}$ mice. This behavioral effect is accompanied by an increased expression of *Bdnf*, but not *Nr4a1-3*, in the prefrontal cortex of these mice, suggesting that this epigenetic intervention restores the expression of a subset of genes by acting downstream of CRTC1. These findings suggest that CRTC1 alterations may be associated with treatment-resistant depression, and support the interesting possibility that targeting HDACs may be a useful therapeutic strategy in antidepressant development.

1. Introduction

Mood disorders (including major depressive disorders and bipolar disorders) are a major cause of disability worldwide, with an estimated lifetime prevalence of 16% (Kessler et al., 2005). Although the underlying etiological mechanisms are complex and still unclear, studies over the past decades have highlighted the importance of the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and one of its target genes brain-derived neurotrophic factor (*Bdnf*) (Blendy, 2006; Krishnan and Nestler, 2008; Martinowich et al., 2007). CREB is a pleiotropic transcription factor involved, in particular, in neuronal growth and survival, neurogenesis, synaptic plasticity and long-term memory (Blendy, 2006; Carlezon et al., 2005; Lonze and Ginty, 2002). BDNF is also implicated in these processes, partly through the induction of its expression by CREB (Martinowich and Lu, 2008; Martinowich et al., 2007). CREB and BDNF have both been involved in depression and antidepressant treatment. Indeed, overexpression of CREB and BDNF in the hippocampus (HIP) results in antidepressant effects (Chen et al., 2001; Shirayama et al., 2002) and inversely, chronic stress reduces *Bdnf* expression in the same structure (Smith et al., 1995). Of note, antidepressants such as

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http://dx.doi.org/10.1016/j.neuropharm.2016.03.012
0028-3908/

fluoxetine and desipramine were also shown to upregulate *Bdnf* expression in the prefrontal cortex (PFC) and HIP of rodents in a CREB-dependent manner (Conti et al., 2002).

CREB-regulated transcription coactivator 1 (CRTC1) is a potent CREB coactivator activated by cAMP and Ca^{2+} signaling that trigger its dephosphorylation and translocation to the nucleus, where it binds to CREB, helps to recruit CREB-binding protein (CBP) and RNA polymerase II, and thus activates CREB-dependent gene transcription (Altarejos and Montminy, 2011; Ch'ng et al., 2012; Conkright et al., 2003; Kovacs et al., 2007; Ravnskjaer et al., 2007; Screaton et al., 2004). CRTC1 is involved in activity-induced dendritic growth and late phase long-term potentiation (L-LTP) (Kovacs et al., 2007; Li et al., 2009; Zhou et al., 2006). Moreover, *Bdnf* expression as well as BDNF-dependent dendritic growth require CRTC1 (Finsterwald et al., 2010; Kovacs et al., 2007; Zhou et al., 2006). Region-specific CRTC1's activation is critical for activity-dependent immediate early genes regulation and long-term fear memory (Nonaka et al., 2014a; Sekeres et al., 2012). CRTC1-dependent transcription of *Bdnf* and orphan nuclear receptors 4a (*Nr4a*) is also involved in cognitive impairments related to Alzheimer's disease (Parra-Damas et al., 2014).

To further investigate the role of CRTC1 in the brain, we generated a CRTC1-deficient mouse line that exhibits several endophenotypes related to mood disorders and a blunted response to the antidepressant fluoxetine in behavioral despair-related paradigms (Breuillaud et al., 2009, 2012). At the molecular level, *Crtc1*$^{-/-}$ mice have a decreased HIP and PFC expression of several CREB-regulated neuroplasticity genes, including notably *Bdnf* and *Nr4a1-3* (Breuillaud et al., 2012). Altogether, this suggests that CRTC1, as a key regulator of neuroplasticity-related genes, is critical for mood regulation and antidepressant response. However, the direct or indirect mechanisms linking CRTC1's deficiency and behavioral consequences are still unclear. As CRTC1 promotes CBP recruitment, which has an intrinsic histone acetyltransferase (HAT) activity, these mechanisms might include CRTC1-related epigenetic gene regulation. Noteworthily, epigenetic mechanisms have been widely associated with depressive disorders and with CREB and *Bdnf* regulation in the frame of stress and antidepressant response (Lin et al., 2012; Tsankova et al., 2006, 2007; Vecsey et al., 2007; Vialou et al., 2013).

In this study, we were interested in further investigating the involvement of CRTC1 in antidepressant response and the underlying molecular mechanisms. We first tested the effects of chronic desipramine on the behavior of *Crtc1*$^{-/-}$ mice, which revealed a blunted behavioral response to this antidepressant. We also observed that desipramine failed to induce *Bdnf* and *Nr4a1-3* expression in the HIP and PFC of *Crtc1*$^{-/-}$ mice. By acting at the epigenetic levels and treating the animals with a HDAC inhibitor, we were able to partially rescue the depressive-like behavior of *Crtc1*$^{-/-}$ mice. This was paralleled by an increased expression of *Bdnf*, but not *Nr4a1-3*, in the PFC of these animals.

2. Materials and methods

2.1. Animals

Crtc1$^{-/-}$ mice and wild-type (WT) littermates were generated and genotyped as previously described (Breuillaud et al., 2009). Mice were housed under a 12-h light-dark cycle with *ad libitum* access to water and standard rodent chow diet. Male mice were weaned at 21 days and group-housed until being isolated at 5 weeks of age in order to avoid wounding of cage mates by aggressive *Crtc1*$^{-/-}$ males (Breuillaud et al., 2012). All animal experiments were conducted in accordance with the Swiss Federal Veterinary Office's guidelines and were approved by the Cantonal Veterinary Service. Behavioral procedures began when mice reached the age of 8 weeks.

2.2. Drugs and treatment

Desipramine hydrochloride was purchased from Sigma (St-Louis, MO, USA). Mice received desipramine in the drinking water at a concentration of 100 mg/l, which corresponds to approximately 20 mg/kg of body weight/day. Desipramine solution was changed every week and concentration was adjusted depending on the weight gain and average water consumption of the mice. Suberoylanilide hydroxamic acid (SAHA, also known as vorinostat) was purchased from Selleck Chemicals (Houston, TX, USA). Mice received SAHA through daily intra-peritoneal injections. SAHA was first dissolved in DMSO at a dose of 50 mg/ml. This stock solution was diluted 1:10 every day in saline solution (final concentration: 5 mg/ml) prior to injections at a dose of 25 mg/kg of body weight. Vehicle groups were injected with a solution of 10% DMSO in saline.

2.3. Repeated open-space forced swim procedure (OSFS)

The repeated OSFS procedure was performed as previously described (Breuillaud et al., 2012; Stone and Lin, 2011). Briefly, swimming was carried out in rat tub cages (24 × 43 × 23 cm) filled with ~14 cm of lukewarm tap water (34 ± 0.5 °C) and colored with ~10 ml of milk. Mice undertook individual daily swim of 15 min during 4 consecutive days (Days −4 to −1). On day 0, desipramine or SAHA treatment started. Swimming sessions were repeated at 3 or 4 days of intervals during 3 weeks (Days 2, 5, 9, 12, 16, 19, 23) under continuous treatment (Fig. 1A). Water was changed after 4 mice had swum, in order to maintain water temperature. Swimming sessions were videotaped from above. Time spent immobile (drifting with no observable movement of limbs or tail) was manually recorded.

2.4. Novelty-induced hypophagia (NIH)

NIH procedure started on day 24, one day after the last swimming session (Fig. 1A). Mice were trained to drink sweetened condensed milk (1:3, condensed milk: water) for two consecutive days (day 24 and 25), 2 × 1 h each day. Milk was presented in homemade drinking tubes made of 10 ml conical tubes (Sarsedt) with a hole at the bottom closed by a glass bead (5 mm diameter) to make a sipper. Tubes were placed through wire cage lids. On the third day (day 26), mice were tested in homecage conditions (light intensity: ~27 lux). Mice were videotaped during 15 min from the side and latency to drink was measured. On the fourth day (day 27), mice were placed in a novel cage without bedding, under bright light (~1200 lux), and the same measurements were made. The cage was cleaned with water and 70% ethanol between each mouse and before the first mouse.

2.5. Brain microdissection and micropunching

On day 28, one day after the end of the NIH procedure, all mice were sacrificed by cervical dislocation and decapitation. The brain was rapidly placed in a stainless steel adult mouse brain slicer matrix with 1 mm coronal section slice intervals. Six coronal slices were made from the second frontal slice channel of the matrix. Brain slices were placed on microscope slides and immediately frozen in dry ice, and then stored at −80 °C. Medial PFC and dorsal HIP were collected with a micropunch (ø 1 mm, Stoelting, Wood Dale, IL, USA) in corresponding brain slices. PFC and HIP samples were kept at −80 °C.

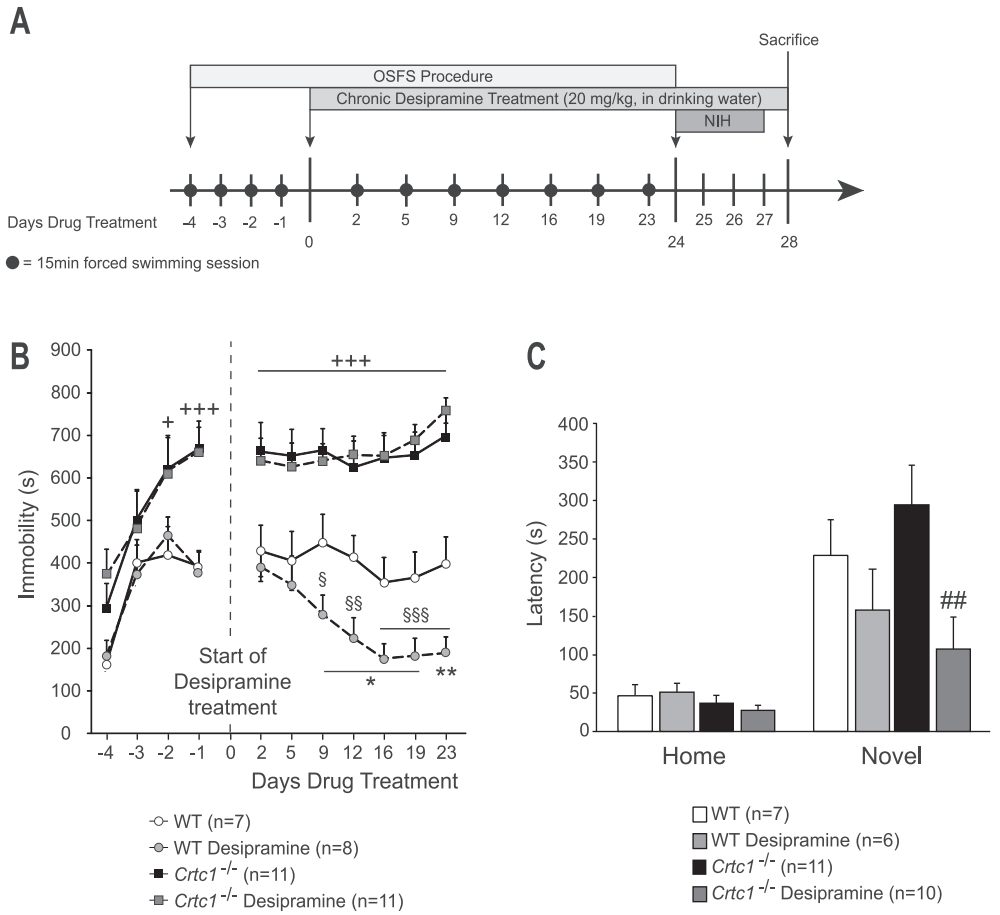

Fig. 1. Behavioral response of *Crtc1*$^{-/-}$ mice to desipramine. (**A**) Experimental design and timeline. The OSFS protocol started when mice reached the age of 8 weeks. Mice underwent forced swimming session during the first four days (day −4 to −1). On day 0, desipramine treatment started and continued until the end of the experiment. Swimming session were regularly repeated until day 24. The NIH test was then applied from day 24 until day 27. On day 28, animals were sacrificed. (**B**) Effects of chronic desipramine in the OSFS model of depression on *Crtc1*$^{-/-}$ mice and WT littermates. During the four consecutive daily swimming sessions prior to treatment (Days −4 to −1), all groups increased their immobility time. *Crtc1*$^{-/-}$ mice were significantly more immobile than WT mice during the two last sessions ($^{+}p < 0.05$, $^{+++}p < 0.001$ vs. WT untreated). From day 0 to day 23, WT and *Crtc1*$^{-/-}$ mice received either water (n = 7 and n = 11 respectively) or chronic desipramine treatment (20 mg/kg in drinking water, n = 8 and n = 11 respectively). *Crtc1*$^{-/-}$ mice were significantly more immobile than WT mice, regardless of the treatment, during the whole procedure ($^{+++}p < 0.001$, vs. WT untreated). Desipramine-treated WT mice showed a progressive decrease in immobility time starting from day 9 of treatment ($^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$, vs. themselves on day 2). Their immobility time was also significantly lower that untreated WT mice ($^{*}p < 0.05$, $^{**}p < 0.01$, vs. WT untreated). No effect of desipramine was seen in *Crtc1*$^{-/-}$ mice. (**C**) Effects of chronic desipramine on the NIH paradigm in *Crtc1*$^{-/-}$ and WT mice. Latencies to drink sweetened condensed milk are shown in the homecage and in the novel environment. Two mice were removed for having latency scores >2 SD from the mean. One mouse was removed for never having drunk the milk during the habituation and test phases. No significant difference of latency between the different groups was observed in the homecage conditions. In the novel environment, desipramine-treated WT mice (n = 7) showed a non-significant trend to a decreased latency as compared to untreated WT mice (n = 6). Desipramine significantly reduced the latency of *Crtc1*$^{-/-}$ mice (n = 10) compared to untreated *Crtc1*$^{-/-}$ mice (n = 11) ($^{\#\#}p < 0.05$ vs. *Crtc1*$^{-/-}$ mice). Data are mean ± SEM.

2.6. Gene expression analysis

Total RNA was extracted and purified from the PFC and HIP micropunches, using the RNAeasy Plus Minikit (Qiagen, Venlo, Netherland) according to the manufacturer's instructions. RNA concentrations were measured by UV spectrophotometry with a NanoDrop Lite (Thermo Scientific, Wilmington, DE, USA). cDNA was prepared in a 50 µl reaction by reverse transcription, using 100 ng of RNA with Taqman Reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). 0.8 µl of cDNA was amplified on a 96-well plate using the SYBR Green PCR Master Mix (Applied Biosystem). Amplification was performed with an ABIPRISM 7500 real-time PCR system (Applied Biosystem). The program was 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative gene expression was quantified using the comparative *ΔΔ Ct* method and normalized with β-actin transcript levels.

The following primers were used, at a concentration of 250 nM: *β-actin* forward 5′-GCTTCTTTGCAGCTCCTTCGT-3′, *β-actin* reverse 5′-ATATCGTCATCCATGGCGAAC-3′, *Crtc1* forward 5′-CAGGACTTGGGCCTGGAA-3′, *Crtc1* reverse 5′-AGACAGACAAGACCCTTTCTAAGCA-3′; *Bdnf* forward 5′-AAAACCATAAGGACGC GGACTT-3′, *Bdnf* reverse 5′-GAGGCTCCAAAGGCACTTGA-3′; *Bdnf exIV* forward 5′-GTAAG AGTCTAGAACCTTGGGGACC-3′, *Bdnf exIV* reverse 5′-GGATGGTCATCACTCTTCTCACCT-3′; *Nr4a1* forward 5′-AAAATCCCTGGCTTCATTGAG-3′, *Nr4a1* reverse 5′-TTAGA TCGGTATGCCAGGCG-3′; *Nr4a2* forward 5′-CGGTTTCAGAAGTGCCTAGC-3′, *Nr4a2* reverse 5′-TTGCCTGGAACCTGGAATAG-3′; *Nr4a3* forward 5′-TGGCTCGACTCCATTAAAGAC-3′, *Nr4a3* reverse 5′-TGCATAGCTCCTCCACTCTCT-3′; *Hdac1* forward 5′-TTCCAACATGACCAACCAGA-3′, *Hdac1* reverse 5′-GGCAGCATCCTCAAGTTCTC-3′, *Hdac2* forward 5′-GGGACAGGCTTGGTTGTTTC-3′, *Hdac2* reverse 5′-GAGCATCAGCAATGGCAAGT-3′, *Hdac3* forward 5′-AGAGAGGTCCCGAGGAGAAC-3′, *Hdac3* reverse 5′-ACTCTTGGGGACACAGCATC-3′, *Hdac4* forward 5′-CAATCCCACAGTCTCCGTGT-3′, *Hdac4* reverse 5′-CAGCACCCCACTAAGGTTCA-3′, *Hdac5* forward 5′-

TGTCACCGCCAGATGTTTTG-3′, *Hdac5* reverse 5′-TGAGCAGAGCCGAGACACAG-3′, *Hdac7* forward 5′-GGTGGACCCCCTTTCAGAAG-3′, *Hdac7* reverse 5′-TGGGTAGCCAGGAGTCTGGA-3′, *Hdac9* forward 5′-GCGAGACACAGATGCTCAGAC-3′, *Hdac9* reverse 5′-TGGGTTTTCCTTCCATTGCT-3′.

2.7. Effects of SAHA on histone acetylation

Mice were weaned and housed according to 2.1. At the age of 8 weeks, *Crtc1*$^{-/-}$ mice and WT littermates received a single injection of either SAHA or vehicle as in 2.2. Animals were sacrificed 2 h after the injection, with a similar procedure as in 2.5. Histone acetylation was measured by Western blotting.

