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**LES REGULATIONS EPIGENETIQUES AU NIVEAU DE LA
SIGNALISATION BDNF-TRKB DANS LA PHYSIOPATHOLOGIE ET
TRAITEMENT DES TROUBLES ANXIO-DEPRESSIFS**

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SIGNALISATION BDNF-TRKB DANS LA PHYSIOPATHOLOGIE ET
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**EPIGENETIC REGULATION OF BDNF-TRKB SIGNALING IN THE
PATHOPHYSIOLOGY AND TREATMENT OF MOOD DISORDERS**

Fabien BOULLE

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CHAPTER I

General Introduction

Fabien BOULLE

Mood disorders, also referred as affective disorders, belong to a group of diagnoses from the Diagnostic and Statistical Manual of Mental Disorder (DSM-IV TR) classification where a disturbance of an individual's mood is the main feature of the disorder (Nettle et al., 2012). Among mood disturbances, depression and mania can be seen as opposite ends of the affective spectrum. Classically, mania refers to the *up* pole of the spectrum, where patients experience elevated and irritable mood often associated with distractibility, racing thoughts and agitation (e.g. a manic or hypomanic episode). In contrast, depression refers to the *down* pole, where patients suffer from low mood, loss of interest, fatigue, feelings of worthlessness and/or suicidal ideation (e.g. major depressive episode). Mania and depression can occur simultaneously (e.g. mixed episodes or bipolar disorder). Epidemiological research indicates that mood disorders are among the major health problems worldwide due to the high prevalence and recurrence in the general population, and the significant burden for individual life quality and the repercussion on healthcare systems and society (WHO, 2001). Today, depression is estimated to affect 350 millions people, with a lifetime prevalence ranging from 3 percent in Japan to 17 percent in the United states - most of the countries showing a prevalence between 8 to 12 percent in the general population - (WHO, 2012). On the other side, the occurrence of manic episodes during a lifetime has been estimated to only 1 to 2 percent (Soldani et al., 2005; Merikangas et al., 2004). Moreover, co-morbidity between mood disorders and somatic or other psychiatric diseases is often observed in patients. For instance, depression frequently co-occurs with anxiety or neuropathic pain, and increases the risk for cardio-vascular problems (Kessler et al., 1996; Bair et al., 2003). In addition, major depression represents a considerable risk factor for suicide, which has been estimated to lead to more than 1 million deaths every year (WHO, 2012). By 2030, major depression is expected to become the largest contributor to disease burden (WHO, 2008).

I – The pathophysiology of mood disorders

Neurotransmitters and circuits in mood disorders: The etiology of mood disorders is still poorly understood, and the complexity and heterogeneity of the diseases hamper adequate diagnosis and treatment (Phillips et al., 2013; Zajecka et al., 2013). Up to date, three principal neurotransmitters have been associated to the pathophysiology and treatment of mood disorders (Krishnan et al., 2008). They are dopamine (DA), noradrenaline (NA) and serotonin (5-HT), the so-called monoamine neurotransmitter systems, and often act in concert to regulate mood. The mesolimbic dopamine pathway comprises neurons projecting from the ventral tegmental area (VTA) in the brainstem to the nucleus accumbens (NAc) in the ventral striatum, and is believed to be involved in behavioral expressions like pleasure, euphoria, delusions or hallucinations. The mesocortical dopamine pathway projects from the VTA to the prefrontal cortex, which is mostly implicated in mediating cognitive and affective functions. Noradrenaline has both ascending and descending pathways. The ascending pathway projects from the locus coeruleus in the brainstem to multiple brain regions including the amygdala, thalamus, striatum, hypothalamus, hippocampus and prefrontal cortex. The ascending noradrenergic projections are mainly

involved in the regulation of attention, decision-making, arousal and the fight or flight response. Serotonin, like noradrenaline, has both ascending and descending projections. The ascending pathway arises from the raphe nucleus in the brainstem, and projects to the amygdala, thalamus, striatum, hypothalamus, hippocampus and prefrontal cortex. In the brain, serotonin is mostly involved in sleep, anxiety, appetite and mood. However, clean-cut evidences for changes of monoamine levels in the brain of mood disorders patients are still lacking.

Pharmacological treatments: Although there is no clear evidence that dysregulation of the monoamine neurotransmitter systems significantly contributes to the etiology of mood disorders, most antidepressant drugs mainly act via the monoaminergic pathways by increasing the levels of the neurotransmitters in the synaptic cleft, either by blocking the reuptake or by inhibiting the breakdown of the neurotransmitters. Tricyclic antidepressants (TCAs) for example, the first class of psychotropic drugs to be clinically used at a large scale, mainly act by blocking serotonin and norepinephrine transporters (SERT and NAT). The downside of these types of antidepressants is the relatively high toxicity and the numerous side effects. Later, more specific reuptake blockers have been developed, like the selective serotonin reuptake inhibitors (SSRIs), the serotonin-norepinephrine reuptake inhibitors (SNRIs) and the norepinephrine reuptake inhibitors (NARIs) that more specifically act on their respective target transporters and generally have fewer side effects. Another way to increase monoaminergic signaling is by inhibiting the breakdown of the neurotransmitters in the synaptic cleft. For example, the monoamine oxidase inhibitors (MAOIs) have shown to reduce the breakdown of monoamines, by inhibiting the monoamine oxidase enzyme family. Thus, MAOIs enhance serotonergic, noradrenergic and dopaminergic transmission. However, all currently available antidepressants encounter notable limitations including a delayed therapeutic effect (from weeks to months), a low response rate (only two third of the patients respond well) and a high relapse rate after treatment termination (Trivedi et al., 2006).

Stress, neurotrophins and depression: Stress has been proposed as a major risk factor to develop affective disorders. Stressful stimuli generally activate the hypothalamus resulting in the production of corticotropin-releasing factor (CRF), which in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary. Finally, ACTH causes the secretion of glucocorticoids, i.e. cortisol (corticosterone in rodents), from the adrenal gland. Cortisol in turn terminated the hormonal stress response by inhibiting the CRF release from the hypothalamus. However, under pathological conditions, such as chronic or excessive stress exposure, the HPA axis fails to exert this negative feedback, resulting in abnormally high glucocorticoid levels. Thus, chronic hypercortisolemia can significantly impact on structural and functional plasticity in various brain areas (Berton et al., 2006). In particular, prolonged stress exposure has been shown to affect the production of growth factors, and among many candidates, brain-derived neurotrophic factor (BDNF) has been reported as a key-role player in the deleterious effects of stress on the brain (see Figure 1). BDNF is a small neurotrophin secreted by neuronal and glial cells in

various regions of the brain, especially in the areas controlling mood and emotions. Upon binding to its tropomyosin-related kinase receptor (TrkB), BDNF induces proliferation, differentiation, survival and neurite extension, which are essential processes in the normal development and functioning of brain cells. However, under situations of chronic stress, BDNF gene expression can be repressed. The resulting decrease in BDNF protein has been suspected to cause cell loss and synaptic dysfunction in the hippocampus and prefrontal cortex of depressed patients (Duman et al., 2012). Antidepressant treatments, via monoamine-related signal transduction cascades, have been shown to increase BDNF and other trophic factors, and potentially restore neuronal loss and atrophy in hippocampal and cortical areas via BDNF (Castren et al., 2008).