2.8. Western blot

Histones were extracted from HIP and PFC micropunches using the Histone Purification Minikit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. Samples with poor histone yield were excluded. 1 μg of extracted histones were diluted 1:1 with sample buffer [65.8 mM Tris–HCl pH 6.8, 26.3% glycerol, 2.1% SDS, 100 mM DTT, 0.01% bromophenol blue], separated on a 15% SDS-polyacrylamide gel, and proteins were transferred to polyvinylidene difluoride (PVDF) membranes with a Transblot Turbo Transfer System (BioRad, Hercules, CA, USA). Blots were blocked for 1 h at room temperature (RT) in TBST [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20], supplemented with 5% skim milk powder. Blots were subsequently incubated with a primary antibody in TBST plus 5% bovine serum albumin (BSA) overnight at 4 °C. Finally, PVDF membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies in TBST plus 5% skim milk powder, and developed using a Pierce ECL Western chemiluminescence detection kit (Thermo Scientific). The following antibodies and dilutions were used: rabbit α-Acetyl-Histone H3, 1:2500 (EMD Millipore, Temecula, CA, USA), rabbit α-Histone H3, 1:2000 (Abcam, Cambridge, UK), rabbit α-Acetyl-Histone H4, 1:2000 (EMD Millipore), rabbit α-Histone H4, 1:2000 (EMD Millipore), donkey HRP-α-rabbit 1:2000 (GE Healthcare, Little Chalfont, UK). Quantification of band intensity was performed with Image J software (National Institute of Health, Bethesda, MD, USA). Acetyl-H3 and acetyl-H4 band intensities were normalized with total H3 and H4 signals, respectively.

2.9. Statistical analyses

Statistical analyses were performed using the Statistica 8.0 Software (StatSoft Inc., Tulsa, OK, USA). All data are presented as mean ± SEM. P-values of $p < 0.05$ were considered as statistically significant. A Shapiro-Wilk test was first performed to assess data normality. All results were found to follow normal distribution; therefore parametric tests could be used. For both behavioral and molecular analyses, a two-way ANOVA was performed to assess statistical differences for two factors (genotype and treatment). A Fisher LSD post-hoc test was carried out afterwards. For analyses of the OSFS data, a two-way ANOVA with repeated measures was performed as immobility time was repeatedly measured. A Fisher LSD post-hoc test was carried out afterwards.

3. Results

3.1. Crtc1$^{-/-}$ mice have a blunted behavioral response to chronic desipramine treatment

Behavioral effects of desipramine were first assessed in the open-space forced swim (OSFS) model of depression (Fig. 1A and B). This protocol induces depressive-like symptoms that are reversed by chronic, but not acute, antidepressant treatments, and thus it has better face and construct validities than the conventional forced swim test (Breuillaud et al., 2012; Stone and Lin, 2011; Stone et al., 2008). All animals presented increased immobility time after the four days of consecutive swimming with a significant effect of time ($F_{(1,37)} = 36.1$, $p < 0.001$) and genotype ($F_{(1,111)} = 13.7$, $p < 0.001$). *Crtc1*$^{-/-}$ mice had higher immobility time than WT mice during the last two sessions of the pre-test period (day −2 and −1) ($p < 0.001$ for both days). This genotype effect remained significant after the beginning of the desipramine treatment ($F_{(1,35)} = 71.34$, $p < 0.001$), indicating that *Crtc1*$^{-/-}$ mice had higher immobility time that WT mice during the whole procedure. WT mice progressively responded to desipramine, as their immobility time significantly decreased over time. As compared to their own immobility time on day 2 of treatment, the effect of desipramine became significant starting from day 9 ($p = 0.029$ on day 9, $p = 0.003$ on day 12, $p < 0.001$ on days 16, 19 and 23, vs. day 2). Their immobility time was also lower than untreated WT mice, starting from day 9 as well ($p = 0.032$ on day 9, $p = 0.032$ on day 12, $p = 0.023$ on day 12, $p = 0.016$ on day 19, $p = 0.009$ on day 23, vs. WT untreated). Desipramine had no effect on the depressive-like behavior of *Crtc1*$^{-/-}$ mice, indicating that these mice were resistant to desipramine in this paradigm. At the end of the procedure, mice were then tested in the novelty-induced hypophagia (NIH) test, a depression- and anxiety-related paradigm sensitive to chronic antidepressant treatment (Dulawa and Hen, 2005) (Fig. 1A and C). In homecage conditions, genotype or treatment had no effect on the latency to consume condensed milk, whereas a significant effect of desipramine was found in the novel environment ($F_{(1,30)} = 6.52$, $p = 0.016$). Desipramine significantly decreased the latency of *Crtc1*$^{-/-}$ mice ($p = 0.005$, vs. untreated *Crtc1*$^{-/-}$ mice), thus showing that these mice responded positively to desipramine in this paradigm. Comparably to their response to fluoxetine (Breuillaud et al., 2012), *Crtc1*$^{-/-}$ mice show a blunted response to chronic desipramine in the OSFS test and a reduced latency to drink sweetened milk in the anxiogenic environment of the NIH, which suggest a differential involvement of CRTC1 in the behavioral response to antidepressants.

3.2. Desipramine fails to induce expression of neuroplasticity-related genes in Crtc1$^{-/-}$ mice

In order to investigate the molecular mechanisms underlying the blunted behavioral response of *Crtc1*$^{-/-}$ mice to desipramine, gene expression analysis was performed in the HIP and PFC of these animals (Fig. 2). *Crtc1*$^{-/-}$ mice have a reduced expression of several neuroplasticity-related genes in these brain regions involved in depressive disorders (Breuillaud et al., 2012). We were first interested in assessing a possible effect of desipramine on *Crtc1* expression in WT mice (Fig. 2A). Desipramine upregulated *Crtc1* mRNA in the PFC of WT mice ($t = -2.35$, $df = 12$, $p = 0.037$, vs. WT untreated), which further suggests a role for CRTC1 in antidepressant response. Total *Bdnf* expression was then measured in WT and *Crtc1*$^{-/-}$ mice (Fig. 2B). In the HIP, genotype had a significant effect on *Bdnf* expression ($F_{(1,27)} = 4.53$, $p = 0.042$), unlike desipramine that had no effect. Indeed, *Crtc1*$^{-/-}$ mice had lower levels of *Bdnf* mRNA ($p = 0.044$, vs. WT untreated) as previously observed in these animals (Breuillaud et al., 2012). In the PFC, a significant effect of treatment was found ($F_{(1,22)} = 9.08$, $p = 0.006$), which revealed that desipramine significantly induced *Bdnf* expression in WT mice ($p = 0.003$, vs. WT untreated), but failed to do so in *Crtc1*$^{-/-}$ mice. Promoter IV-driven *Bdnf* expression (*BdnfIV*) analysis showed a significant effect of treatment in the HIP ($F_{(1,22)} = 13.46$, $p = 0.001$), as desipramine induced an increased expression of *BdnfIV* mRNA in

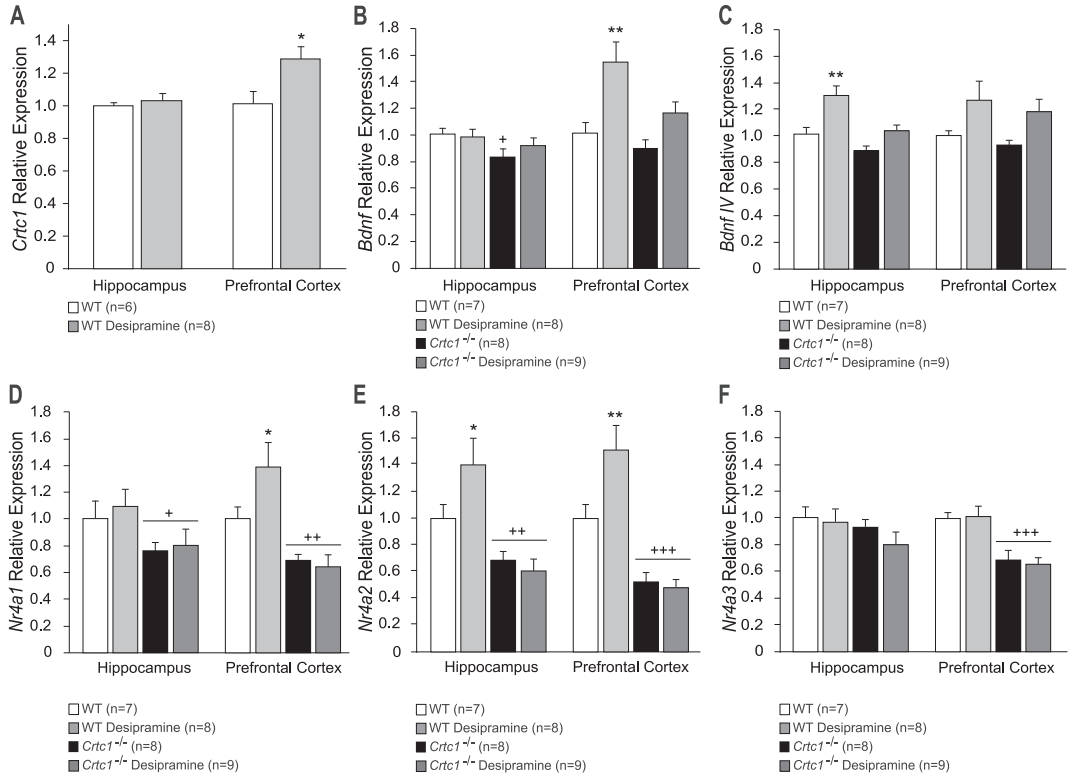

Fig. 2. Molecular effects of desipramine in the HIP and PFC of *Crtc1*$^{-/-}$ mice and WT littermates. (**A**) Desipramine had no effect on *Crtc1* expression in the HIP of WT mice. In the PFC, WT mice treated with desipramine (n = 8) showed increased levels of *Crtc1* expression as compared to untreated WT mice (n = 6) (*$p < 0.05$,vs. WT untreated). (**B**) Untreated *Crtc1*$^{-/-}$ mice (n = 8) displayed lower hippocampal *Bdnf* expression compared to WT mice (n = 7) ($^{+}p < 0.05$, vs. WT untreated). Desipramine had no effect on *Bdnf* expression of WT and *Crtc1*$^{-/-}$ mice (n = 8 and n = 9 respectively). In the PFC, desipramine significantly increased *Bdnf* levels of WT mice (*$p < 0.01$ vs. WT untreated) but not *Crtc1*$^{-/-}$ mice. (**C**) Hippocampal *BdnfIV* expression was increased in desipramine-treated WT mice (**$p < 0.01$, vs. WT untreated), but not in *Crtc1*$^{-/-}$ mice. In the PFC, desipramine did not have a significant effect on both genotypes. (D–F) Effects of desipramine on the expression of *Nr4a1-3*. (**D**) *Crtc1*$^{-/-}$ mice displayed lower levels of *Nr4a1* expression than WT mice in the HIP and PFC ($^{+}p < 0.05$, $^{++}p < 0.01$, vs. WT untreated). Desipramine increased *Nr4a1* levels of WT mice in the PFC but had no effect in the HIP (*$p < 0.05$, vs. WT untreated). (**E**) *Nr4a2* expression was found to be lower in the HIP and PFC of *Crtc1*$^{-/-}$ mice compared to WT mice ($^{+}p < 0.05$, $^{++}p < 0.01$, vs. WT untreated). Desipramine had no effect on *Nr4a2* expression in *Crtc1*$^{-/-}$ mice but increased its expression in the PFC of WT mice (*$p < 0.05$, vs. WT untreated). (**F**) Desipramine increased *Nr4a3* expression in the HIP of WT animal but had no effect on *Crtc1*$^{-/-}$ mice (*$p < 0.05$, vs. WT untreated). *Crtc1*$^{-/-}$ mice displayed lower *Nr4a3* expression than WT animals in the PFC ($+p < 0.05$, vs. WT untreated). Data are mean ± SEM.

WT mice ($p = 0.002$ vs. WT untreated), but not in *Crtc1*$^{-/-}$ mice (Fig. 2C). No significant effect of genotype or treatment was observed in the PFC. We were then interested in measuring *Nr4a1-3* gene expression, as these orphan nuclear receptors are down-regulated in *Crtc1*$^{-/-}$ mice (Breuillaud et al., 2012). Furthermore, NR4A1-3 have been shown to be involved in *Bdnf* regulation, in mood disorders and in neuroprotection (Schaffer et al., 2010; Volakakis et al., 2010; Volpicelli et al., 2007). Analysis of *Nr4a1* expression (Fig. 2D) revealed a significant effect of genotype in the HIP ($F_{(1,27)} = 6.71$, $p < 0.015$), showing that, independently of the treatment, *Crtc1*$^{-/-}$ mice presented decreased levels of *Nr4a1* ($p = 0.025$ vs. WT untreated). In the PFC, genotype had a significant effect ($F_{(1,26)} = 23.84$, $p < 0.001$). Desipramine significantly increased *Nr4a1* expression in WT mice ($p = 0.026$ vs. WT untreated), but not in *Crtc1*$^{-/-}$ mice, which also presented decreased *Nr4a1* levels ($p = 0.003$ vs. WT untreated). Similar results were observed for *Nr4a2* expression (Fig. 2E). A significant effects of genotype was observed in both structures (HIP: $F_{(1,27)} = 22.79$, $p < 0.001$; PFC: $F_{(1,27)} = 39.04$, $p < 0.001$), as *Crtc1*$^{-/-}$ mice presented lower levels of *Nr4a2* expression (HIP: $p = 0.002$; PFC: $p < 0.001$, vs. WT untreated). Furthermore, desipramine induced *Nr4a2* expression in the HIP and PFC of WT mice (HIP: $p = 0.035$; PFC: $p = 0.008$, vs. WT untreated). A significant genotype*treatment interaction was found in the PFC ($F_{(1, 27)} = 5.12$, $p = 0.031$) as desipramine had no effect in *Crtc1*$^{-/-}$ mice. Finally, no effect of genotype or treatment was observed for *Nr4a3* in the HIP (Fig. 2F), whereas only a genotype effect was found in the PFC, ($F_{(1,27)} = 27.59$, $p < 0.001$), as *Crtc1*$^{-/-}$ mice presented decreased *Nr4a3* expression ($p < 0.001$, vs. WT untreated). Altogether, these data show that desipramine fails to induce the expression of *Bdnf*, *Bdnf IV*, *Nr4a1* and *Nr4a2* in the PFC and HIP of *Crtc1*$^{-/-}$ mice, therefore possibly explaining their abnormal behavioral response to this antidepressant.

3.3. SAHA partially rescues the depressive-like phenotype of *Crtc1*$^{-/-}$ mice

As CRTC1 is helping phosphorylated CREB to recruit the histone acetyltransferase CBP, we reasoned that HDAC inhibition might compensate for the possible deficit of CBP recruitment in *Crtc1*$^{-/-}$ mice, and thus rescue the expression of neuroplasticity genes involved in mood regulation. Moreover, several lines of evidence both in humans and in animal models have suggested that pathological conditions may alter the expression of *Hdac* family members, mainly of class I and IIa (Covington et al., 2009; Gräff et al., 2012; Han et al., 2014; Morris and Monteggia, 2013; Tsankova et al., 2006; Uchida et al., 2011). Therefore, we first measured the expression of class I (*Hdac1,2,3)* and class IIa (*Hdac4,5,7,9*) members in the HIP and PFC of WT and *Crtc1*$^{-/-}$ mice to determine whether mutant mice may present a possible altered epigenetic profile

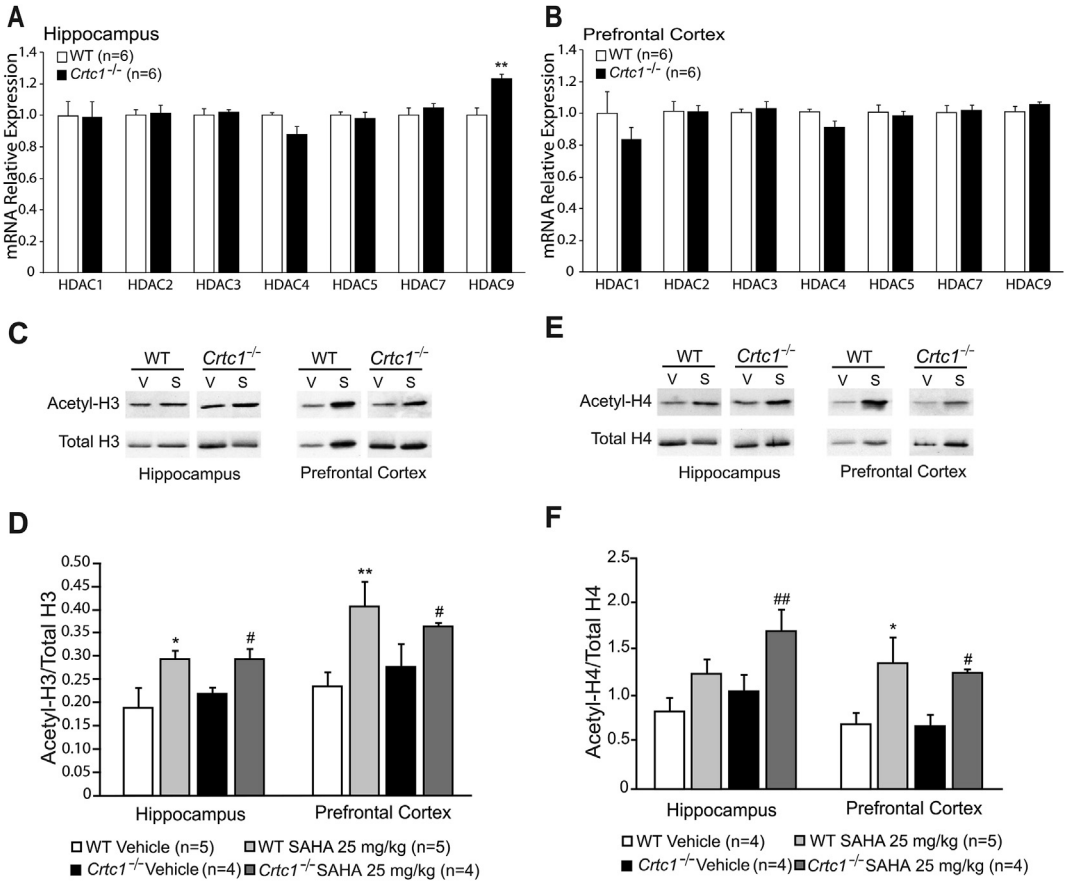

Fig. 3. Expression of *Hdac1,2,3,4,5,7,9* and effects of SAHA in the HIP and PFC of *Crtc1*$^{-/-}$ and WT mice. (**A**) Hippocampal mRNA expression of most *Hdac* class I (1,2,3) and IIa (4,5,7,9) was unchanged in *Crtc1*$^{-/-}$ mice (n = 6) with the exception of *Hdac9* which was upregulated as compared to WT mice (n = 6) (**$p < 0.01$, vs. WT mice). (**B**) No difference of *Hdac* expression in the PFC of *Crtc1*$^{-/-}$ mice. (**C**–**F**) Histone H3 and H4 acetylation 2 h after a single SAHA injection (25 mg/kg) in *Crtc1*$^{-/-}$ and WT mice. Immunoblot analysis of acetylated histones H3 and H4 (acetyl-H3 and acetyl-H4) and total H3 and H4 revealed single bands at the expected sizes of 15 kDa and 10 kDa, respectively. Panel (**C**) shows a representative western blot for acetylated histone H3 (Acetyl-H3) and total histone H3 (total H3) in the HIP and PFC of WT and *Crtc1*$^{-/-}$ mice. (**D**) SAHA-treated WT mice (n = 5) presented higher levels of acetyl-H3 than vehicle-treated WT mice (n = 5) in the hippocampus (*$p < 0.05$, vs. WT Vehicle) and PFC (**$p < 0.01$, vs. WT vehicle). SAHA-treated *Crtc1*$^{-/-}$ mice (n = 4) also presented increased histone H3 acetylation compared to vehicle-treated *Crtc1*$^{-/-}$ mice (n = 4) in the HIP and PFC ($^{\#}p < 0.05$, vs. *Crtc1*$^{-/-}$ Vehicle). Panel (**E**) shows a representative western blot for acetylated histone H4 (Acetyl-H4) and total histone H4 (total H4) in the HIP and PFC of WT and *Crtc1*$^{-/-}$ mice. (**F**) SAHA-treated WT mice (n = 4) presented a non-significant tendency to higher levels of acetyl-H4 than vehicle-treated WT mice (n = 5) in the HIP. In the PFC, SAHA increased levels of H4 acetylation in WT mice (**$p < 0.01$, vs. WT Vehicle). SAHA-treated *Crtc1*$^{-/-}$ mice (n = 4) presented increased histone H4 acetylation compared to vehicle-treated *Crtc1*$^{-/-}$ mice (n = 4) in the HIP and PFC ($^{\#}p > 0.05$, $^{\#\#}p < 0.01$, vs. *Crtc1*$^{-/-}$ Vehicle). Data are mean ± SEM.

(Fig. 3A and B). We found that expression of most *Hdac* tested was globally unchanged in the HIP and PFC of *Crtc1*$^{-/-}$ mice, except for *Hdac9* that was slightly upregulated in the HIP of mutant mice ($t = -4.29$, $df = 10$, $p = 0.002$, vs. WT mice) (Fig. 3A). However, this increased expression of *Hdac9* in the HIP of *Crtc1*$^{-/-}$ mice did not influence global acetylation of histone H3 and H4, as revealed by Western blot analysis (Fig. 3C–F). This suggested that the behavioral and molecular phenotypes of *Crtc1*$^{-/-}$ mice did not result from a major alteration of *Hdac* expression and histone acetylation.

HDAC inhibitors have been shown to have antidepressant effects in rodents and to regulate similar patterns of genes as antidepressants (Covington et al., 2009; Schroeder et al., 2007). SAHA is a hydroxamic acid compound that inhibits class I and II HDACs (Gräff and Tsai, 2013). Systemic administration of SAHA increases histone acetylation in the brain and has antidepressant effects in mice (Hockly et al., 2003; Uchida et al., 2011). Moreover, in primary neuronal cultures, SAHA induces a rapid increase in histone acetylation at *Bdnf* promoters I and IV, and an upregulation of *Bdnf* expression (Koppel and Timmusk, 2013). Therefore, SAHA was chosen to assess the behavioral response of WT and *Crtc1*$^{-/-}$ mice to chronic HDAC inhibition. We first controlled that an intraperitoneal injection of SAHA indeed triggered an increase of histone acetylation in the brain of WT and *Crtc1*$^{-/-}$ mice (Fig. 3C–F). Acetyl-H3 and Acetyl-H4 signals were quantified and normalized over total H3 and total H4 signals. Analysis of histone H3 acetylation (Fig. 3C and D) showed a significant effect of SAHA in the HIP ($F_{(1, 14)} = 11.17$, $p = 0.004$) and in the PFC ($F_{(1,14)}$, $= 13.86$, $p = 0.002$) of WT (HIP: $p = 0.014$, PFC: $p = 0.003$, vs. WT vehicle) and *Crtc1*$^{-/-}$ mice (HIP: $p = 0.017$, PFC: $p = 0.032$, vs. *Crtc1*$^{-/-}$ vehicle). Similarly, SAHA had a significant effect on H4 acetylation (Fig. 3E and F) in the HIP ($F_{(1, 14)} = 9.28$, $p = 0.008$) and in the PFC ($F_{(1,13)} = 13.97$, $p = 0.001$) of WT (PFC: $p = 0.003$, vs. WT vehicle) and *Crtc1*$^{-/-}$ mice (HIP: $p = 0.003$, PFC: $p = 0.034$, vs. *Crtc1*$^{-/-}$ vehicle).

Having shown that SAHA crosses the blood-brain barrier and increases histone acetylation in the brain, we then tested the behavioral response of WT and *Crtc1*$^{-/-}$ mice to chronic SAHA treatment. Animals were first tested in the OSFS protocol (Fig. 4A and B). During the initial four consecutive days (from day −4 to −1), all groups showed an increase in their immobility time, with a significant interaction of time and genotype ($F_{(3,96)} = 3.89$, $p = 0.011$), and *Crtc1*$^{-/-}$ mice progressively displayed increased

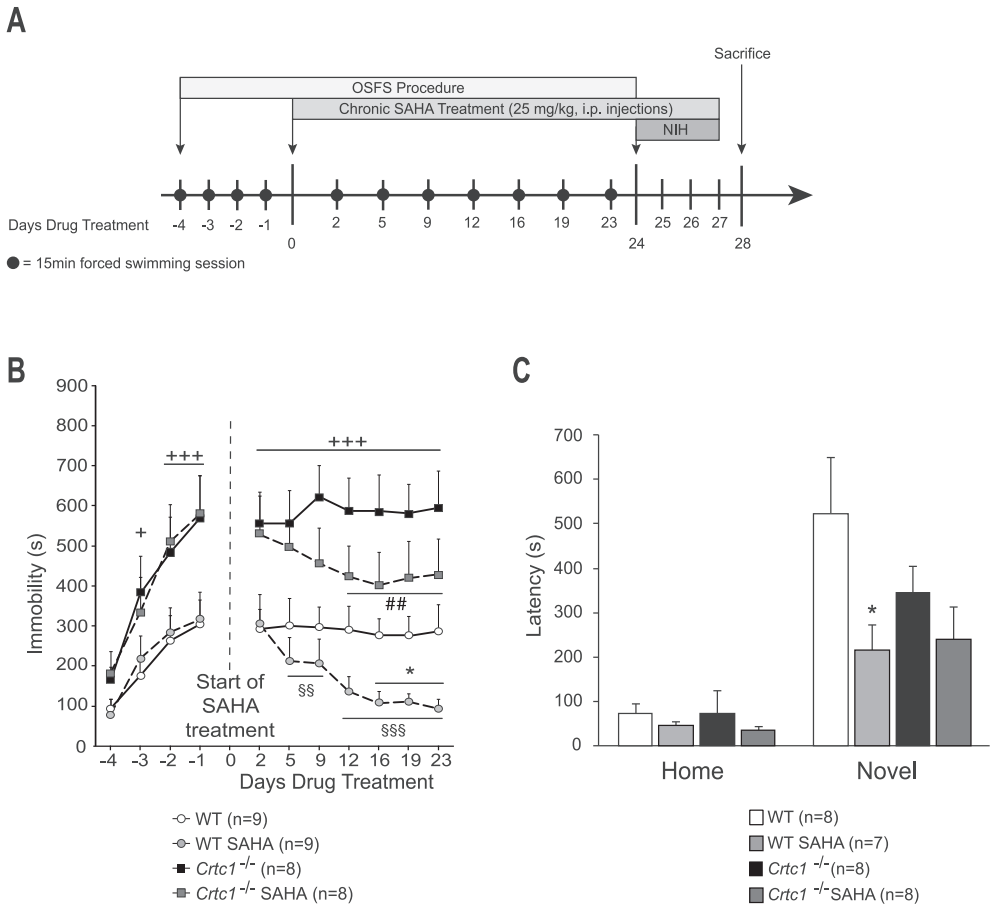

Fig. 4. Behavioral response of *Crtc1*$^{-/-}$ mice to SAHA. (**A**) Experimental design and timeline. The OSFS protocol started when mice reached the age of 8 weeks. Mice underwent forced swimming session during the first four days (day −4 to −1). On day 0, SAHA treatment started and continued until the end of the experiment (last injection on day 27). Swimming session were regularly repeated until day 24. The NIH test was then applied from day 24 until day 27. On day 28, animals were sacrificed. (**B**) Effects of chronic SAHA in the OSFS model of depression on *Crtc1*$^{-/-}$ mice and WT littermates. All groups increased their immobility time during the pre-treatment period (Day −4 to day −1). *Crtc1*$^{-/-}$ mice presented higher immobility time than WT mice starting from day −2 ($^{+}p < 0.05$, $^{+++}p < 0.001$, vs. WT mice). From day 0 until day 23, WT and *Crtc1*$^{-/-}$ mice were daily injected i.p. with either vehicle (n = 9 and n = 8 respectively) or 25 mg/kg SAHA (n = 9 and n = 8 respectively). Vehicle-treated *Crtc1*$^{-/-}$ mice were significantly more immobile that vehicle-treated WT mice during the whole procedure ($^{+++}p < 0.001$, vs. WT Vehicle). SAHA-treated WT mice significantly decreased their immobility time starting from day 2 of treatment ($^{§§}p < 0.01$, $^{§§§}p < 0.001$, vs. themselves on day 2). Their immobility time was also significantly lower than vehicle-treated WT mice during the last three sessions ($^{*}p < 0.05$, vs. WT Vehicle). SAHA-treated *Crtc1*$^{-/-}$ mice significantly decreased their immobility time starting from day 12 of treatment ($^{\#\#}p < 0.01$, vs. themselves on day 2). (**C**) Effects of chronic SAHA on the NIH paradigm. Latencies to drink sweetened condensed milk are shown in the homecage and in the novel environment. Two mice were removed for having latency scores >2 SD from the mean. One mouse was removed for never having drunk the milk during the habituation and test phases. In the homecage conditions, no effect of SAHA was observed in any groups. In the novel environment, SAHA-treated mice (n = 7) presented significant shorter latencies to drink the milk, as compared to vehicle-treated WT mice (n = 8) ($^{*}p < 0.05$, vs. WT Vehicle). SAHA-treated *Crtc1*$^{-/-}$ mice showed a non-significant trend to a shorter latency than vehicle-treated *Crtc1*$^{-/-}$ mice (n = 8 for each group). Data are means ± SEM.

immobility time as compared to WT mice (day −3: $p = 0.02$, day-2: $p = 0.001$, day-4: $p < 0.001$, vs. WT mice). After the beginning of SAHA treatment, a significant effect of genotype ($F_{(1,30)} = 20.21$, $p < 0.001$) and a significant interaction of time and treatment ($F_{(6,180)} = 6.23$, $p < 0.001$) were observed. SAHA-treated mice rapidly decreased their immobility time, as compared to their score at the beginning of the treatment (day 5: $p = 0.01$, day 9: $p = 0.007$, day 12–23: $p < 0.001$, vs. themselves on day 2). Their immobility time was also significantly lower than vehicle-treated WT mice on the last three sessions (day 16: $p = 0.037$, day 19: $p = 0.049$, day 23: $p = 0.046$, vs. WT Vehicle). Vehicle-treated *Crtc1*$^{-/-}$ mice displayed higher immobility time than WT mice during the whole procedure (day 2–23: $p < 0.001$, vs. WT Vehicle). *Crtc1*$^{-/-}$ mice responded to SAHA, as they progressively decreased their immobility time (day 12: $p = 0.007$, day 16: $p = 0.001$, day 19: $p = 0.005$, day 23: $p = 0.009$, vs. themselves on day 2). Animals were then tested in the NIH paradigm (Fig. 4A and C). In the homecage conditions, there was no effect of genotype or treatment, whereas a significant effect of genotype was observed in the novel environment ($F_{(1,26)} = 4.55$, $p = 0.042$), where SAHA decreased the latency of WT mice ($p = 0.039$, vs. WT Vehicle). There was not however a significant effect of SAHA on *Crtc1*$^{-/-}$ mice, although SAHA-treated mutant mice presented a trend to a decreased latency. Taken together, these results show that SAHA improves the phenotype of *Crtc1*$^{-/-}$ mice, as it partially rescued their depressive-like behavior in the OSFS paradigm.

3.4. SAHA partially restores normal gene expression in *Crtc1*$^{-/-}$ mice

Gene expression analysis of *Crtc1*, *Bdnf*, *BdnfIV* and *Nr4a1-3* was performed in order to investigate the effects of chronic SAHA on these genes (Fig. 5). SAHA had no effect on *Crtc1* expression in the HIP and PFC of WT mice (Fig. 5A). A significant effect of genotype on total *Bdnf* expression (Fig. 5B) was seen in the HIP ($F_{(1,26)} = 18.06$, $p < 0.001$), as *Crtc1*$^{-/-}$ mice surprisingly displayed higher *Bdnf*

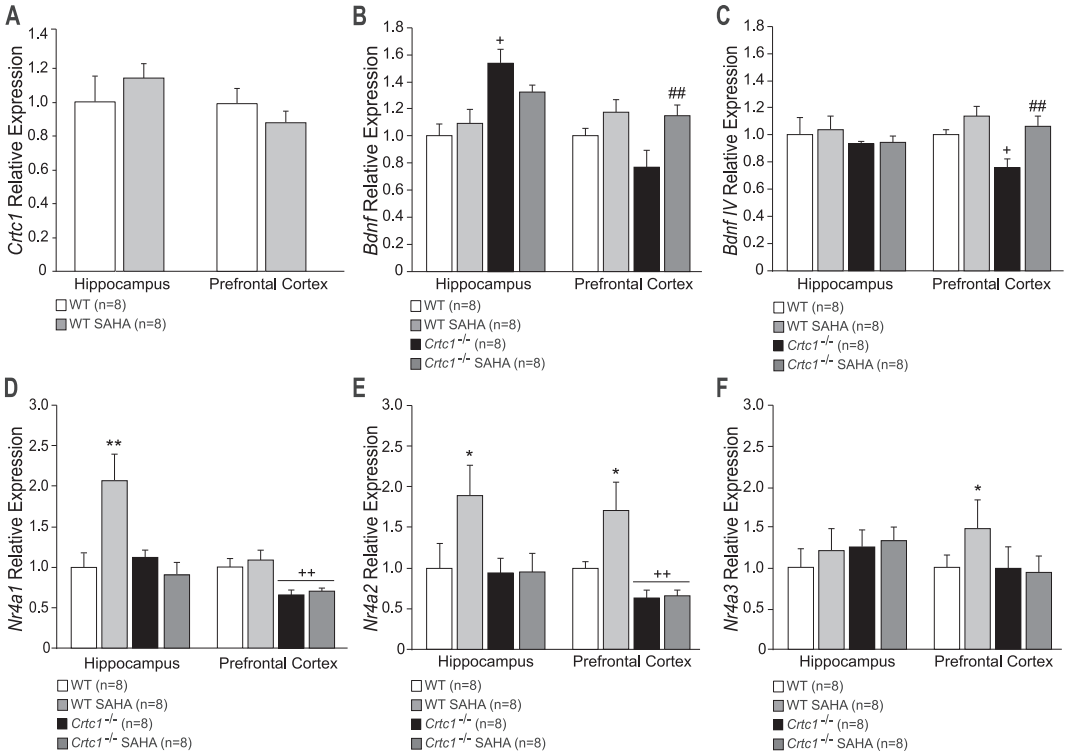

Fig. 5. Molecular effects of SAHA in the HIP and PFC of *Crtc1*$^{-/-}$ mice and WT littermates. (**A**) SAHA had no effect on *Crtc1* expression in the HIP and PFC of WT mice (n = 8 for both group). (**B**) Untreated *Crtc1*$^{-/-}$ mice (n = 8) displayed higher hippocampal *Bdnf* expression compared to WT mice ($^{+}p < 0.05$, vs. WT mice). SAHA had no effect on *Bdnf* expression of WT and *Crtc1*$^{-/-}$ mice (n = 8 for each group). In the PFC, SAHA significantly increased *Bdnf* levels of *Crtc1*$^{-/-}$ mice ($^{\#\#}p < 0.01$ vs. *Crtc1*$^{-/-}$ Vehicle). (**C**) Hippocampal *BdnfIV* expression was unchanged among the different groups. In the PFC, vehicle-treated *Crtc1*$^{-/-}$ mice showed a decreased expression of *BdnfIV* ($^{+}p < 0.05$, vs. WT Vehicle). SAHA treatment significantly restored *BdnfIV* expression in *Crtc1*$^{-/-}$ mice ($^{\#\#}p < 0.01$, vs. *Crtc1*$^{-/-}$ mice Vehicle). (**D**–**F**) Effects of SAHA on the expression of *Nr4a1-3*. (**D**) *Crtc1*$^{-/-}$ mice displayed lower levels of *Nr4a1* expression than WT mice in the PFC ($^{++}p < 0.01$, vs. WT Vehicle). Desipramine increased *Nr4a1* levels of WT mice in the HIP but had no effect in the PFC ($^{**}p < 0.01$, vs. WT Vehicle). (**E**) *Nr4a2* expression was found to be lower in the PFC of *Crtc1*$^{-/-}$ mice compared to WT mice ($^{++}p < 0.01$, vs. WT Vehicle). SAHA had no effect on *Nr4a2* expression in *Crtc1*$^{-/-}$ mice but increased its expression in the HIP and PFC of WT mice ($^{*}p < 0.05$, vs. WT Vehicle). (**F**) SAHA had no effect on *Nr4a3* expression in the HIP of all groups. In the PFC, SAHA increased *Nr4a3* expression of WT animal but had no effect on *Crtc1*$^{-/-}$ mice ($^{*}p < 0.05$, vs. WT Vehicle). Data are mean ± SEM.