II – BDNF/TrkB signaling

Neurotrophins in the brain: Neurotrophins are a family of small-secreted proteins that play a key role in the development and maintenance of the vertebrate nervous system (Chao, 2003). To mediate their effects, neurotrophins can bind to two distinct classes of receptors, displaying opposite effects. The high affinity interaction with the “tropomyosin-related kinase” receptor family (Trks) is usually associated with cell survival, differentiation and synaptic plasticity, whereas the low affinity interaction with the p75 (p75NTR) promotes proteolysis and apoptosis (Lu et al., 2005). The neurotrophin tyrosine kinase receptor 2 (NTRK2), also known as TrkB, is specifically activated by several neurotrophins including BDNF, neurotrophin 3 (NT 3) and neurotrophin 4/5 (NT 4/5), thereby exerting their trophic effects (Klein et al., 1991; Klein et al., 1992; Soppet et al., 1991). Among all the neurotrophins, the interaction of BDNF with TrkB has been characterized most thoroughly. The expression of BDNF, as well as of TrkB, is very abundant in the cortico-limbic structures of the brain and both are also present at lower levels in different peripheral tissues (Maisonpierre et al., 1990; Pruunsild et al., 2007). BDNF/TrkB signaling has been reported as critical in various physiological processes such as learning, memory and reward. In particular, BDNF is required for the maturation, differentiation and survival of neural progenitor cells, e.g. neurogenesis, in the dentate gyrus (DG) of the hippocampus. Abnormal BDNF-mediated activation of TrkB has been reported in various neurological and psychiatric disorders (Pezet and Malcangio, 2004).

BDNF-mediated activation of TrkB: The activation of TrkB by BDNF generally follows the common pattern associated with tyrosine kinase receptor activation (Friedman and Greene, 1999). Neurotrophins exist preferentially as homodimer complexes, and bind to the extracellular interface of a TrkB monomer resulting in receptor dimerisation (Jing et al., 1992). The dimerisation increases the catalytic activity of the intracellular domain of the receptor enabling the phosphorylation of tyrosine residues inside the activation loop (tyrosine Y701, Y705 and Y706) and subsequently the autophosphorylation of tyrosine residues situated outside of the activation loop (tyrosine Y515 and Y816) (Cunningham and Greene,

1998). Then, activation and recruitment of partner proteins and adaptators to the different tyrosine sites will lead to activation of three main intracellular signaling pathways that are strongly interconnected (Huang and Reichardt, 2003). Briefly, phosphorylation of tyrosine Y515 activates 1) the Ras-mitogen-activated protein kinase (MAPK) signaling cascade, which promotes neuronal differentiation and growth, and 2) the phosphatidylinositol 3-kinase (PI3-K) signaling cascade, which promotes neuronal survival and growth. Phosphorylation of tyrosine Y816 activates 3) the phospholipase C γ (PLC γ) pathway generally associated with synaptic plasticity and neurotransmission (Minichiello, 2009). The neurotrophin/Trk complex is internalized by endocytosis and the intracellular domain of the receptor, present on the outside of the endosome, is still phosphorylated and susceptible to binding of signaling molecules (Howe et al., 2001). However, the exact mechanism underlying TrkB internalization and recycling is still poorly understood. In addition, different alternative TrkB mRNA splice variants can lead to the translation of different TrkB isoforms including two truncated forms (Middlemas et al., 1991). It has been described that the two truncated forms of TrkB (TrkB-T1 and TrkB-T2) lacking the intrinsic tyrosine kinase activity, were able to inhibit full-length TrkB (TrkB-FL) signaling via the binding of a truncated form with a full form (Dorsey et al., 2006).