mRNA levels than WT mice ($p = 0.02$, vs. WT Vehicle). In the PFC, a significant effect of treatment was observed ($F_{(1,26)} = 8.52$, $p = 0.007$), as chronic SAHA increased *Bdnf* mRNA levels in *Crtc1*$^{-/-}$ mice ($p = 0.009$, vs. *Crtc1*$^{-/-}$ Vehicle). Analysis of *Bdnf* promoter IV expression (Fig. 5C) did not reveal any effect of genotype or treatment in the HIP, whereas a significant genotype and treatment effect was observed in the PFC (genotype: $F_{(1,25)} = 4.82$, $p = 0.037$; treatment: $F_{(1,25)} = 10.11$, $p = 0.003$), where *Crtc1*$^{-/-}$ mice displayed lower expression of *BdnfIV* ($p = 0.039$, vs. WT Vehicle). SAHA treatment successfully restored *BdnfIV* levels in *Crtc1*$^{-/-}$ mice ($p = 0.005$, vs. *Crtc1*$^{-/-}$ Vehicle). Measure of *Nr4a1* mRNA expression (Fig. 5D) showed a significant interaction of genotype and treatment ($F_{(1,25)} = 9.58$, $p = 0.004$), as SAHA increased *Nr4a1* expression in the HIP of WT mice ($p = 0.001$, vs. WT Vehicle), but had no effect in *Crtc1*$^{-/-}$ mice. In the PFC, a significant effect of genotype was observed ($F_{(1,27)} = 15.57$, $p < 0.001$), as *Crtc1*$^{-/-}$ mice showed lower levels of *Nr4a1* (p = 0.005, vs. WT Vehicle). SAHA had no effect on both genotypes in this structure. Similar effects of SAHA on *Nr4a2* expression were observed in the HIP (Fig. 5E), where SAHA increased *Nr4a2* levels in WT mice ($p = 0.001$, vs. WT Vehicle), but had no effect in *Crtc1*$^{-/-}$ mice. SAHA also increased *Nr4a2* levels in the PFC of WT mice ($p = 0.013$, vs. WT mice). A significant effect of genotype was also visible in the same structure ($F_{(1,24)} = 14.12$, $p < 0.001$), as *Crtc1*$^{-/-}$ mice displayed decreased expression of *Nr4a2* ($p = 0.005$, vs. WT Vehicle). Genotype or treatment had no effect on *Nr4a3* mRNA levels in the HIP (Fig. 5F). In the PFC, a significant interaction of genotype and treatment ($F_{(1,25)} = 4.54$, $p < 0.042$) was observed, as SAHA increased *Nr4a3* expression in WT mice only ($p = 0.012$, vs. WT Vehicle).

Taken together, these results interestingly show that chronic SAHA treatment restored normal *Bdnf* and *BdnfIV* expression in the PFC of *Crtc1*$^{-/-}$ mice, and that it increased *Nr4a1-3* expression in WT mice, but not in *Crtc1*$^{-/-}$ mice. However, it is worth noting that the relative gene expression levels of vehicle-injected *Crtc1*$^{-/-}$ mice (Fig. 5, black bars) displayed several discrepancies, mostly in the HIP, as compared with those of mice that were exposed to the same behavioral paradigms, but not chronically injected (Fig. 2, black bars). The most striking difference was the surprising increased *Bdnf* expression in the HIP of vehicle-injected *Crtc1*$^{-/-}$ mice (Fig. 5B). In addition, contrary to our previous observations and the results of Fig. 2, *Nr4a1* and *Nr4a2* levels were not reduced in the HIP of *Crtc1*$^{-/-}$ mice. These inconsistencies prompted us to compare the expression levels of *Bdnf*, *BdnfIV*, and *Nr4a1-3* in WT and *Crtc1*$^{-/-}$ mice control groups of Figs. 2 and 5 (supplementary Fig. S1). The apparent increase of *Bdnf* expression in the HIP of vehicle-injected *Crtc1*$^{-/-}$ mice (Fig. 5B) appeared to be rather due to a decreased *Bdnf* expression in chronically injected WT mice (Fig. S1A). Similarly, hippocampal *Nr4a1-3* levels seemed to be reduced mostly in WT mice chronically injected with vehicle (Fig. S1C and E). With a few exceptions, *Crtc1*$^{-/-}$ mice appeared to be less affected by this chronic injection stress, which might explain the inconsistent data of Fig. 5. In conclusion, the behavioral effect of chronic SAHA administration in *Crtc1*$^{-/-}$ mice might be related to the restoration of normal *Bdnf* and *BdnfIV* expression in the PFC. However, unlike its effect in WT mice, chronic SAHA was not able to induce *Nr4a1-3* expression in *Crtc1*$^{-/-}$ mice, which suggests that the upregulation

of these genes is not required to improve the behavioral despair of these animals in the OSFS paradigm.

4. Discussion

The generation and characterization of *Crtc1*$^{-/-}$ mice highlighted a key role of CRTC1 as a mediator of neuroplasticity-related genes expression potentially involved in the pathophysiology of mood disorders (Breuillaud et al., 2012). Moreover, we showed that CRTC1 is required for proper behavioral response to chronic fluoxetine treatment. In the present study, we further investigated the role of CRTC1 in behavioral and molecular response to antidepressants. We were first interested to assess the effect of another class of antidepressants. The tricyclic antidepressant desipramine was selected as this type of compounds mainly acts on the noradrenergic system, while fluoxetine is a selective serotonin reuptake inhibitor. We observed that *Crtc1*$^{-/-}$ mice responded similarly to desipramine and fluoxetine; i.e. desipramine had no effect on these animals in the OSFS protocol, whereas it had anxiolytic effects in the NIH paradigm. These results further suggest that CRTC1 is involved in behavioral response to antidepressants, mainly in behavioral despair-related paradigms, and that both noradrenergic and serotonergic pathways activated by antidepressants converge on CRTC1. We also observed that desipramine induced the expression of *Crtc1* in the PFC of WT mice, which strengthens the hypothesis of a pivotal role of CRTC1 in antidepressant response.

Interestingly, desipramine, like fluoxetine in our previous study, still elicited behavioral response in *Crtc1*$^{-/-}$ mice in the NIH paradigm. This test has the advantage to be sensitive to chronic, but not acute antidepressant treatment. However, hyponeophagic behavior can be related to depressive but also anxious behavior (Dulawa and Hen, 2005) and both fluoxetine and desipramine have anxiolytic effects (Zohar and Westenberg, 2000). Therefore, the behavioral response of *Crtc1*$^{-/-}$ mice observed in the NIH might be due to the anxiolytic effects of these treatments. The fact that these drugs still have anxiolytic effect in the absence of CRTC1 suggests that these effects are either CRTC1-independent, or that alternative pathways can be used to counteract the absence of CRTC1.

Gene expression analyses revealed that desipramine induced the expression of *Bdnf*, *BdnfIV, Nr4a1,* and *Nr4a2* in the HIP and PFC of WT mice, but failed to do so in *Crtc1*$^{-/-}$ mice. While desipramine has been known for a long time to induce the expression of *Bdnf* (Nibuya et al., 1995), we report here that it also induces the expression of *Nr4a1 and Nr4a2*, which have been linked with mood disorders and *Bdnf* regulation (Buervenich et al., 2000; Volpicelli et al., 2007; Xing et al., 2006). Furthermore, we show that CRTC1 is required for the induction of *Nr4a1 and Nr4a2* by chronic desipramine, thus suggesting a role for these nuclear receptors in the behavioral response to antidepressants.

Considering this hypothesis, we attempted to counteract CRTC1 deletion and restore normal gene expression in *Crtc1*$^{-/-}$ mice by treating them with a HDAC inhibitor. Epigenetic gene regulation has been widely shown to be involved in mood disorders. Mechanisms such as DNA methylation and histone acetylation (mechanisms that respectively repress and activate gene transcription) were found to be altered in depressed patients and in response to chronic stress (Sun et al., 2013; Vialou et al., 2013). Antidepressants also induce epigenetic changes, such as histone H3 hyperacetylation (Tsankova et al., 2006). HDAC inhibitors were found to have antidepressant effects in rodents, and to regulate a subset of genes in a similar way as antidepressants drugs (Covington et al., 2009; Schroeder et al., 2007; Uchida et al., 2011). Of particular interest for this study, *Bdnf* and *Nr4a1-3* have been shown to be induced by HDAC inhibitors (Hawk et al., 2012; Schroeder et al., 2007; Vecsey et al., 2007). In the light of these results, we injected intraperitoneally the HDAC inhibitor SAHA to WT and *Crtc1*$^{-/-}$ mice, and confirmed that a systemic administration of SAHA increased histone acetylation in the brain. Mutant mice responded to chronic SAHA in the OSFS paradigm, as their immobility time decreased over time, indicating a successful antidepressant effect of SAHA in these animals. However, the effect was not complete, as treated *Crtc1*$^{-/-}$ mice did not reach the level of WT animals. This suggests that SAHA was not able to completely rescue the depressive-like behavior of these animals. It is important to keep in mind that *Crtc1*$^{-/-}$ mice are complete knock-out animal, therefore developmental effects of CRTC1 deficiency cannot be excluded. Thus, some behavioral and molecular impairment might not be completely reversible, and might explain the partial behavioral effects of SAHA. Nevertheless, this result indicates that the depressive-like behavior of *Crtc1*$^{-/-}$ mice can be partially rescued by acting at the epigenetic level.

In the NIH, the effect of SAHA was less clear. While it reduced the latency of WT mice, it only produced a trend to a decreased latency in *Crtc1*$^{-/-}$ mice. However, basal latency of vehicle-treated WT animals was abnormally high, which might indicate that the animals had higher basal anxiety, as compared with the NIH experiment of Fig. 1C. This might be explained by a possible effect of the three-week stressful OSFS procedure combined with chronic injections. All in all, NIH results suggest that SAHA had no effect on the anxiety of *Crtc1*$^{-/-}$ mice, yet as WT animals seemed to present unusual higher anxiety, results of this test should be taken with caution.

Molecular investigation of SAHA effects allowed us to better understand the behavioral effects of this treatment on *Crtc1*$^{-/-}$ mice. In the PFC, the decreased *Bdnf* and *BdnfIV* expression was successfully rescued by SAHA treatment. This rescue of *Bdnf* expression may be related to the decreased immobility of *Crtc1*$^{-/-}$ mice in the OSFS, as BDNF alterations have often been linked with behavioral despair (Borsoi et al., 2014; Koponen et al., 2005; Shirayama et al., 2002; Siuciak et al., 1997). While no effect of SAHA was observed in the HIP, *Crtc1*$^{-/-}$ mice elusively displayed higher *Bdnf* expression in this structure, instead of their usual lower levels. The comparison of *Bdnf* levels in control groups of WT and *Crtc1*$^{-/-}$ mice of Figs. 2 and 5 suggested that the stress generated by chronic daily vehicle injections decreased *Bdnf* expression only in WT and not in *Crtc1*$^{-/-}$ mice (Fig. S1). While it is generally suggested that acute and chronic stress decrease *Bdnf* expression (Duman and Monteggia, 2006), several studies have shown opposite findings (Charrier et al., 2006; Marmigere et al., 2003; Nair et al., 2007), thus revealing a complex interplay between stress and BDNF. *Crtc1*$^{-/-}$ mice might thus present an altered reactivity to stress, evidenced by differential *Bdnf* regulation. This highlights again the importance of CRTC1 in the regulation of *Bdnf* and stress response, and should be a focus for future investigations.

Interestingly, SAHA was found to increase *Nr4a1-3* expression in the HIP and/or PFC of WT mice, which could be correlated with its antidepressant effect in these mice. This is in line with previous studies showing that HDAC inhibitors increase *Nr4a1-3* expression via the CREB-CBP pathway, which is a key mechanism for memory enhancement (Hawk et al., 2012; Vecsey et al., 2007). However, SAHA treatment was not able to increase *Nr4a1-3* expression in *Crtc1*$^{-/-}$ mice, and therefore these genes are apparently not involved in the partial rescue of their depressive-like behavior. These results suggest that CRTC1 is required for the CREB-CBP-mediated *Nr4a1-3* transcription induced by HDAC inhibitors. As this pathway is involved in learning processes, these findings are in keeping with the recently characterized important role of CRTC1 in memory and cognitive behavior (Nonaka et al., 2014a, 2014b; Parra-Damas et al., 2014). Further characterization of cognitive and learning behaviors of *Crtc1*$^{-/-}$ mice should better characterize

CRTC1 function in these processes.

5. Conclusions

In this study, we provide evidence that CRTC1 is a key regulator of behavioral and molecular response to antidepressant. We show that CRTC1 is required for complete behavioral antidepressant response and for desipramine-induced expression of *Bdnf* and *Nr4a1-2*. By acting downstream of CRTC1, the HDAC inhibitor SAHA improves the depressive-like behavior of *Crtc1*$^{-/-}$ mice. This partial rescue was paralleled by a restoration of *Bdnf* expression in the PFC, whereas SAHA was unable to increase *Nr4a1-3* expression in these mice, thus suggesting that these orphan nuclear receptors do not play a major role in the improvement of their depressive-like behavior. In conclusion, several lines of evidence strongly suggest that CRTC1-deficient mice are a valuable animal model of depressive phenotypes and treatment resistance. As an important regulatory mediator of depression-related genes, CRTC1 provide a new focus for future research aiming at a better understanding of depression and new therapeutic approaches.

Acknowledgements

This work was funded by a grant from the Swiss National Science Foundation (grant number 31003A-135692), and partly supported by the National Centre of Competence in Research (NCCR) Synapsy.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2016.03.012.

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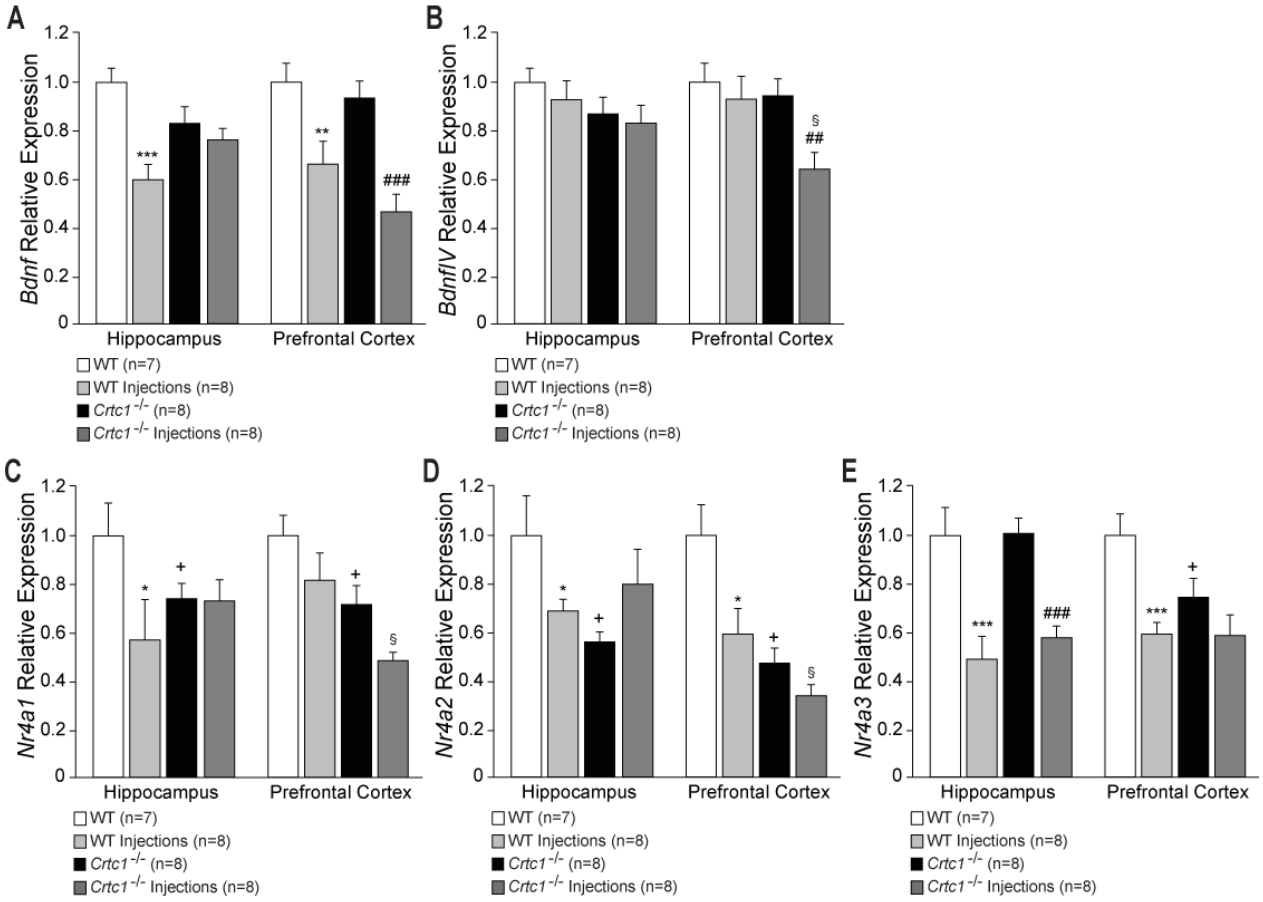

Fig. S1 Effects on gene expression of chronic intraperitoneal injections of vehicle solution (controls of Figure 5) compared to uninjected WT and *Crtc1*$^{-/-}$ mice (controls of Figure 2). **(A)** Effects of chronic injections on total *Bdnf* expression in the hippocampus (HIP) and prefrontal cortex (PFC). Chronic injections reduced *Bdnf* levels of WT in the HIP and PFC (**p<0.01, ***p<0.001) and of *Crtc1*$^{-/-}$ mice in the PFC ($^{\#\#\#}p$<0.001, vs. *Crtc1*$^{-/-}$ mice). **(B)** Effects of chronic injections on *BdnfIV* expression in the HIP and PFC. Chronic injections had no effect on *BdnfIV* levels in the HIP. Injections significantly downregulated *BdnfIV* in the PFC of *Crtc1*$^{-/-}$ mice ($^{\#\#}p$<0.01, vs. *Crtc1*$^{-/-}$ mice), and they also presented lower *BdnfIV* levels than injected WT mice in the same structure ($^{\S}p$<0.05, vs. WT injected). **(C-F)** Effects of chronic injections on *Nr4a1-3* expression in the HIP and PFC. Chronic injections significantly decreased the expression of *Nr4a1-3* in the HIP and of *Nr4a2-3* in the PFC of WT mice (*p<0.05, ***p<0.001, vs. WT mice). Non-injected *Crtc1*$^{-/-}$ mice presented lower *Nr4a1-3* levels in the HIP and PFC than non-injected WT mice (^{+}p<0.05, vs. WT mice), except for *Nr4a3* in the HIP. Injected *Crtc1*$^{-/-}$ mice also displayed lower levels of *Nr4a1* and *Nr4a2* in the PFC ($^{\S}p$<0.01, vs. WT injected). In *Crtc1*$^{-/-}$ mice, chronic injections only reduced the levels of *Nr4a3* in the HIP ($^{\#\#\#}p$<0.001). *: WT vs. WT injected, $^{+}$: WT vs. *Crtc1*$^{-/-}$, $^{\#}$: *Crtc1*$^{-/-}$ vs. *Crtc1*$^{-/-}$ injected, §: WT injected vs. *Crtc1*$^{-/-}$ injected.

OPEN

Citation: Transl Psychiatry (2016) 6, e852; doi:10.1038/tp.2016.116

www.nature.com/tp

ORIGINAL ARTICLE

Involvement of the agmatinergic system in the depressive-like phenotype of the *Crtc1* knockout mouse model of depression

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Recent studies implicate the arginine-decarboxylation product agmatine in mood regulation. Agmatine has antidepressant properties in rodent models of depression, and agmatinase (Agmat), the agmatine-degrading enzyme, is upregulated in the brains of mood disorder patients. We have previously shown that mice lacking CREB-regulated transcription coactivator 1 (CRTC1) associate behavioral and molecular depressive-like endophenotypes, as well as blunted responses to classical antidepressants. Here, the molecular basis of the behavioral phenotype of *Crtc1*$^{-/-}$ mice was further examined using microarray gene expression profiling that revealed an upregulation of *Agmat* in the cortex of *Crtc1*$^{-/-}$ mice. Quantitative polymerase chain reaction and western blot analyses confirmed *Agmat* upregulation in the *Crtc1*$^{-/-}$ prefrontal cortex (PFC) and hippocampus, which were further demonstrated by confocal immunofluorescence microscopy to comprise an increased number of Agmat-expressing cells, notably parvalbumin- and somatostatin-positive interneurons. Acute agmatine and ketamine treatments comparably improved the depressive-like behavior of male and female *Crtc1*$^{-/-}$ mice in the forced swim test, suggesting that exogenous agmatine has a rapid antidepressant effect through the compensation of agmatine deficit because of upregulated *Agmat*. Agmatine rapidly increased brain-derived neurotrophic factor (BDNF) levels only in the PFC of wild-type (WT) females, and decreased eukaryotic elongation factor 2 (eEF2) phosphorylation in the PFC of male and female WT mice, indicating that agmatine might be a fast-acting antidepressant with N-methyl-D-aspartate (NMDA) receptor antagonist properties. Collectively, these findings implicate Agmat in the depressive-like phenotype of *Crtc1*$^{-/-}$ mice, refine current understanding of the agmatinergic system in the brain and highlight its putative role in major depression.

Translational Psychiatry (2016) **6,** e852; doi:10.1038/tp.2016.116; published online 12 July 2016

INTRODUCTION

Major depressive disorder (MDD) is a complex neuropsychiatric disease comprising one of the leading causes of disability worldwide, with an estimated lifetime prevalence of 16%.[1] However, the etiological mechanisms underlying MDD are not clearly established. Studies over the past decades have suggested that altered neuroplasticity is a cardinal feature of MDD,[2] leading to the network hypothesis of depression. This latter proposes that impaired neuroplasticity related to problems in activity-dependent neuronal communication might alter information processing in the affected neural networks, and ultimately cause MDD.[3] In line with this hypothesis, antidepressants have been shown to promote synaptogenesis, neurogenesis and dendritic growth in the hippocampus (HIP) of rodents.[4,5] These neurotrophic effects correlate with positive behavioral responses to antidepressants and are thought to rely, at least partly, on the activation of cAMP-response element-binding protein (CREB)-regulated genes, including increased signaling of the brain-derived neurotrophic factor (BDNF)-TrkB pathway.[4] We and others have previously shown that CREB-dependent *Bdnf* expression requires CREB-regulated transcription coactivator 1 (CRTC1).[6–8] CRTC1 has been shown to act as a neuronal calcium- and cAMP-sensitive coincidence detector and to promote CREB-dependent transcription.[6,9] In addition to its important role in *Bdnf* expression, CRTC1 has also been shown to be critical for specific aspects of neuroplasticity, as evidenced by its role in dendritic growth of developing cortical neurons[10,11] and its requirement for maintenance of long-term potentiation in the HIP.[6,8]

To further understand the role and function of CRTC1, we generated a *Crtc1*-deficient mouse line.[12] These mice present behavioral and molecular features mirroring mood disorders, such as increased behavioral despair, anhedonia, increased irritability/aggressiveness, decreased sexual motivation, social withdrawal, decreased dopamine and serotonin turnover in the prefrontal cortex (PFC), as well as decreased HIP and PFC expression in several neuroplasticity-related genes including *Bdnf* and its receptor *TrkB*.[13] Furthermore, *Crtc1*$^{-/-}$ mice exhibit a blunted antidepressant response to the selective serotonin reuptake inhibitor fluoxetine and to the tricyclic antidepressant desipramine in a behavioral despair paradigm.[13,14] Taken together, these findings suggest an important role for CRTC1 in the etiology of MDD and a possible involvement in treatment-resistant depression.

Substantial evidence supports the involvement of the arginine-decarboxylation product agmatine in MDD. This metabolite is

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Received 17 November 2015; revised 27 April 2016; accepted 29 April 2016

widely expressed in several mammalian organs, including the brain. Agmatine synthesis competes with other arginine-dependent pathways, such as the urea cycle and nitric oxide (NO) synthesis.[15] It is degraded by the enzyme agmatinase (Agmat) into putrescine, a key precursor for polyamine synthesis.[15,16] Accumulating evidence suggests that polyamines and their precursors have a role in the etiology and pathology of mental disorders, notably in mood disorders and suicidal behavior.[17,18] Agmatine has also been proposed to function as a neurotransmitter: it is stored in synaptic vesicles and released upon depolarization, followed by selective reuptake or degradation.[19] In addition, agmatine has the ability to bind a wide range of receptors, including nicotinic receptors, imidazoline I_1 and I_2 receptors, α_2-adrenergic receptors and serotoninergic 5HT-2A and 5HT-3 receptors.[20–23] Remarkably, agmatine also acts as a glutamate N-methyl-D-aspartate receptor (NMDAR) antagonist.[24] This property is particularly interesting in light of the recent and growing interest in glutamate-based rapid-acting antidepressants, whose prototype is the NMDAR antagonist ketamine.[25–29]

Importantly, humans affected by depression show altered blood levels of agmatine, and post-mortem studies have shown increased Agmat levels in brain tissues from depressed individuals.[30,31] In rodents, cortical and hippocampal agmatine levels are decreased by restraint stress, and agmatine demonstrates neuroprotection against acute and chronic stress effects.[32–35] Furthermore, acute agmatine treatment has rapid antidepressant activities in depression-related paradigms such as the forced swim test (FST) and tail suspension test.[36] These effects have been attributed to agmatine actions on monoaminergic and opioid systems, imidazoline and α_2-adrenergic receptors, and NMDAR blockade.[36–39] Moreover, agmatine has the ability to modulate pro- and anti-oxidative balance in the HIP, which might also underlie its behavioral effects.[34] Finally, a recent study has shown that in parallel to its antidepressant activity, agmatine increases HIP CREB phosphorylation and BDNF levels, and induces cell survival pathways.[40] Altogether, these data suggest that agmatine stimulates several endogenous mood-regulating mechanisms known to be altered in MDD, leading to the conclusion that dysregulation of the agmatinergic system could play a role in the etiopathogenesis of MDD and agmatine supplementation might have a positive outcome on the disease.

In this study, we investigated the molecular basis for the depressive-like phenotype of *Crtc1*$^{-/-}$ mice, which led us by differential expression analysis to discover a cortical upregulation of *Agmat* expression. Immunohistochemical studies revealed that mutant mice have an increased number of Agmat-expressing cells in the PFC and HIP, particularly parvalbumin (PV)- and somatostatin (Sst)-positive interneurons. Based on this result, we hypothesized that increased Agmat levels would result in reduced agmatine bioavailability in the brains of *Crtc1*$^{-/-}$ mice, and that supplementation with exogenous agmatine would improve their depressive-like behavior. Indeed, we found that acute agmatine administration had a rapid antidepressant effect both in wild-type (WT) and *Crtc1*$^{-/-}$ mice, the latter requiring a higher dose than WT, in accordance with their increased brain Agmat levels. Finally, we also investigated the molecular mechanisms underlying the rapid antidepressant effects of exogenous agmatine in WT and *Crtc1*$^{-/-}$ mice. We found that agmatine induced BDNF translation in the PFC of WT mice, paralleled by dephosphorylation of eukaryotic elongation factor 2 (eEF2), suggesting NMDAR-mediated antidepressant mechanisms.

MATERIALS AND METHODS

Animals

Crtc1$^{-/-}$ mice and WT littermates were generated and genotyped as previously described.[12] Mice were housed under a 12-h light–dark cycle with *ad libitum* access to water and standard rodent chow diet. All animal experiments were conducted in accordance with the Swiss Federal Veterinary Office's guidelines and were approved by the Cantonal Veterinary Service. All behavioral tests were carried out in the dark phase of the reverse light cycle according to the standard procedures. Male and female mice were weaned at 21 days and housed in same-sex sibling groups until being isolated at 5 weeks of age in order to avoid wounding of cage mates by aggressive *Crtc1*$^{-/-}$ male mice.[13] At the age of 8 weeks, animals were randomly assigned into treatment groups and either killed for molecular experiments or used for behavioral assessments.

Brain microdissection

Male and female mice were killed by cervical dislocation, and the brain was rapidly placed in a stainless steel adult mouse brain slicer matrix with 1-mm coronal section slice intervals. A first cut included the PFC from which the olfactory bulbs and associated structures were removed. Total hippocampi were unrolled from the cortex. All the structures were sequentially quick-frozen in dry ice for mRNA and protein extraction and stored at − 80 °C until further processing.

Gene expression analysis

Total RNA was extracted and purified from the PFC and HIP using the RNAeasy Plus Minikit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturers' instructions. RNA concentrations were measured by Ultraviolet spectophotometry with a NanoDrop Lite (Thermo Scientific, Wilmington, DE, USA). Complementary DNA was prepared in a 50-µl reaction by reverse transcription, using 200 ng of RNA with Taqman Reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). Complementary DNA (0.8 µl) was amplified on a 96-well plate using the SYBR Green PCR Master Mix (Applied Biosystem). Amplification was performed with an ABIPRISM 7500 real-time PCR system (Applied Biosystem). The program was 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative gene expression was quantified using the comparative *ΔΔ Ct* method and normalized with β-actin transcript levels.

The following primers were used at a concentration of 250 nM: *β-actin* forward 5′-GCTTCTTTGCAGCTCCTTCGT-3′, *β-actin* reverse 5′-ATATCGTCATCCATGGCGAAC-3′, *Agmat* forward 5′-TGGACAGCAAGCGAGTGGTACA-3′, *Agmat* reverse 5′-GGACCAGTGACTTCATCCAACAG-3′.

Affymetrix gene expression arrays

Gene expression levels were evaluated using DNA microarrays (GeneChip Mouse Genome 430 version 2.0, Affymetrix, Santa Clara, CA, USA) and RNA from the cerebral cortices of female mice ($n = 5$ for WT and $n = 5$ for *Crtc1*$^{-/-}$). Biotinylated cRNAs were prepared from 300 ng total RNA using the GeneChip 3′ IVT Express Kit (Affymetrix) following the manufacturer's instructions. cRNA (15 µg) was hybridized to GeneChip arrays and processed, stained and scanned according to the manufacturer's recommendations. The quality of input RNAs and cRNAs was verified with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) before use. Microarray quality control was performed using the software package provided on RACE.[41] Chips with a median-normalized unscaled s.e. greater than 1.05 were excluded. Affymetrix annotations (version 3.0) were used for probeset-to-gene assignments. Mod *t*-statistics and false discovery rate corrections for multiple testing with a significance threshold of $P < 0.05$ were used as criteria for differential expression, as described in Hochberg and Benjamini.[42] Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE80633 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE80633).

Western blot

PFC and HIP samples were manually homogenized with a microtube pestle in RIPA buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA (pH 7.5), 2.5 mM EGTA (pH 8.0), 10% glycerol, 1% NP-40, 1% deoxycholate, 0.1% SDS, with a protease inhibitor cocktail (Sigma, St Louis, MO, USA) and a phosphatase inhibitor cocktail (PhosSTOP, Roche, Rotkreuz, Switzerland) and extracted for 20 min at 4 °C. Protein quantification was performed with the Pierce BCA Protein Assay Kit (Thermo Scientific). Samples with low-protein extract (< 2 mg ml^{-1}) were excluded. Fifty µg of tissue homogenates were diluted 1:1 with sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 9% glycerol, 1% bromophenol blue), separated on a 12% SDS-polyacrylamide gel and proteins were transferred to polyvinylidene difluoride membranes with a semi-dry blotting system

(Bio-Rad, Hercules, CA, USA). Blots were blocked for 1 h at room temperature (RT) in TBST (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20), supplemented with 5% skim milk powder. Blots were subsequently incubated with a primary antibody in TBST plus 5% bovine serum albumin overnight at 4 °C. Finally, polyvinylidene difluoride membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies in TBST plus 5% skim milk powder, and were developed using a Pierce ECL Western Chemiluminescence Detection Kit (Thermo Scientific). The following antibodies and dilutions were used: rabbit α-Agmat 1:400 (sc-98802, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit α-BDNF 1:500 (sc-546, Santa Cruz Biotechnology), rabbit α-phospho-eEF2 1:1000 (#2331, Cell Signaling, Danvers, MA, USA), rabbit α-eEF2 1:1000 (#2332, Cell Signaling), mouse α-β-actin 1:10 000 (Ab-6276, Abcam, Cambridge, UK), donkey HRP-α-rabbit 1:2000 (#NA934, GE Healthcare, Little Chalfont, UK) and sheep HRP-α-mouse 1:2000 (#NA931, GE Healthcare). Quantification of band intensity was performed with the Image J software (National Institute of Health, Bethesda, MD, USA). Agmat and BDNF band intensities were normalized with β-actin signals; phospho-eEF2 band intensities were normalized with total eEF2 signals.