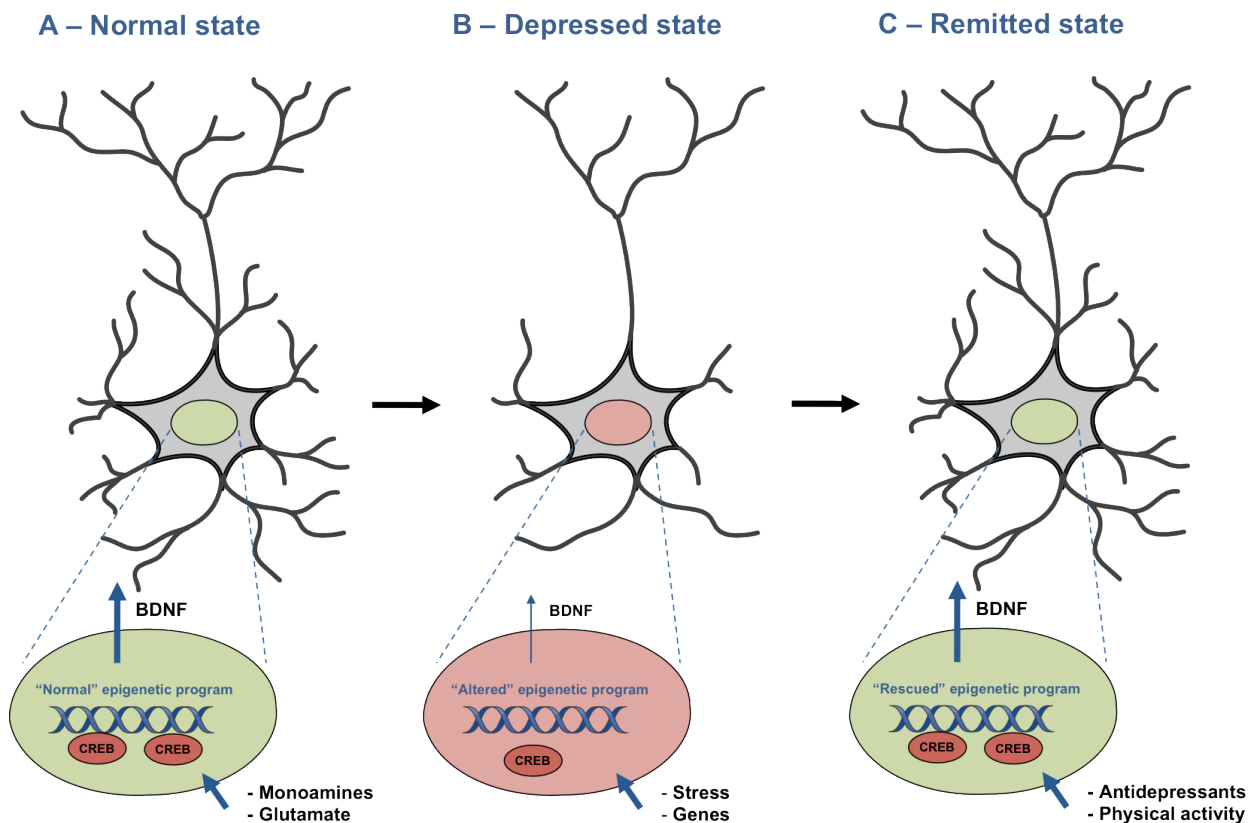


Figure 1: Schematic representation of epigenetic and neuroplastic mechanisms in the hippocampus of depressed patients and antidepressant action.

(A) Stimulation of hippocampal pyramidal neurons by glutamate or monoaminergic neurotransmitters, possibly via the activation of cAMP responsive element binding (CREB), is critical for the regulation of BDNF expression. In addition, epigenetic mechanisms control the expression of BDNF, and an appropriate epigenetic programming during development and adulthood ensures an adequate expression and function of BDNF. (B) Chronic stress exposure, as well as genetic variations, can interfere with epigenetic regulation and reduce BDNF-mediated plasticity in the hippocampus. (C) Interestingly, it has been shown that antidepressant treatments and physical activity can reverse epigenetic alterations and restore a normal expression of BDNF and enhance neuroplasticity in hippocampal neurons. Adapted from Berton et al (2006).

III – Epigenetic regulation in mood disorders

Definition and basic principles: Epigenetics refers to mechanisms that regulate gene expression independently of the DNA sequence. Hence, the modulation of gene expression by epigenetic mechanisms is essential during development, where a “cellular memory” is created enabling an appropriate pattern of gene expression and a specific cellular phenotype (Probst et al., 2009). Diverse stimuli such as life events, behavioral experience or circadian rhythms can induce cellular responses that involve epigenetic remodeling, and it is believed that stable epigenetic imprints at particular genetic loci may shape neuronal plasticity and thereby influence behavior. The two best-described molecular mechanisms of epigenetic regulation are DNA methylation and post-translational histone modifications in the cell nucleus.

DNA methylation is the process of chemical addition of a methyl group to the carbon atom at position 5 of a cytosine residue, which typically occurs at CpG nucleotides (i.e. where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases, separated by a phosphate). In general, DNA methylation in promoter regions disables transcription factors to bind to their recognition sites, thereby silencing gene expression. DNA methylation needs the recruitment of specific enzymes called DNA methyltransferases (DNMTs), which mediate the addition of the methyl group on cytosine residues. In addition to DNMTs, some proteins are able to interact with methyl-CpG sites and increase the repressive potential of methylated DNA. These molecules can carry a methyl-CpG binding domain (MBD proteins) or other specific domains that recognise methylated DNA. Several pathways leading to DNA demethylation have been proposed involving the growth arrest and DNA-damage-inducible protein 45 alpha (GADD45a) (Barreto et al., 2007). In addition, ten-eleven-translocation (TET) enzymes can oxidize the 5-methylcytosine into a 5-hydroxymethylcytosine, which can be further oxidized into 5-formylcytosine and 5-carboxylcytosine (Tahiliani et al., 2009; Ito et al., 2011).

In the eukaryotic nucleus, DNA is compacted and wrapped around histone and non-histone proteins to form a dynamic structure called chromatin. The nucleosome consists of an octamer of core histones (two copies of histone H2A, H2B, H3 and H4) around which DNA is wound, and represents the basic repeated unit of chromatin. Two types of chromatin can be distinguished based upon their condensation states. Heterochromatin is inactive and condensed, which means that transcription factors are not able to approach and recognise their specific DNA-binding sites to activate gene transcription. On the other hand, euchromatin reflects an “open” state of chromatin, and is associated with active transcription. Chromatin remodelling enables the transition between a permissive and a repressive state, and vice versa, enabling a temporal and spatial modulation of gene expression. Post-translational changes encompass covalent modification of histones at distinct amino acid residues on their amino-terminal tails, including lysine acetylation, methylation, SUMOylation and ubiquitinylation; arginine methylation; serine

phosphorylation and proline isomerisation (Berger et al., 2007). The effect of these chemical alterations on gene transcription depends on the type of histone modification but also on the amino acid residue being modified. Generally, histone acetylation is associated with euchromatin, while histone methylation is linked to heterochromatin. The enzymes responsible for acetylation or deacetylation are histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. Regarding methylation, histone methyltransferases (HMTs) catalyse the addition of the methyl group, whereas histone demethylases (HDMs) reverse the methylated state. Interestingly, it has been shown that HDACs and HMTs were associated with MBDs and act as co-repressor molecules to form complexes that further repress gene transcription (Jones et al., 1998). All these findings support a link between DNA methylation and chromatin remodelling in transcriptional regulation.

Clinical and preclinical evidence of epigenetic mechanisms in mood disorders: The episodic nature of mood disorders suggests that they are unlikely to be pure genetic diseases, which generally are responsible for the genesis of life-long illnesses. Genome-wide association studies have revealed hundreds of potential candidates for severe mental illness, but the effective contribution of each individual gene appears to be very small. Epidemiological evidence also links environmental factors, especially early life events, with an increased risk for mood disorders (Hammen et al., 2005; Kessler et al., 1997). Hence, the notion of gene x environment (GxE) interaction has emerged as a prevalent theory to explain the inter-individual variations underlying susceptibility and resilience to mental disorders. As a possible mediator of GxE interactions, epigenetic reprogramming during brain development may be of critical importance. A multitude of clinical studies have investigated epigenetic signatures in blood-derived lymphocytes and post-mortem brain tissues from mood disorder patients. For example, post-mortem brain analysis revealed a decreased expression of G9a histone methyltransferase, HDAC2 and H3K9 methylation, all pointing toward a permissive state of chromatin, in the NAc of depressed patients (Covington et al., 2009; Covington et al., 2011). In the periphery, mRNA profiling in white blood cells revealed a decrease of HDAC2 and HDAC5 in the depressive state of major depressive disorder (MDD), and a decrease of HDAC6 and HDAC8 in the depressive state of bipolar disorder (BPD) (Hobara et al., 2010). Interestingly, the alterations at HDAC2 and HDAC5 were normalized in the remitted state. Similarly, the mRNA expression in peripheral white blood cells of the HDACs Sirt1, Sirt2 and Sirt6 was decreased in the depressed state of MDD and BPD, whereas no differences were observed in the remitted state (Abe et al., 2011). In addition, a recent study showed that monozygotic twins affected with MDD have greater variance in methylation in white blood cells as compared to their unaffected siblings (Byrne et al., 2013). Next to the above-mentioned clinical evidence, various animal studies have brought further support for the involvement of epigenetics in the vulnerability to stress and mood disorders. Meaney and colleagues were the first to demonstrate a mechanism of epigenetic regulation for stress vulnerability in rodents. They found that variation in maternal care could regulate the expression of the glucocorticoid receptor (GR) in the hippocampus by inducing changes in DNA methylation and histone acetylation, an