Immunofluorescence

Eight-week-old male mice were deeply anesthetized using sodium pentobarbital and intracardially perfused with saline followed by 4% buffered paraformaldehyde. Brains were dissected out, postfixed for 1 h in 4% paraformaldehyde and cryoprotected in 30% sucrose. Brain sections of 35 µm were cut with a freezing microtome (Microm, Thermo Fisher Scientific, Waltham, MA, USA) and stored at − 20 °C in a cryoprotectant solution. Blocking (1 h, RT) as well as primary (overnight, 4 °C) and secondary antibody incubation (1 h, RT) were performed in phosphate-buffered saline+0.3% Triton X-100+2% normal horse serum+0.2% bovine serum albumin. Slices were washed three times in phosphate-buffered saline+0.3% Triton X-100 after each incubation. The following antibodies and dilutions were used: rabbit α-Agmat 1:100 (sc-98802, Santa Cruz Biotechnology), mouse α-Parvalbumin 1:2500 (PV235, Swant, Marly, Switzerland), mouse α-Calretinin 1:2500 (CR7697, Swant), goat α-Somatostatin 1:500 (sc-7819, Santa Cruz Biotechnology), Cy3-conjugated donkey α-rabbit 1:500 (#711-165-152, Jackson Immunoresearch, West Grove, PA, USA), Alexa Fluor 488-conjugated goat α-mouse 1:500 (A-21121, Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-conjugated donkey α-goat 1:500 (A11055, Invitrogen, Carlsbad, CA, USA). After the secondary antibody incubation, slices were washed, stained with 4,6-diamidino-2-phenylindole 1:30 000 (Invitrogen), mounted on glass slides with the antifade Vectashield medium (Vector Laboratories, Burlingame, CA, USA) and analyzed with a Zeiss LSM 710 Quasar Confocal Microscope (Carl Zeiss, Oberkochen, Germany). Image processing and cell counting were carried out with the Image J Software (National Institute of Health).

Agmatine and ketamine treatment

Agmatine sulphate salt and ketamine (Ketanarkon) were, respectively, purchased from Sigma and Streuli Pharma (Uznach, Switzerland) and dissolved in saline solution. Male and female mice were intraperitoneally injected with 10 ml kg^{-1} of agmatine (10 or 50 mg kg^{-1}) or ketamine (3 mg kg^{-1}). Controls were injected with saline. Injections were performed 30 min before the FST for two consecutive days.

FST

A 2-day test was performed (days 1 and 2). Mice were put during 5 min in a 5-l glass beaker (26 cm tall, ø18 cm) filled to a depth of 22 cm with tap water (25 ± 1 °C). Sessions were videotaped from above and manually analyzed non-blindly by the experimenter with the Ethovision 3.1 Software (Noldus Information Technology, Wageningen, The Netherlands) for immobility and climbing time. Mice were judged immobile when no detectable movement was observed, except for minor movements to keep their head above the water. The experiment was conducted in a room with a light intensity of ~ 35 lux. Immediately after the test on day 2, mice were killed for BDNF and phospho-eEF2 measurements.

Statistical analyses

The number of animals tested in each group is specified in the figure legends. Owing to the small number of WT and *Crtc1*$^{-/-}$ mice per litter and a limited breeding cage space, all experiments were performed sequentially with several batches of mice, and their data were combined. Sample sizes were determined based on power analysis and common practice in behavioral experiments (~10 animals per group). Statistical analyses (other than those employed for microarray analyses (see above)) were performed using the Statistica 8.0 Software (StatSoft, Tulsa, OK, USA). All data are presented as mean ± s.e.m. $P < 0.05$ were considered statistically significant. A Shapiro–Wilk test and a Levene test were first performed to assess data normality and variance homogeneity. All results were found to follow normal distribution and to display similar variance. For immunofluorescence, quantitative PCR and western blot data, a two-tailed Student's *t*-test was performed when only two groups were compared (WT versus *Crtc1*$^{-/-}$ mice). For behavioral data, BDNF and phospho-eEF2 data, a two-way analysis of variance (ANOVA; with genotype and treatment as independent variables) was performed, followed by a Fisher's Least Significant Difference (LSD) *post hoc* test.

RESULTS

Male and female *Crtc1*$^{-/-}$ mice exhibit increased levels of *Agmat* mRNA and protein in the PFC and HIP

To identify gene expression changes associated with *Crtc1* deficiency, we performed genome-wide transcriptomic profiling analyses of cortical samples from *Crtc1*$^{-/-}$ and WT female mice using oligonucleotide microarrays (Table 1). Among the down-regulated genes were CREB target genes that we previously showed to have a decreased expression in the PFC and HIP of *Crtc1*$^{-/-}$ male mice.[13] Interestingly, a few genes were upregulated in *Crtc1*$^{-/-}$ mice, and amidst them *Agmat*, whose expression was increased by 1.67-fold. To follow up and confirm this finding, we measured *Agmat* messenger RNA (mRNA) and protein levels in male and female *Crtc1*$^{-/-}$ mice (Figure 1). We focused our investigations on the PFC and HIP, as these two regions are widely implicated in mood disorders and are known to have high levels of agmatine.[43] Quantitative PCR analyses of *Agmat* mRNA levels found a significant ($t = -3.31$, degree of freedom (df) = 9, $P = 0.013$) 1.5-fold increase of *Agmat* mRNA in the HIP and a threefold increase ($t = -6.72$, df = 8, $P < 0.001$) in the PFC of male *Crtc1*$^{-/-}$

Table 1. Selection of genes differentially expressed in the cortex of *Crtc1*$^{-/-}$ mice

Symbol	*Gene*	*GenBank*	*Fold change*	*FDR* P
Cartpt	CART prepropeptide	NM_013732	0.42	0.0086
Nr4a1	Nuclear receptor subfamily 4, group A, 1	NM_010444	0.50	0.0181
Nr4a3	Nuclear receptor subfamily 4, group A, 3	NM_015743	0.52	0.0103
Crem	cAMP responsive element modulator	NM_001110859	0.59	0.0028
Bdnf	Brain-derived neurotrophic factor	NM_007540	0.61	0.0131
Nr4a2	Nuclear receptor subfamily 4, group A, 2	NM_013613	0.70	0.0476
Ntrk2	Neurotrophic tyrosine kinase receptor, 2 (TrkB)	NM_008745	0.81	0.0108
Agmat	Agmatine ureohydrolase (agmatinase)	NM_001081408	1.67	0.0099

Abbreviation: FDR *P*, false discovery rate corrected *P*-value.

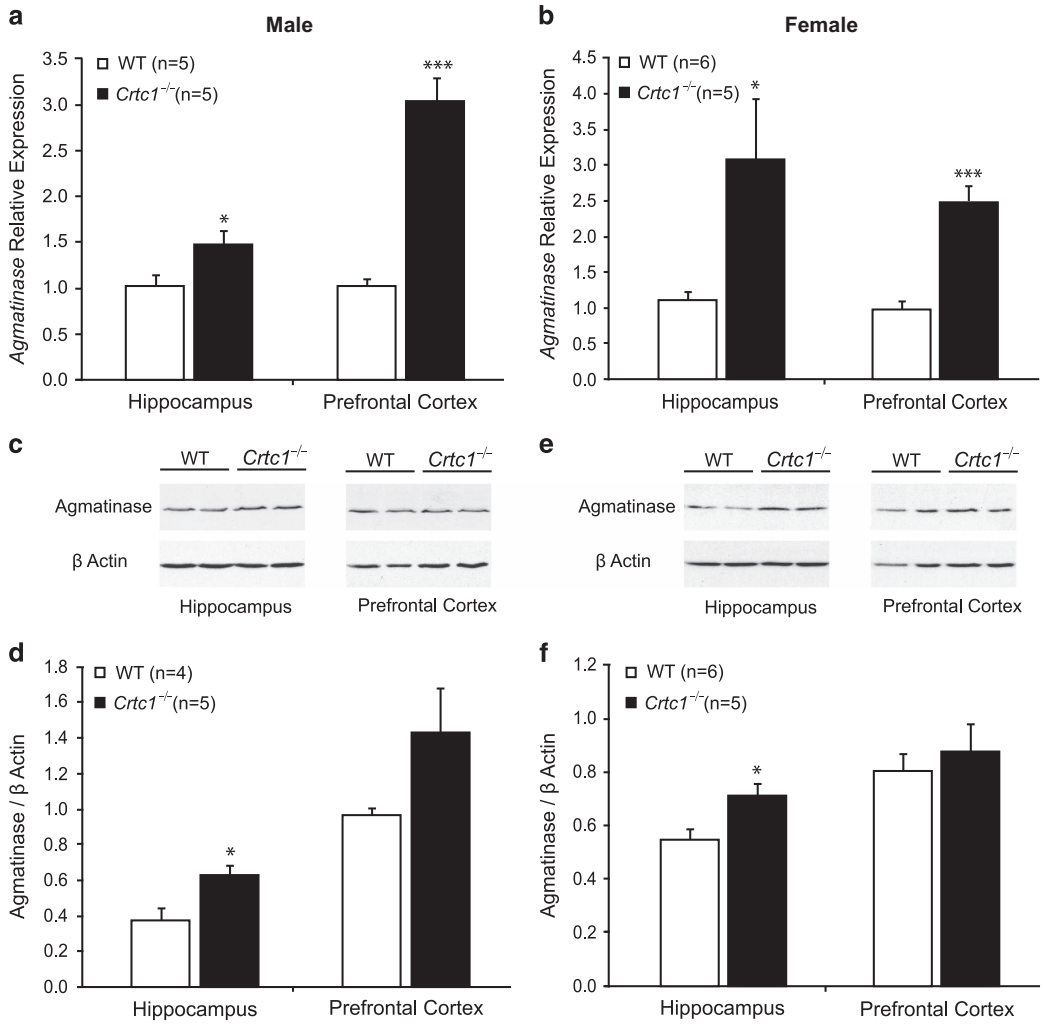


Figure 1. Increased levels of agmatinase (Agmat) in *Crtc1*$^{-/-}$ mice. (**a**) Real-time quantitative PCR measurements showed an increased expression of *Agmat* in the hippocampus (HIP) and prefrontal cortex (PFC) of male *Crtc1*$^{-/-}$ mice ($n=5$) compared with wild-type (WT) littermates ($n=5$). (**b**) *Agmat* was also overexpressed in the HIP and PFC of female *Crtc1*$^{-/-}$ mice ($n=6$) compared with WT control mice ($n=5$). Representative western blot of Agmat and β-actin is shown in **c** for male mice and in **e** for female mice. Quantitative analyses of western blot showed increased protein levels of Agmat in the HIP of male *Crtc1*$^{-/-}$ mice ($n=4$) compared with WT mice ($n=5$**; d**). Protein levels of Agmat were also increased in the HIP of female *Crtc1*$^{-/-}$ mice ($n=6$) compared with WT mice ($n=5$; **f**). Results are presented as ratio between Agmat and β-actin signals. Data are mean ± s.e.m. $^{*}P<0.5$, $^{***}P<0.001$.

mice (Figure 1a). In female *Crtc1*$^{-/-}$ mice, a significant ($t=-2.65$, df = 9, $P=0.029$) threefold increase in *Agmat* mRNA was observed in the HIP and a 2.5-fold increase ($t=5.45$, df = 9, $P<0.001$) in the PFC (Figure 1b). Western blot analysis of extracts from these same structures showed an Agmat protein band at the expected size of ~35 kDa (Figures 1c and e), which was subsequently quantified (normalized to β-actin signal). *Crtc1*$^{-/-}$ male mice had increased levels of Agmat protein in the HIP ($t=-2.72$, df = 6, $P=0.036$), whereas a nonsignificant trend ($t=-1.51$, df = 9, $P=0.152$) of increased Agmat was observed in the PFC (Figure 1d). In female *Crtc1*$^{-/-}$ mice, a similar increase was found in the HIP ($t=-2.58$, df = 9, $P=0.027$), whereas no change in Agmat protein content could be seen in the PFC ($t=-0.61$, df = 9, $P=0.553$; Figure 1f). Taken together, these results confirm an upregulation of *Agmat* gene expression in the HIP and PFC of *Crtc1*$^{-/-}$ mice, independently of gender. Although Agmat protein levels only partially correlated with the gene expression data (which might reflect translational regulation or a complex subcellular protein localization), these results strongly suggest that *Crtc1*$^{-/-}$ mice have an altered agmatinergic system.

Crtc1$^{-/-}$ mice have an increased number of Agmat-expressing cells in the PFC and in several regions of the HIP

To determine whether the increased *Agmat* expression in *Crtc1*$^{-/-}$ mice was because of higher Agmat levels or an increased number of Agmat-expressing cells, we visualized Agmat protein expression using immunofluorescence in the PFC and in the CA1, CA3 and dentate gyrus (DG) subregions of the HIP (Figure 2). Staining revealed numerous cells in the PFC (Figure 2a), and in the DG (Figure 2b), CA1 (Figure 2c) and CA3 (Figure 2d) regions of the HIP, and its subcellular localization appeared mainly perinuclear within those structures. In the HIP, staining could be observed in the pyramidal cell layer, but appeared stronger in interneuron-like cells. Agmat-expressing cells were counted and results were normalized to total numbers of cells, counted with 4,6-diamidino-2-phenylindole staining (Figure 2e). Agmat-positive cells counting revealed a significant increased number of cells in the PFC of *Crtc1*$^{-/-}$ mice (+60%, $t=-4.23$, df = 9, $P=0.001$) as compared with WT littermates. This could also be observed in the DG (+45%, $t=-2.55$, df = 9, $P=0.029$) and CA1 regions of the HIP (+70%,

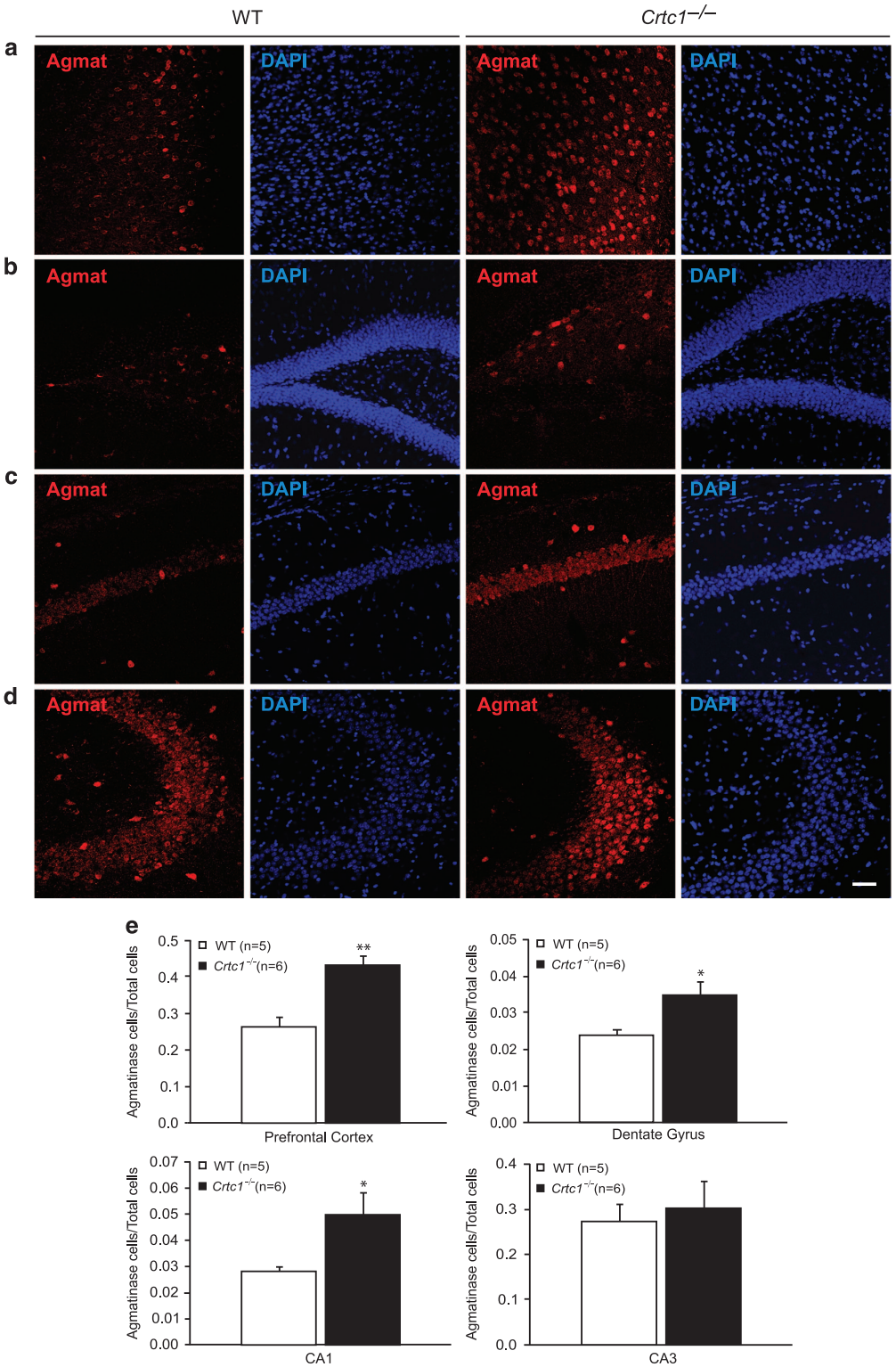


Figure 2. Increased number of agmatinase (Agmat)-expressing cells in male *Crtc1*$^{-/-}$ mice. Representative immunofluorescence staining of Agmat-expressing cells (red) and total cells (4,6-diamidino-2-phenylindole (DAPI) staining) in the prefrontal cortex (PFC; **a**), and in the dentate gyrus (DG; **b**), CA1 (**c**) and CA3 (**d**) regions of the hippocampus (HIP). Cell counting resulted in an increased number of Agmat-expressing cells in the PFC, DG and CA1 regions of male *Crtc1*$^{-/-}$ mice ($n = 6$) compared with wild-type (WT) littermates ($n = 5$; **e**). No difference in number of Agmat-expressing cells was found in the CA3 region. Results are expressed as ratio between number of Agmat-expressing cells and total number of cells. Data are mean ± s.e.m. *$P < 0.5$, **$P < 0.01$. Scale bar, 50 μm.

$t = -2.28$, df = 9, $P = 0.049$). No differences in the numbers of Agmat-expressing cells could be seen in the CA3 region of the HIP ($t = -0.40$, df = 9, $P = 0.698$). Overall, these data suggest that the increased Agmat expression found in *Crtc1*$^{-/-}$ mice would be the result of a higher number of Agmat-expressing cells in the PFC and selected HIP subregions. Moreover, the morphology and localization of the hippocampal cells expressing higher Agmat levels indicated that they could be GABAergic interneurons.