effect that seemed to be stable into adulthood. Offspring who received higher levels of maternal care showed less anxiety-like behavior and better recovery from stressful situations (Weaver et al., 2004). This result was the first to indicate the importance of early life events in epigenetic programming and susceptibility for mood disorders later in life. Moreover, using a model of adult stressful life events, Nestler and colleagues showed that an inbred population of mice could be separated into susceptible and unsusceptible subpopulations after exposure to social defeat stress (Krishnan et al., 2007), and that the molecular signature underlying the resistance to stress seems to reside in epigenetic regulations. Direct evidence for a possible role of epigenetic mechanisms in the development of mood disorders is further supported by the work on the Circadian Locomotor Output Cycles Kaput (Clock) gene. Clock has been shown to display histone acetyl transferase properties, and is critical in regulating circadian rhythms (Nakahata et al., 2008). Interestingly, mutations in the Clock gene resulted in manic-like behavior in mice (Roybal et al., 2007), and strongly support the implication of circadian rhythms and associated epigenetic regulations in the pathophysiology of BPD (Milhiet et al., 2011). Altogether, clinical and preclinical studies suggest that the interplay between life events and genetic variation might play a critical role in epigenetic reprogramming, and may be one of the most plausible explanations underlying differential susceptibility towards mood disorders.

Epigenetic mechanisms in the treatment of mood disorders: Antidepressants are known to increase levels of monoamine neurotransmitters at the synapse, and, after prolonged exposure, to induce neuroplastic changes. Hence, the modulation of monoamine-related signaling cascades might trigger various cellular events involving epigenetic remodeling and subsequent gene transcription. Fluoxetine, one of the most prescribed SSRI antidepressants, was shown to directly induce the expression of MBDs (MeCP2 and MBD2) in adult rat brain (Cassel et al., 2006). In addition, chronic treatment with fluoxetine could significantly upregulate H3 acetylation at the BDNF gene in the hippocampus of a mouse model of depression (Onishchenko et al., 2008). Similarly, escitalopram - another SSRI antidepressant - could decrease DNMT expression and reduce the excessive DNA methylation at the P11 gene in the frontal cortex of the Flinders Sensitive Line genetic rodent model of depression (Melas et al., 2011). Imipramine, a TCA, was shown to reverse the decreased H3 acetylation levels in the hippocampus induced by chronic stress in rodents (Covington et al., 2011; Hollis et al., 2011; Hollis et al., 2010). Furthermore, imipramine treatment reversed the large majority of histone methylation changes in the NAc induced by social defeat stress in mice (Wilkinson et al., 2009). Additional experiments further supported the notion that imipramine exerts its therapeutic effects by increasing BDNF levels, at least partly through downregulation of HDAC5 (Tsankova et al., 2006). Moreover, electroconvulsive shock therapy (ECT), still the most effective treatment for refractory depressed patients, has been shown to increase H3 acetylation at the BDNF gene in naïve rats (Tsankova et al., 2004). Regarding mood stabilizers, valproate, an effective treatment for mania and bipolar disorders, was shown to act as an HDAC inhibitor (Phiel et al., 2001). Interestingly, administration of HDAC inhibitors directly into the NAc exerted antidepressant

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properties in mice (Covington et al., 2009). This same study showed that HDAC inhibition produced a similar pattern of gene expression in the NAc as compared to fluoxetine (Covington et al., 2009). Moreover, infusion of DNMT inhibitors such as RG108 and zebularine into the NAc could reverse the depressive-like behaviors induced by chronic stress in mice (LaPlant et al., 2010).

Altogether, the current literature indicates that epigenetic remodeling might be necessary for the therapeutic action of antidepressants.

IV – Aim and outline of the thesis

The research presented in this thesis aims to explore the role of BDNF signaling and its downstream epigenetic regulations in the pathophysiology and treatment of mood disorders.

First of all, an overview of current literature is provided in the first part of this dissertation. **Chapter 2** gives an overview about epigenetic regulations at the BDNF gene in psychiatric phenotypes, and discusses potential implications for psychiatric neuroscience and development of novel psychotropic drugs. Further, **Chapter 3** summarizes the literature about the impairment of neuroplasticity in depression and potential implications for the development of novel antidepressant drugs. In particular, a detailed overview of the different trophic factors (including BDNF) and intracellular signaling pathways implicated in the pathophysiology of depression is presented. In addition, the neurotrophic actions of current antidepressant medications are reviewed. Finally, **Chapter 4** describes the upregulation of TrkB signaling in brain disorders and proposes different methods and approaches to model BDNF/TrkB interaction and develop novel TrkB modulators.

Further, the original research of this thesis explored the involvement of BDNF/TrkB signaling and epigenetic regulations in the pathophysiology and treatments of mood disorders. **Chapter 5** provides further insights into the epigenetic and neuroplastic regulations of a novel TrkB inhibitor cyclotraxin-B. In **Chapter 6**, the effect of developmental exposure to fluoxetine was investigated using a model of prenatal stress in rats. In particular, the effects of maternal fluoxetine on affective behavior and epigenetic regulations at BDNF/TrkB signaling were addressed. **Chapter 7** brings further evidence on the requirement of TrkB in the anxiolytic effects of the new generation antidepressant agomelatine in a transgenic mouse model of depression, while **Chapter 8** focusses on the effects of agomelatine in a mouse model of hyperactivity and manic-like behavior. More specifically, the ability of agomelatine to restore stress-induced alterations at histone modifications, intracellular signaling cascades and plasticity-related gene expression was investigated.

Finally, **Chapter 9** summarizes and discusses the main findings of this dissertation, while also providing future directions and perspectives.

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CHAPTER II

Epigenetic regulation of the BDNF gene: implications for psychiatric diseases

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Abstract

Abnormal brain-derived neurotrophic factor (BDNF) signaling seems to play a central role in the course and development of various neurological and psychiatric disorders. In addition, positive effects of psychotropic drugs are known to activate BDNF-mediated signaling. Although the *BDNF* gene has been associated with several diseases, molecular mechanisms other than functional genetic variations can impact on the regulation of *BDNF* gene expression and lead to disturbed BDNF signaling and associated pathology. Thus, epigenetic modifications, representing key mechanisms by which environmental factors induce enduring changes in gene expression, are suspected to participate in the onset of various psychiatric disorders. More specifically, various environmental factors, particularly when occurring during development, have been claimed to produce long-lasting epigenetic changes at the *BDNF* gene, thereby affecting availability and function of the BDNF protein. Such stable imprints on the *BDNF* gene might explain, at least in part, the delayed efficacy of treatments as well as the high degree of relapses observed in psychiatric disorders. Moreover, *BDNF* gene has a complex structure displaying differential exon regulation and usage, suggesting a subcellular- and brain region-specific distribution. As such, developing drugs that modify epigenetic regulation at specific *BDNF* exons represents a promising strategy for the treatment of psychiatric disorders. Here, we present an overview of the current literature on epigenetic modifications at the *BDNF* locus in psychiatric disorders and related animal models.

Keywords: *BDNF* gene, environmental factors, epigenetic, psychiatric disorders, treatments

CHAPTER III

Growth factors and neuroplasticity pathways in the pathophysiology and treatment of depression

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Abstract

Depression is considered as a major health problem with a high prevalence and a heavy economic burden in western societies. At the anatomical level, major depression corresponds to atrophy or impaired functioning of cortico-limbic regions involved in mood and emotions including the hippocampus and the prefrontal cortex. Especially, it is suspected that alterations in growth factors and associated signaling pathways might underlie neuronal loss and synaptic dysfunction in cortico-limbic structures, which may be causally related to the development and course of depression. Accordingly, mounting evidences suggest that antidepressant treatments may exert their therapeutic effects by enhancing trophic signalling and their downstream neuroplastic signaling. However, current therapies still have significant limitations including a delayed onset of action, as well as a considerable lack of efficacy. Hence, a deeper understanding about the molecular and cellular mechanisms involved in the pathophysiology, as well as in the action of antidepressants might lead to the identification of key-role effectors, and provide further insight into the development of novel fast-acting and more effective therapies. Here, we summarized the current literature about the involvement of growth factors and associated signalling in the pathophysiology and treatment of depression, and provide future directions for the development of novel antidepressant drugs.

Key words: growth factors, neurocircuits, plasticity, antidepressant, neurotrophic signalling, mood

I – Introduction

Depression has emerged over the past decades as a major debilitating disease with a high prevalence in occidental populations, resulting in profound social and economic burden (Kessler *et al.*, 2003; Lopez and Murray, 1998; Pincus and Pettit, 2001). The molecular and cellular mechanisms underlying the pathophysiology of depression remain poorly understood. Furthermore, the development and course of the illness are likely to be mediated by a complex interaction between genetic and environmental factors, and the associated heterogeneity of the disease makes it difficult to develop effective therapeutic treatments (Keers and Uher, 2012). So far, many classes of antidepressant medication have been discovered and marketed for the treatment of depression. However, currently available antidepressants display significant limitations, including low response rates and relapse after treatment cessation, which remain major drawbacks for a disease where suicide rate are relatively high (Angst *et al.*, 2002). Up to date, clinical and preclinical studies have revealed that depression could be linked to alterations in cortico-limbic structures controlling mood and emotions, including neuronal loss and synaptic dysfunction (Duman and Aghajanian, 2012). Among many candidates, growth factors and related signalling pathways constitute major players in neuroplasticity, and current evidence indicate that impairment of such regulations might be associated with depressed mood (Pittenger and Duman, 2008). Interestingly, currently prescribed antidepressants, predominantly functioning via monoaminergic signalling cascades, have been shown to modulate neuroplastic signalling to exert their therapeutic effects (Tardito *et al.*, 2006). Hence, a deeper understanding of the exact molecular and cellular mechanisms involved in antidepressant action might lead to the identification of key effectors, and provide further insight into the development of novel fast-acting and more effective therapies. Here, we summarized the current literature on the implication of trophic factors and associated signalling pathways in depression and antidepressant treatments. First, a brief overview of brain regions and circuits implicated in the pathophysiology of depression, and in the response to antidepressant is highlighted. Further, evidences for the involvement of growth factors and associated signalling pathways in depression and its treatment are described. Finally, perspectives and future directions for the development of new antidepressant drugs are suggested.

II – Brain region and neurocircuits

Neuroanatomical evidences: Many brain regions, especially within the limbic system, has been associated with mood and emotions, and the impairment in those areas might play a role in the pathophysiology of depression. For instance, the prefrontal cortex (PFC) has many functional connections within the brain, processing sensory input and mediating executive motor functions, and its links with the

limbic system (e.g. hippocampus and amygdala) form an integral part of memory consolidation and retrieval (Ongur and Price, 2000; Price, 1999). Especially, the prefrontal cortex has been associated with decision-making, personality expression and social behaviour. Neuroimaging studies found a reduction in size of multiple areas of the PFC in subjects diagnosed with major depression (Bremner *et al.*, 2002; Drevets, 2000). In line with those studies, post mortem brain analysis of depressed patients revealed reduced neural cell size and neural and glial cell densities in the dorsolateral and subgenual PFC (Cotter *et al.*, 2002; Öngür *et al.*, 1998; Rajkowska *et al.*, 1999). In rodents, chronic restraint stress caused a significant reduction in number and length of apical dendritic branches in some areas of the PFC (Cook and Wellman, 2004). Similar results were observed after chronic administration of corticosterone, where drastic dendritic reorganization of pyramidal neurons was reported in the medial PFC (mPFC) (Wellman, 2001). Dendritic arborisation in the mPFC induced by stress in rats was found to be predictive of impaired attentional set-shifting performance, a measure of cognitive performance related to mPFC functioning (Liston *et al.*, 2006).