Characterization of Agmat-expressing cells in the PFC and HIP

As hippocampal Agmat-expressing cells have an interneuron-like morphology, we characterized Agmat-expressing cells with markers for specific GABAergic interneuron types. Bernstein *et al.*[44] studied regional and cellular expression of *Agmat* in the rat brain and found that Agmat colocalized with calretinin (CR)-expressing interneurons in the cortex and CA1 region of the HIP.[44] We therefore performed double immunolabeling of CR and Agmat in the PFC and HIP of WT mice (Figure 3 and Supplementary Figure S1). In the PFC, we observed very little colocalization between CR and Agmat staining (Figure 3a, double-labeled cells indicated with arrows). Little colocalization was also seen in the CA3 region of the HIP (Figure S1b). Moreover, no colocalization was observed in the DG and CA1 regions of the HIP (Figure 3b and Supplementary Figure S1a, respectively). In order to further investigate which type of interneurons expressed Agmat, we performed double immunostaining of Agmat- and PV-expressing cells. We observed high colocalization of Agmat and PV cells in all studied structures: PFC, DG, CA1 and CA3 regions of the HIP (Figures 3c and d and Supplementary Figure S1c and d, respectively). Indeed, nearly all PV-expressing cells were also Agmat-positive. On the other hand, there were some Agmat-expressing cells that were not PV-positive, therefore suggesting that other types of cells express Agmat.

Several lines of evidence involved *somatostatin* (*Sst*) in mood disorders,[45] and we previously observed that *Sst* was down-regulated in the brain of *Crtc1*$^{-/-}$ mice.[13] Therefore, we investigated whether *Agmat* might be expressed in somatostatinergic interneurons by performing double immunostaining of Agmat- and Sst-expressing cells. As for PV staining, most of Sst-expressing cells were colocalized with Agmat in all regions observed: PFC, DG, CA1 and CA3 regions of the HIP (Figures 3e and f and Supplementary Figure S1e and f, respectively). Colocalization was, however, less extended in the PFC; as some Sst-expressing cells were not colocalized with Agmat staining.

Altogether, these data confirm the expression of Agmat in specific GABAergic interneuron subpopulations, with apparent high expression in PV and Sst interneurons, and slight colocalization with CR interneurons.

Rapid ketamine-like antidepressant effect of acute agmatine in male and female WT and *Crtc1*$^{-/-}$ mice

Given the possible role of an agmatine deficit in depression and the observed increase in Agmat levels in the brain of *Crtc1*$^{-/-}$ mice, we postulated that their depressive-like behavior is due, at least in part, to a dysregulated agmatinergic system. To test this hypothesis, we treated WT and *Crtc1*$^{-/-}$ mice with acute intraperitoneal (IP) injections of agmatine and tested antidepressant effects in the FST, a classical test for rodent depression-related behavior. We hypothesized that restoring agmatine levels by exogenous supplementation would normalize behavioral response to the helplessness-inducing effects of FST. We first treated the animals with agmatine at 10 mg kg^{-1} and compared their depressive-like behavior in the FST with saline-injected control animals. This protocol was repeated a second time on the next day. The antidepressant effects of agmatine were assessed by measuring the floating (immobility) time of the mice. Agmatine had a significant antidepressant effect on WT mice but failed to significantly reduce *Crtc1*$^{-/-}$ mouse immobility time (data not shown). However, a tendency to decrease the immobility time of *Crtc1*$^{-/-}$ mice prompted us to repeat this experiment with an increased dose of agmatine (50 mg kg^{-1}, IP; Figures 4a and b). For male mice (Figure 4a), a significant effect of genotype on immobility could be seen on both days of test, as shown by two-way ANOVA (Day 1: $F_{(1,29)} = 6.74$, $P = 0.014$; Day 2: $F_{(1,29)} = 19.4$, $P < 0.001$). *Post hoc* analyses revealed that vehicle-treated *Crtc1*$^{-/-}$ mice presented higher immobility time than WT mice (Day 1: +13%, $P = 0.031$, Day 2: +22%, $P = 0.004$). A significant treatment effect could be observed on the second day of test ($F_{(1,29)} = 12.38$, $P = 0.001$) as agmatine significantly decreased the immobility time of WT and *Crtc1*$^{-/-}$ mice ($P = 0.012$ and $P = 0.026$, respectively). For female mice (Figure 4b), the analysis of immobility time by two-way ANOVA revealed a significant treatment effects for both days of test (Day 1: $F_{(1,28)} = 10.28$, $P = 0.003$; Day 2: $F_{(1,28)} = 23.46$, $P < 0.001$). *Post hoc* analyses showed that agmatine significantly reduced immobility time in WT female mice on day 1 (−30%, $P = 0.014$) and of both genotypes on day 2 (WT: − 30%, $P = 0.003$; *Crtc1*$^{-/-}$: − 30%, $P = 0.001$). Thus, 50 mg kg^{-1}of agmatine reduced the immobility time of male and female *Crtc1*$^{-/-}$ mice. Overall, these data confirm the antidepressant effect of an acute agmatine treatment in WT animals. *Crtc1*$^{-/-}$ mice also respond to the antidepressant effect of agmatine, but require a higher dose (50 mg kg^{-1}).

Ketamine and other NMDAR antagonists have been shown to have rapid and long-lasting antidepressant effects in behavioral despair paradigms such as the FST.[27] As agmatine also acts as a NMDAR antagonist,[24] we assessed the antidepressant effect of ketamine in male and female WT and *Crtc1*$^{-/-}$ mice (Supplementary Figure S2). Interestingly, ketamine (3 mg kg^{-1}, IP) significantly decreased the depressive-like behavior of WT and *Crtc1*$^{-/-}$ mice of both sexes in a very similar way as agmatine did, which suggested that the rapid antidepressant action of agmatine and ketamine may involve the same molecular pathways.

Characterization of pathways involved in agmatine antidepressant effect

Although agmatine antidepressant effects have been established, the underlying molecular mechanisms remain unclear. A recent study suggested that chronic agmatine treatment induces an increase of BDNF protein levels, as well as an increased phosphorylation of CREB, PKA and other kinases involved in pathways associated with neuroplasticity.[40] Although the underlying cellular and molecular mechanisms of ketamine's antidepressant action are not completely understood, they involve the rapid induction of BDNF translation via activation of the mammalian target of rapamycin (mTOR) pathway as seen by dephosphorylation of eEF2.[27] Therefore, we investigated whether agmatine's rapid antidepressant effect involved NMDAR blockade-associated changes in BDNF levels and eEF2 phosphorylation. We measured the levels of phospho-eEF2 and BDNF proteins in the PFC and HIP of male and female mice treated with 50 mg kg^{-1} (Figures 4c-j). Western blot for phospho-eEF2 and total eEF2 showed a single band at the expected size of 95 kDa (Figures 4c and d). Phospho-eEF2 signal was quantified and normalized over total eEF2 signal. In male mice (Figure 4e), a two-way ANOVA showed no effect of genotype or treatment in the HIP. A significant effect of genotype could be seen in the PFC ($F_{(1,23)} = 13.34$, $P = 0.001$) as both groups of *Crtc1*$^{-/-}$ mice presented lower levels of phospho-eEF2 ($P = 0.001$ and $P = 0.002$). Agmatine treatment had no effect on p-eEF2 levels of *Crtc1*$^{-/-}$ mice, but it significantly reduce p-eEF2 in WT mice ($P = 0.04$). In female mice (Figure 4f), a two-way ANOVA showed no effect of genotype or treatment in the HIP. A trend to decreased levels of phosphorylated eEF2 was present in agmatine-treated WT mice and in both groups of *Crtc1*$^{-/-}$ animals. In the PFC, significant effects of

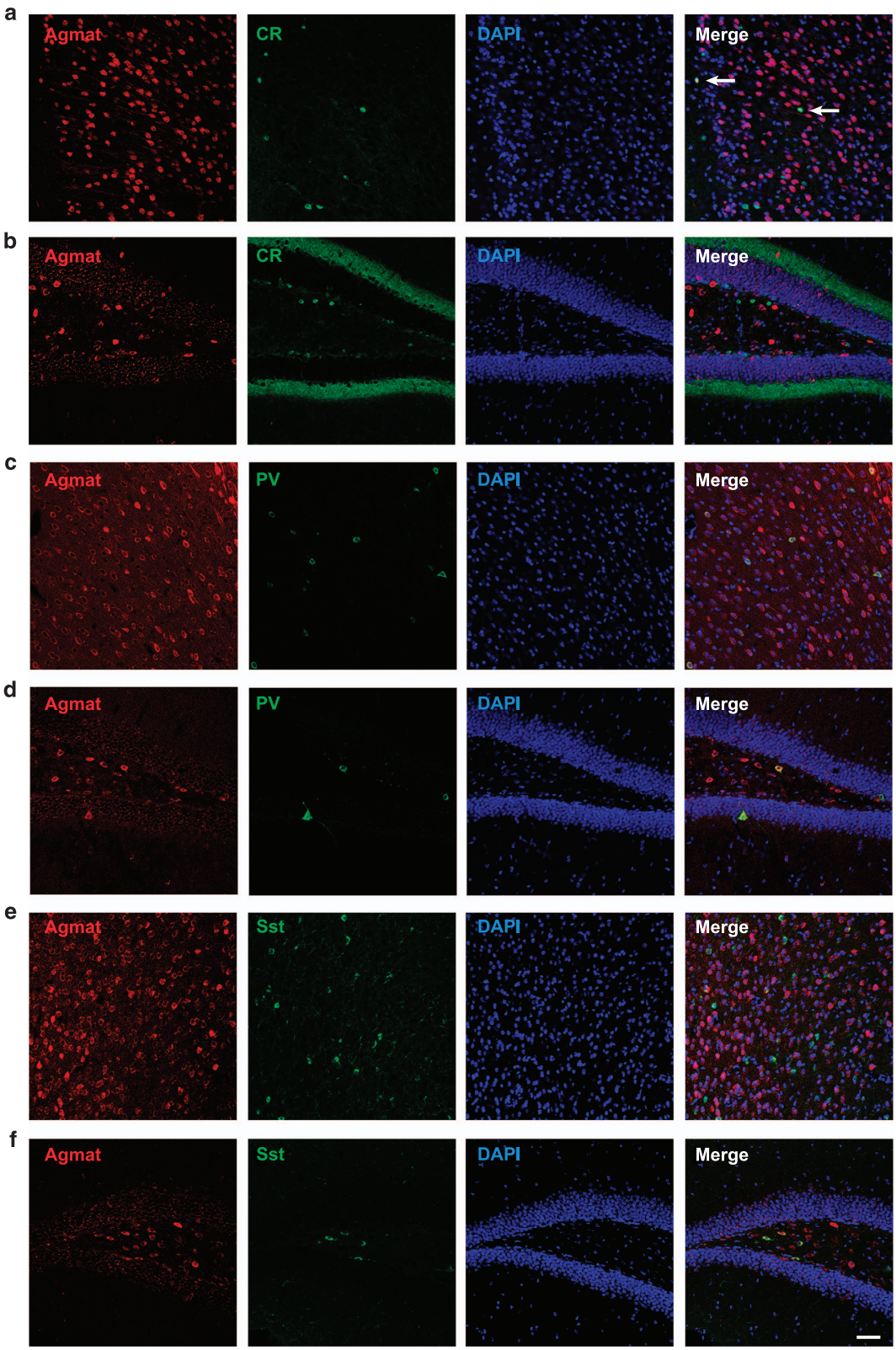
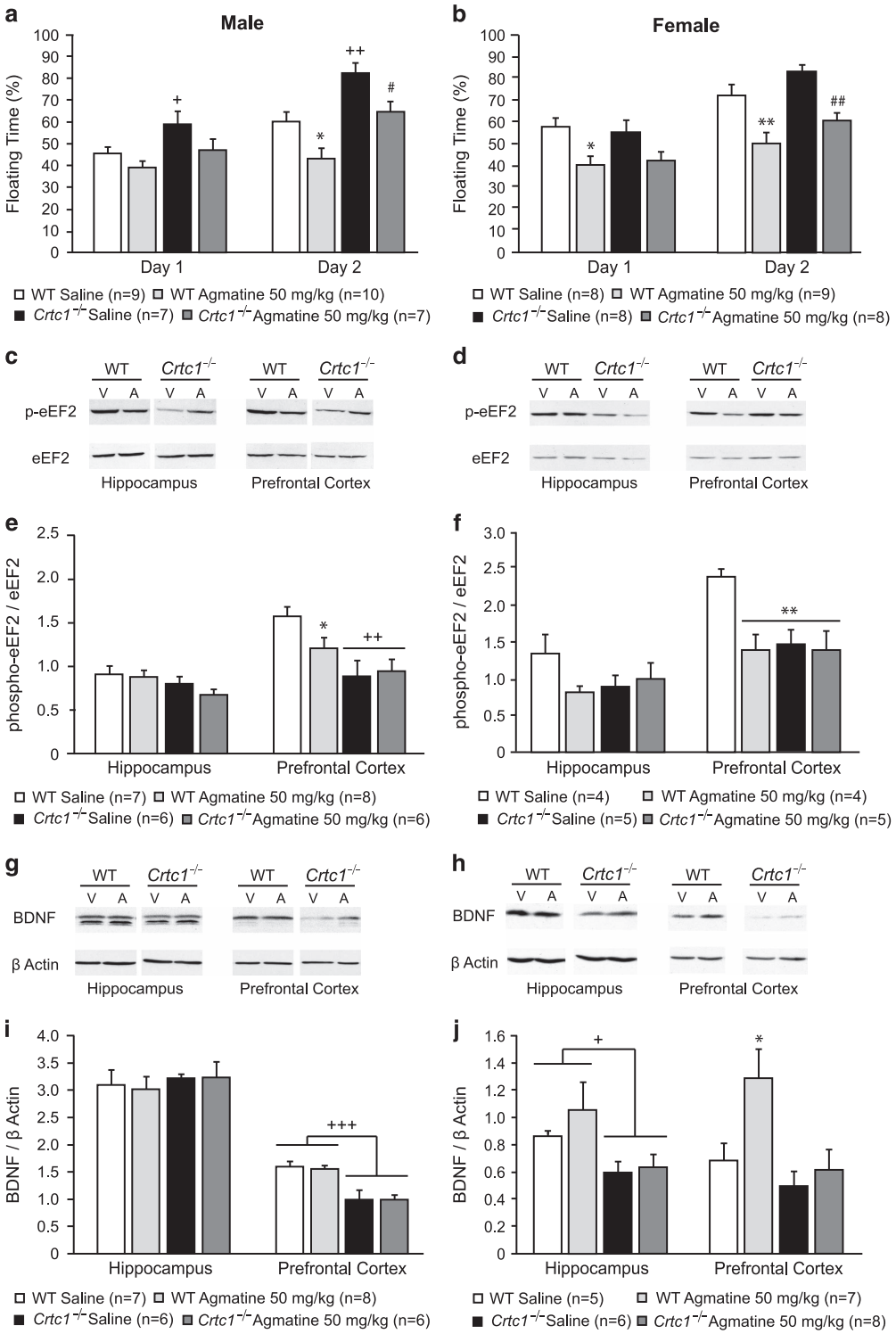


Figure 3. Characterization of GABAergic interneuron subpopulations expressing agmatinase (Agmat) in the prefrontal cortex (PFC) and hippocampus (HIP) of wild-type (WT) male mice. Double immunofluorescence labeling of (**a**, **b**) Agmat and calretinin (CR; **c, d**) Agmat and parvalbumin (PV), and (**e**, **f**) Agmat and somatostatin (Sst) in (**a**, **c**, **e**) PFC and (**b**, **d**, **f**) dentate gyrus (DG) of the HIP. Total cells were identified by nuclear 4,6-diamidino-2-phenylindole (DAPI) staining. Merged images showed few colocalization of Agmat and CR staining in the PFC (**a**) as indicated by arrows. No colocalization could be observed in the DG (**b**). All PV and Sst interneurons also expressed Agmat in the PFC (**c**, **e**) and DG (**d**, **f**). Scale bar, 50 μm.

genotype ($F_{(1,13)}$, $P = 0.011$), treatment ($F_{(1,13)}$, $P = 0.043$) and genotype × treatment ($F_{(1,13)}$, $P = 0.011$) were observed. *Post hoc* analyses revealed that agmatine significantly decreased the phosphorylation of eEF2 in WT mice ($P = 0.003$). Both groups of *Crtc1*$^{-/-}$ mice also presented decreased levels of phospho-eEF2 as compared with vehicle-treated WT mice (*Crtc1*$^{-/-}$ Vehicle: $P = 0.001$; *Crtc1*$^{-/-}$ Agmatine: $P = 0.002$). Agmatine had no effect on the phosphorylation of eEF2 in *Crtc1*$^{-/-}$ mice.

Western blot for BDNF revealed a single band at the expected size of 14 kDa (Figures 4g–h). BDNF signal was quantified and normalized with β-actin signal. In male mice (Figure 4i), no effect of genotype or treatment could be observed in the HIP. In the PFC, a two-way ANOVA showed an effect of genotype ($F_{(1,\ 23)} = 29.12$, $P < 0.001$). *Post hoc* analysis revealed that both vehicle- and agmatine-treated *Crtc1*$^{-/-}$ mice presented lower levels of BDNF protein than WT mice ($P < 0.001$ for both groups). Agmatine



treatment had no effect on BDNF levels of WT and *Crtc1*$^{-/-}$ mice. In female mice (Figure 4j), a two-way ANOVA revealed a significant effect of genotype in both structures (PFC: $F_{(1,22)} = 6.78$, $P = 0.016$; HIP: $F_{(1,22)} = 6.27$, $P = 0.02$). *Post hoc* analyses showed that both groups of *Crtc1*$^{-/-}$ mice had significantly lower levels of BDNF than WT animals in the HIP, independently of the treatment ($P = 0.01$). No effect of agmatine treatment could be seen in the HIP, whereas it was significant in the PFC ($F_{(1,22)} = 4.63$, $P = 0.042$). *Post hoc* analyses indicated that agmatine significantly increased BDNF protein levels in the PFC, however, only in WT mice ($P = 0.024$). Taken together, these results provide evidence that agmatine induces eEF2 dephosphorylation in WT male and female mice, thus suggesting activation of the mTOR pathway, through its NMDAR antagonist property. Agmatine effects on BDNF levels are gender-dependent, as agmatine rapidly induces BDNF translation in WT female mice, but not in WT male mice.