Further, the amygdala is an integrant part of the limbic system implicated in cognitive and emotional processing, especially involving fear response (Aggleton, 1993; LeDoux, 2000). Volumetric MRI studies so far revealed contrasting results, with studies showing both an increase in volume (Frodl *et al.*, 2003; Lange and Irle, 2004; Vassilopoulou *et al.*, 2013) as well as a decrease in amygdala volume in depressed patients (Bellani *et al.*, 2011; Kronenberg *et al.*, 2009; Lorenzetti *et al.*, 2009). Functional magnetic resonance imaging (fMRI) studies in depressed patients have shown exaggerated amygdala activity when confronted with emotional facial expressions (Canli *et al.*, 2005; Sheline *et al.*, 2001), and increased memory sensitivity to negative stimuli (Hamilton and Gotlib, 2008). Depressed patients also showed an overall increased activity of the left amygdala (Drevets *et al.*, 1992). Similarly, Drevets and colleagues used PET imaging to find an increase in amygdala activation and metabolism in MDD patients (Drevets, 2003). A reduction in glial cell number, probably due to a decrease in oligodendrocytes, was discovered in post-mortem amygdala tissues of major depressive disorder (MDD) patients (Bowley *et al.*, 2002; Hamidi *et al.*, 2004). Increased fractional anisotropy suggesting improved connectivity was observed mainly from the left amygdala to the brain stem, cerebellum and hippocampus (Arnold *et al.*, 2012). Accordingly, experiments using rats exposed to chronic stress showed enhanced dendritic arborisation, elongation and spine density, providing evidence for increased synaptic connectivity in this brain region (Vyas *et al.*, 2006; Vyas *et al.*, 2002). However, functional coupling was reduced between the amygdala and the supragenual PFC (Matthews *et al.*, 2008). The heterogeneity of the depressed subjects used in the aforementioned studies is likely to be the cause of the contrasting results with factors such as medication, gender and onset and disease development. The data however strongly support the notion that disease severity correlates with more pronounced neurological alterations (Bellani *et al.*, 2011; Lorenzetti *et al.*, 2009), so there is no doubt that amygdala functioning is of particular interest in view of major depression.

Next to the PFC and amygdala, a preponderant role for the ventral striatum has been reported in major depression. The basic fundamentals of the natural reward system are attributed to the dopaminergic connections between the ventral tegmental area (VTA) and the nucleus accumbens (NAc). In this respect, the NAc and the VTA may play a role in the hedonic symptoms of depression (Nestler and Carlezon Jr, 2006; Yadid and Friedman, 2008). Depressed patients show an attenuated activation of the VTA-NAc pathway or the NAc itself when compared to normal patients using fMRI analysis (Epstein *et al.*, 2006; Furman *et al.*, 2011; Pizzagalli *et al.*, 2009; Smoski *et al.*, 2009). Deep brain stimulation (DBS) targeting the NAc was shown to have antidepressant, anxiolytic and hedonic effects, most notably in treatment resistant depression (Bewernick *et al.*, 2010; Giacobbe *et al.*, 2009; Schlaepfer *et al.*, 2008). These effects have been proven to be long lasting and stable up to four years (Bewernick *et al.*, 2012; Malone Jr *et al.*, 2009). Rat models indicate that NAc DBS influences the neurobiology of efferent projections of the NAc to the hippocampus. In response to NAc DBS, more neuronal precursors were found in the dentate gyrus of the hippocampus, hinting at an enhanced adult neurogenesis (Schmuckermair *et al.*, 2013). DBS may also alter the morphology of the PFC, with increased apical and basilar dendrite length (Falowski *et al.*, 2011). Both the alteration of the PFC and hippocampus in response of NAc DBS show the influence of the NAc in depression over the long term.

Finally, the hippocampus is a major part of the limbic system known to be highly vulnerable to stress and environmental variations. This region is critical in mood and cognition. MRI analyses revealed reduced hippocampal volumes in patients suffering from both recurrent and first episode depression (Bremner *et al.*, 2000; Cole *et al.*, 2011; Frodl *et al.*, 2007), and a correlation between volume reductions and total duration of major depression has been reported (Sheline *et al.*, 1996). It has also been proposed that reductions in hippocampal volume may not antedate illness onset, but volume may decrease at the greatest rate in the early years after illness onset (MacQueen *et al.*, 2003). Post mortem analysis in MDD patients suggested an increase in the density of pyramidal, granule and glial cells combined with a decrease of soma size of pyramidal cells (Stockmeier *et al.*, 2004). This might indicate a decrease in cellular neuropil which might provide an explanation for the reduced hippocampal volumes found in depressed subjects. In rodents, chronic stress caused atrophy of apical dendrites of pyramidal neurons in the CA3 region of the hippocampus (Magarinos *et al.*, 1996; Vyas *et al.*, 2002; Watanabe *et al.*, 1992). Exposure to excess glucocorticoids in rats showed decreased apical branching numbers and apical dendrite length (Woolley *et al.*, 1990), suggesting a role of the activation of the hypothalamic-pituitary-adrenal (HPA) axis in the remodelling of hippocampal morphology and neural cell loss associated with depression. Accordingly, various models of chronic stress exposure in rodents have indicated a decrease of neurogenesis in the dentate gyrus, involving a reduced proliferation, survival and differentiation of neural stem cells (Eisch and Petrik, 2012). Moreover, evidence that adult hippocampal neurogenesis is required for the behavioural effects of antidepressants (Sahay and Hen, 2007; Santarelli *et al.*, 2003) provides further proof of the crucial role of the hippocampus in depression.

Hippocampal plasticity and neurogenesis: Hippocampal neurogenesis is believed to be required in the therapeutic action of antidepressant drugs (David *et al.*, 2009; Djavadian, 2004; Klempin *et al.*, 2013; Santarelli *et al.*, 2003). Accordingly, chronic treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine increased hippocampal neurogenesis, involving the generation of newborn cells in the DG (Malberg *et al.*, 2000; Santarelli *et al.*, 2003; Surget *et al.*, 2011) (Boldrini *et al.*, 2009; Encinas *et al.*, 2006). In addition, chronic fluoxetine administration increased the dentate connectivity and synaptic plasticity in naive rats (Stewart and Reid, 2000). A report proving that serotonin depletion reduced neurogenesis in the DG and SVZ in adult rats also supports the theory linking antidepressant treatment to neurogenesis (Brezun and Daszuta, 1999). The different serotonin receptors in the brain showed differential region specific neurogenesis patterns. Activation of 5-HT_{1A} resulted in increased neurogenesis in the SGL and SVZ whereas activation of 5-HT_{2A} increased proliferation in the SGL but not the SVZ. The opposite was the case regarding 5-HT_{2C} activation, with an increased neurogenesis in the SVZ but not the SGL of the DG (Banasz *et al.*, 2004). Further, serotonin and noradrenaline reuptake inhibitor (SNRI) antidepressants, like SSRIs, have been shown to modulate neurogenesis and plasticity. Neurogenesis in the DG of the hippocampus was increased following chronic venlafaxine administration to rats (Mostany *et al.*, 2008). Similarly, chronic venlafaxine treatment proved to be efficient in preventing the deleterious effects of restraint stress on hippocampal neurogenesis and BDNF protein expression (Xu *et al.*, 2006). However, a recent study reported that duloxetine treatment did not significantly increase the number of new cells in the hippocampus of mice (Marlatt *et al.*, 2010). Finally, tricyclic antidepressants (TCA) have also proved to modulate hippocampal neurogenesis. Analysis of post-mortem brains of MDD patients treated with nortriptyline and clomipramine showed an increase of neural progenitor cells and dividing cells in the DG, as compared to healthy controls (Boldrini *et al.*, 2009). In animal models, clomipramine was able to counteract the stress-induced inhibition of proliferation in the hippocampus (Liu *et al.*, 2008). Chronic imipramine and desipramine treatment increased cell proliferation in multiple brain regions of the hippocampus of rodents (Pechnick *et al.*, 2011; Santarelli *et al.*, 2003; Schiavon *et al.*, 2010). 5-HT_{1A} knockout in mice abolished the antidepressant effects of fluoxetine but not desipramine and imipramine (Santarelli *et al.*, 2003). In addition, imipramine could restore the decrease in neurogenesis caused by chronic stress and chronic corticosteroid upregulation (Diniz *et al.*, 2013; Doi *et al.*, 2010; Hitoshi *et al.*, 2007; Kitamura *et al.*, 2011; Van Bokhoven *et al.*, 2011).

III – Neurotrophins and growth factors

BDNF: Brain-derived neurotrophic factor (BDNF) is involved in the growth, differentiation and survival of neurons (McAllister *et al.*, 2001) and is also an important factor in the regulation of neurogenesis (Rossi *et al.*, 2006) and synaptic plasticity (McAllister *et al.*, 1999). BDNF is abundantly expressed in the mammalian brain, with the highest concentrations found in the hippocampus and cortex (Hofer *et al.*,

1990). Reduction of BDNF and its tropomyosin-related kinase receptor B (TrkB) expression in the hippocampus and PFC has been reported in post-mortem brain analysis of suicide victims (Dwivedi *et al.*, 2003b; Pandey *et al.*, 2008), and lowered serum concentration of BDNF has been associated with MDD (Karege *et al.*, 2005; Karege *et al.*, 2002; Sen *et al.*, 2008). Interestingly, serum concentration could be largely restored with antidepressant treatment (Aydemir *et al.*, 2005; Gonul *et al.*, 2005; Piccinni *et al.*, 2008; Sen *et al.*, 2008; Shimizu *et al.*, 2003), indicating that serum BDNF levels could perhaps provide an indication of antidepressant response (Gass and Hellweg, 2010). One of the most common functional single nucleotide polymorphism (SNP) in the BDNF gene is the val66met. This polymorphism affects the activity-dependent secretion of BDNF (Egan *et al.*, 2003) and negatively influences hippocampal volume (Frodl *et al.*, 2007), and may in this way increase susceptibility to MDD. Expression of BDNF was also increased in the post mortem brain of depressed patients treated with antidepressant drugs as compared to non-treated patients (Chen *et al.*, 2001b; Dunham *et al.*, 2009). In rodents, direct infusion of BDNF protein in the hippocampus (Deltheil *et al.*, 2008; Deltheil *et al.*, 2009; Shirayama *et al.*, 2002; Sirianni *et al.*, 2010) and midbrain (Siuciak *et al.*, 1997) showed antidepressant-like effects. Contrastingly, the opposite effect was reported after infusion of BDNF in the VTA of the NAc (Eisch *et al.*, 2003). The same disparity was present using region specific knockdown of BDNF expression. The impairment of BDNF signalling in the DG of the hippocampus (Taliaz *et al.*, 2010) elicited pro-depressive behaviour whereas knockdown of BDNF in the NAc had an anti-depressive effect (Berton *et al.*, 2006). Conditional knockout in the forebrain resulted in an increase in depressive behaviour in female but not male mice (Monteggia *et al.*, 2007) and decreased the efficacy of the antidepressant desipramine (Monteggia *et al.*, 2004). A conditional knockout of BDNF in the forebrain displayed the same gender-specific incongruity in stress-induced depressive-like behaviour (Autry *et al.*, 2009). A different study using adeno-associated viral mediated knockout of BDNF in the DG and CA1 of the hippocampus proved that a loss of BDNF function in the hippocampus attenuated antidepressant drug treatment efficacy (Adachi *et al.*, 2008). Lee and colleagues developed a val66met mouse analogue (Chen *et al.*, 2006) and subsequent experiments showed that the val66met genetic variant decreased fluoxetine efficacy through impaired synaptic plasticity in the DG (Bath *et al.*, 2012; Ninan *et al.*, 2010) and PFC (Pattwell *et al.*, 2012). In addition, BDNF expression in the brain of rats was upregulated after chronic antidepressant drug and electroconvulsive therapy (ECT) (Altar *et al.*, 2003; Balu *et al.*, 2008; Jacobsen and Mørk, 2004; Nibuya *et al.*, 1995).

FGF: The fibroblast growth factor (FGF) family has been described as a major player in proliferation and maturation of neurons in the main neurogenic areas of the brain, the subgranular (SGZ) and subventricular (SVZ) zone of the dentate gyrus in the hippocampus. In addition, these growth factors (FGF1 and FGF2) were evidenced to play a critical role in the regulation of synaptic plasticity (Reuss *et al.*, 2003). Post mortem brain analysis revealed a lowered expression of FGF1 and FGF2 in the dorsolateral PFC and the anterior cingulate cortex of patients with MDD (Evans *et al.*, 2004). In addition,

FGF2 was decreased in the hippocampus of depressed patients, whereas fibroblast growth factor receptor 1 (FGFR1) was increased (Gaughran *et al.*, 2006). In rodents, Turner and colleagues reported reduced mRNA expression of FGF2 and its main receptor FGFR1 in the CA1, CA2, CA3 and DG following social defeat stress, a well-established model of depression (Turner *et al.*, 2008a). Moreover, intracerebroventricular (ICV) injection of FGF2 induced neurogenesis in both the SVZ and SGZ (Jin *et al.*, 2003; Mudo *et al.*, 2009; Rai *et al.*, 2007), and was also shown to improve depressive-like behaviour in rats (Turner *et al.*, 2008b). A more recent study also reported that increased neural proliferation in the PFC following FGF2 infusions might also be involved in the antidepressant actions of FGF2 (Elsayed *et al.*, 2012). Functional FGF2 knockout mice showed a significant decrease in newly generated neurons but no reduction in proliferating cells (Werner *et al.*, 2011). The additional increase in cell death in the hippocampus indicated a faulty neurogenesis following FGF2 knockout (Werner *et al.*, 2011). Conditional knockout experiments with FGFR1-*null* mice show defective long-term potentiation (LTP) and neurogenesis (Zhao *et al.*, 2007), suggesting that the FGF2/FGFR1 interaction might represent an important foundation of neurogenesis.