DISCUSSION

In this study, we showed for we believe the first time a link between downregulation of the CRTC1–CREB pathway and alteration of the agmatinergic system in the context of a rodent model of depression. We found that CRTC1-deficient mice exhibit increased mRNA and protein levels of Agmat in the HIP and PFC. We also determined that these higher Agmat levels are mainly because of an increased number of *Agmat*-expressing cells in the PFC and HIP of *Crtc1*$^{-/-}$ mice. These findings suggest that *Crtc1*$^{-/-}$ mice have a dysregulated agmatinergic system resulting in increased *Agmat* expression and ensuing decreased agmatine levels. Hence, the depressive-like phenotype of these animals would be in keeping with the protective and antidepressant role of endogenous agmatine.

Immunofluorescent detection of Agmat revealed that this enzyme is mainly expressed in interneurons in accordance with a previous characterization in the rat brain.[44] As *Crtc1*$^{-/-}$ mice have more *Agmat*-expressing cells, these mice might also have overall GABAergic system alteration. In line with this, agmatine and GABA seem to be closely related because agmatine is degraded into putrescine, whose derived polyamines can be used as GABA precursors.[16] Therefore, we hypothesize that dysregulation of agmatine metabolism might lead to abnormal GABA regulation and ultimately to overall impaired interneuronal circuitry.

A characterization of the subpopulations of GABAergic interneurons expressing *Agmat* showed that it mainly colocalizes with PV and Sst interneurons, whereas no or little colocalization with CR interneurons was observed. These findings are in contradiction with the study of Bernstein *et al.*,[44] which showed that Agmat was mainly found in CR interneurons. The reason for this discrepancy is unclear. Future studies should focus on a deeper characterization of *Agmat*-expressing cells, as Agmat staining revealed that it is present in many cells in the mouse brain and thus probably in a wide range of cell types.

The depressive-like behavior of *Crtc1*$^{-/-}$ mice was successfully normalized by acute agmatine treatment as efficiently as ketamine's effect. This suggests that their altered agmatinergic system contributes to their phenotype. It is noteworthy that only a higher dose of agmatine (50 mg kg^{-1}) was effective, suggesting that the 10 mg kg^{-1} dose was not sufficient to compensate for a possible decrease in agmatine levels and to restore normal agmatinergic functions.

When looking at the molecular effects of agmatine, we found that it was able to induce an increase in BDNF protein levels in the PFC of WT females. This agmatine-induced BDNF upregulation was paralleled by eEF2 dephosphorylation, which stimulates protein translation. This mechanism has been shown to underlie the rapid antidepressant effect of NMDAR antagonists such as ketamine and MK-801.[26,27,46] Therefore, our results suggest that agmatine acts as an antidepressant, possibly through this pathway. This is in line with the ability of agmatine to block NMDAR and the involvement of this function in its antidepressant effects.[36,47,48] In contrast, the behavioral effects of agmatine in WT males were apparently not mediated by BDNF because agmatine did not increase its levels in HIP and PFC. The mechanisms that underlie these sex differences are still unclear, but not completely unexpected. These gender-specific effects are actually of much interest in the light of the female preponderance in major depression. Sex differences have been reported in animal models of depression. For instance, the impact of BDNF signaling on depression-like behavior is different in male and female mice.[49] Moreover, it has been shown that hippocampal NO may contribute to sex difference in depressive-like behaviors.[50] This study showed that stress promotes hippocampal NO production in male mice, whereas stress suppresses it in female ones. Worthy of note, both NO excess in male mice and shortage in female mice resulted in depressive-like behaviors through affecting CREB activation.

Figure 4. Behavioral and molecular effects of agmatine treatment on male and female wild-type (WT) and *Crtc1*$^{-/-}$ mice. (**a, b**) Effects of acute agmatine treatment (50 mg kg^{-1}, intraperitoneal (IP)) 30 min before a forced swim test (FST) during two consecutive days. In male mice (**a**) vehicle-treated *Crtc1*$^{-/-}$ mice ($n = 7$) showed higher immobility levels than vehicle-treated WT mice ($n = 9$) on both days of test ($^{+}P < 0.05$, $^{++}P < 0.01$). On Day 2, agmatine significantly decreased the immobility time of WT mice ($^{*}P < 0.05$) and *Crtc1*$^{-/-}$ mice ($^{\#}P < 0.05$; $n = 10$ and $n = 7$, respectively). In female mice (**b**) WT mice treated with agmatine ($n = 8$) showed significantly decreased immobility time ($^{*}P < 0.05$, $^{**}P < 0.01$) compared with vehicle-treated WT mice ($n = 9$) on both days of test. On day 2, agmatine-treated *Crtc1*$^{-/-}$ mice ($n = 8$) also presented significantly decreased immobility time ($^{\#\#}P < 0.01$) than vehicle-treated *Crtc1*$^{-/-}$ mice ($n = 8$). (**c**–**f**) Effects of acute agmatine treatment (50 mg kg^{-1}, IP) on eukaryotic elongation factor 2 (eEF2) phosphorylation. (**c**, **d**) A representative western blot for phospho-eEF2 and total eEF2 in the hippocampus (HIP) and prefrontal cortex (PFC) of WT and *Crtc1*$^{-/-}$ mice (V, vehicle-treated; A, agmatine-treated) in male (**c**) and female (**d**) mice. (**e**, **f**) Quantitative analyses of p-eEF2 western blot in male (**e**) and female (**f**) mice. In male mice, no effect of agmatine could be seen in the HIP of WT and *Crtc1*$^{-/-}$ mice. In the PFC, agmatine-treated WT mice ($n = 8$) presented lower levels of p-eEF2 than vehicle-treated WT mice ($n = 7$; $^{*}P < 0.05$). Both vehicle- and agmatine-treated *Crtc1*$^{-/-}$ mice ($n = 6$ for both) displayed lower p-eEF2 levels than WT mice ($^{++}P < 0.01$); agmatine treatment had no effect on p-eEF2 levels in these animals (**e**). In female mice, quantification showed no effect of agmatine treatment in the HIP of WT and *Crtc1*$^{-/-}$ mice (**f**). In the PFC, agmatine-treated WT mice ($n = 4$) presented lower levels of eEF2 phosphorylation compared with vehicle-treated WT animals ($n = 4$; $^{**}P < 0.01$). *Crtc1*$^{-/-}$ mice treated with vehicle ($n = 5$) or agmatine ($n = 5$) also displayed lower levels of eEF2 phosphorylation than WT mice ($^{**}P < 0.01$). Agmatine treatment had no effect on *Crtc1*$^{-/-}$ mice. Results are presented as ratio between phospho-eEF2 and total eEF2 signals. (**g**–**j**) Effects of acute agmatine treatment (50 mg kg^{-1}, IP) on brain-derived neurotrophic factor (BDNF) protein level. (**g, h**) A representative western blot for BDNF and β-actin in the HIP and PFC of WT and *Crtc1*$^{-/-}$ mice in male (**g**) and female (**h**) mice. (**i, j**) Quantitative analyses of BDNF western blot in male (**i**) and female (**j**) mice. In male mice, no effect of agmatine could be seen in the HIP of WT and *Crtc1*$^{-/-}$ mice. In the PFC, both vehicle- and agmatine-treated *Crtc1*$^{-/-}$ mice ($n = 6$ for both) displayed lower BDNF levels than vehicle- and agmatine-treated WT mice ($n = 7$ and $n = 8$, respectively; $^{+++}P < 0.001$) (**i**). In female mice, quantitative analyses of western blot showed decreased levels of BDNF in the HIP of all *Crtc1*$^{-/-}$ animals ($n = 14$) compared with WT animals ($n = 13$), independently of the treatment ($^{+}P < 0.05$). In the PFC, agmatine-treated WT mice ($n = 7$) presented higher levels of BDNF compared with vehicle-treated WT animals ($n = 5$; $^{*}P < 0.05$). *Crtc1*$^{-/-}$ mice treated with agmatine ($n = 8$) did not present different BDNF levels than those treated with saline ($n = 6$; **j**). Results are presented as ratio between BDNF and β-actin signals. Data are mean ± s.e.m.

Interestingly, the effects of agmatine on BDNF and eEF2 were restricted to WT animals. *Crtc1*$^{-/-}$ male and female mice displayed basal lower levels of phospho-eEF2 in the PFC, and agmatine did not decrease them further nor did it increase BDNF levels, which suggest a dysregulation of this pathway and the involvement of alternative mechanisms underlying agmatine's antidepressant effects in these animals.

In conclusion, our results provide evidence for the involvement of the agmatinergic system in the *Crtc1*$^{-/-}$ mouse model of depression, and lend support to previous reports of the antidepressant properties of agmatine. The comparable rapid antidepressant effects of agmatine and ketamine in WT and *Crtc1*$^{-/-}$ mice, as well as the molecular effects that acute agmatine treatment causes in the brain of WT mice suggest that agmatine possibly functions as a fast-acting antidepressant through NMDAR blockade. The relationship between the CRTC1–CREB pathway and agmatine regulation merits further investigation, as it will bring better knowledge of these systems and their contribution to MDD etiology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Suzanne Badoux for her initial work with an agmatinase antibody that was not used in this study. This work was funded by a grant from the Swiss National Science Foundation (31003A-135692) and partly supported by the National Centre of Competence in Research (NCCR) Synapsy.

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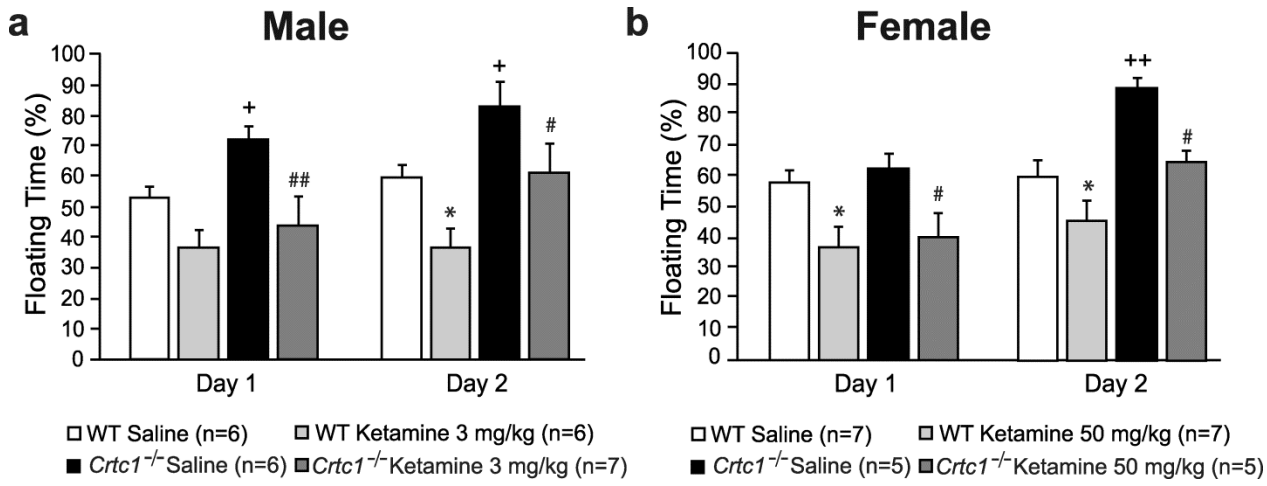
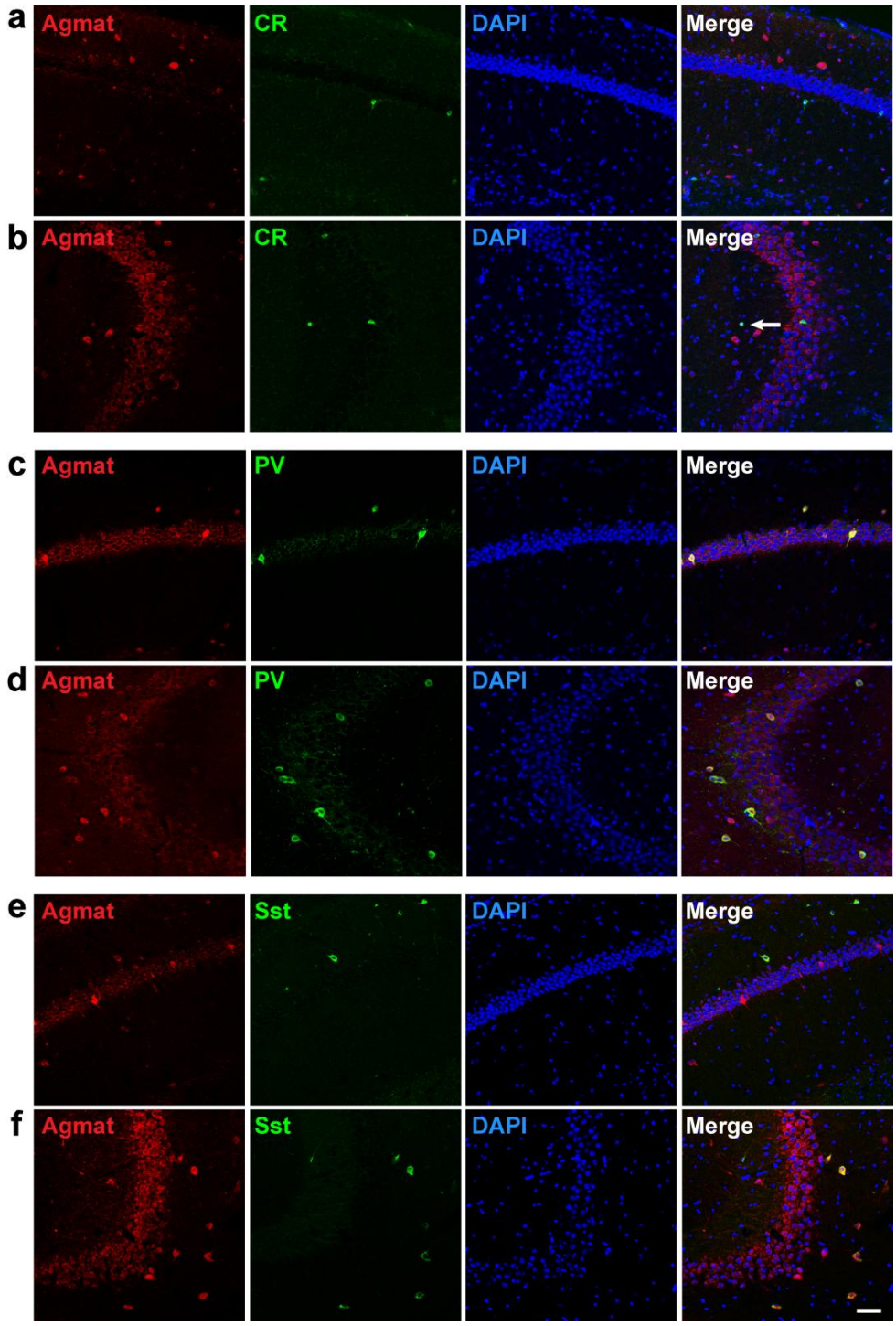
Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)



Supplementary Figure S2 Behavioral effects of acute ketamine treatment on male and female WT and *Crtc1*$^{-/-}$ mice. (a-b) Effects of acute ketamine treatment (3 mg/kg, IP) 30 min prior to a forced swim test (FST) during two consecutive days. In male mice (a) vehicle-treated *Crtc1*$^{-/-}$ mice (n=6) showed higher immobility levels than vehicle-treated WT mice (n=6) on both days of test ($^{+}p < 0.05$). Ketamine significantly decreased the immobility time of WT mice ($^{*}p < 0.05$, n=6) on day 1, and *Crtc1*$^{-/-}$ mice ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, n=7) on both days of test. In female mice (b) ketamine-treated WT and *Crtc1*$^{-/-}$ mice (n=7, n=5 respectively) showed significantly decreased immobility time ($^{*}p < 0.05$,$^{\#}p<0.05$) compared to vehicle-treated WT and *Crtc1*$^{-/-}$ mice (n=7, n=5 respectively) on both days of test. On day 2, vehicle-treated *Crtc1*$^{-/-}$ mice also presented significantly increased immobility time ($^{++}p < 0.01$) than vehicle-treated WT mice. Data are mean ± SEM.



Supplementary Figure S1 Characterization of GABAergic interneurons subpopulations expressing agmatinase (Agmat) in the CA1 and CA3 regions of the hippocampus (HIP) of WT male mice. Double immunofluorescence labeling of (a, b) Agmat and calretinin (CR), (c, d) Agmat and parvalbumin (PV), and (e, f) Agmat and somatostatin (Sst) in WT (a, c, e) CA1 and (b, d, f) CA3 regions of the HIP. Total cells were identified by nuclear DAPI staining. Merged images showed no colocalization of Agmat and CR staining in CA1 (a). Few colocalization could be observed in CA3, as indicated by the arrow. All PV- and most of Sst- interneurons also express Agmat in the CA1 (c, e) and CA3 (d, f) regions of the HIP. Scale bar 50 μm.