VEGF: Vascular endothelial growth factor (VEGF) is primarily known for its induction of angiogenesis and modulation of vascular permeability during embryogenesis and growth, as well as pathological events such as tumorigenesis (Ferrara *et al.*, 2003). In addition, mounting evidence suggests that VEGF can be considered as a potent neurotrophic factor as well (Nowacka *et al.*, 2012, Storkebaum *et al.*, 2003). A multitude of studies have investigated the plasma concentrations of vascular endothelial growth factor (VEGF) in MDD patients, but the data and interpretation remain conflicting possibly due to the differences in study design (Clark-Raymond and Halaris, 2013). However, experimental studies showed that VEGF displayed robust neuroprotective effects in cell models of ischemia and hypoxia (Jin *et al.*, 2000) as well as a positive effect on neuronal growth, maturation and proliferation under normoxic conditions (Khaibullina *et al.*, 2004; Rosenstein *et al.*, 2003; Silverman *et al.*, 1999; Sondell *et al.*, 1999; Zhu *et al.*, 2003). A role in the development of dendrites and axons has also been described for VEGF (Khaibullina *et al.*, 2004; Licht *et al.*, 2010; Rosenstein *et al.*, 2003; Sondell *et al.*, 1999). Chronic stress in rats decreased the expression of VEGF and its receptor in the hippocampus (Heine *et al.*, 2005). Moreover, ICV administration of VEGF increased neuroprotection and neurogenesis in the adult rat brain after ischemia (Sun *et al.*, 2003). More specifically, ICV administration of VEGF increased neurogenesis in both the SVZ and the SGZ of the DG with enhanced proliferation of neurons, astroglia and endothelial cells (Jin *et al.*, 2002), while VEGF-B knockout mice showed impaired neurogenesis (Sun *et al.*, 2006). Hence, the overexpression of VEGF in the hippocampus using an adeno-associated viral vector in rats resulted in increased neurogenesis and was associated with improved learning and memory (Cao *et al.*, 2004; During and Cao, 2006). Furthermore, VEGF is required for the proliferation of neural stem-like cells in the hippocampus following electroconvulsive therapy (ECT) treatments (Elfving and Wegener,

2012; Segi-Nishida *et al.*, 2008). In a similar manner, VEGF also seems to be required for the behavioural action of various antidepressants drugs in rodent models of depression (Greene *et al.*, 2009; Sun *et al.*, 2012; Warner-Schmidt and Duman, 2007; Warner-Schmidt and Duman, 2008).

GDNF: Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor first discovered in a glial cell line, and is expressed in many brain regions (Golden *et al.*, 1998). It is a member of the transforming growth factor β (TGF- β) superfamily and is important for neuronal survival (Pascual *et al.*, 2008). Only a few clinical studies investigating the role of GDNF in depression have been reported, and contrasting findings between brain and blood expression remain. A recent post-mortem brain analysis showed an increased of (GDNF) expression in the parietal cortex of depressed patients (Michel *et al.*, 2008). In contrast, decreased GDNF serum levels have been associated with depression (Diniz *et al.*, 2012b; Pallavi *et al.*, 2013; Zhang *et al.*, 2008), and negatively correlated with disease severity in adolescents (Pallavi *et al.*, 2013). Similarly, lowered expression of GDNF was also reported in peripheral white blood cells of depressed patients (Otsuki *et al.*, 2008). Interestingly, antidepressant treatment significantly increased the serum concentration of GDNF in depressed patients (Zhang *et al.*, 2008), as well as ECT in patients with drug-resistant depression (Zhang *et al.*, 2009). Experimental studies in animal models evidenced a neuroprotective role of GDNF, and ICV infusion of GDNF increased progenitor cell proliferation in the DG (Dempsey *et al.*, 2003) and SVZ (Kobayashi *et al.*, 2006). Similarly, infusion of GDNF in the striatum of rats increased progenitor cell proliferation in the hippocampus and substantia nigra (Chen *et al.*, 2005). Moreover, GDNF induced differentiation of DG-derived neural precursors into astrocytes *in vitro* (Boku *et al.*, 2013). Further, adeno-associated viral vector-induced overexpression of GDNF in the rat cortex provided neuroprotection against ischemia-induced injury (Tsai *et al.*, 2000). In addition, antidepressant treatment increased GDNF release in a rat C6 glioblastoma cell line (Hisaoaka *et al.*, 2001), whereas lithium treatment in rats resulted in increased GDNF concentration in the PFC and occipital cortex but a decrease in the hippocampus (Angelucci *et al.*, 2003b). Thus far, the exact involvement of GDNF in the aetiology of depression is not fully understood, but its neuroprotective capacity might make it an interesting future target for antidepressant treatment.

IGF-1: Insulin-like growth factor (IGF-1) has been designated as a potential therapeutic target for neurodegenerative disease such as depression (Carro *et al.*, 2003, Paslakis *et al.*, 2012). IGF-1 is a growth factor for many tissues and IGF-1 and its receptor IGF-1R are produced in many body tissues including the brain (Bondy *et al.*, 1993). Decreased serum concentrations of IGF-1 have been associated with increased neurodegeneration (Busiguina *et al.*, 2000). Studies using selective knockout mice showed that a decrease in both systemic and hippocampal IGF-1 levels could increase susceptibility to depression (Mitschelen *et al.*, 2011). IGF-1 induced differentiation of neuronal precursors (Anderson *et al.*, 2002; Arsenijevic and Weiss, 1998), and proved to be neuroprotective in cerebellar granule neurons *in vitro* (D'Mello *et al.*, 1993). IGF-1 knockout mice showed a decrease in total brain size and DG granular cell

layer volume, further supporting the importance of IGF-1 in neurodevelopment (Beck *et al.*, 1995). Developmental research in mice has revealed the importance of IGF-1 in hippocampal neurogenesis and synaptogenesis (O'Kusky *et al.*, 2000). ICV infusion of IGF-1 ameliorated the age-related decline in hippocampal neurogenesis (Lichtenwalner *et al.*, 2001), and peripheral administration of this growth factor could selectively induce hippocampal neurogenesis in rat (Åberg *et al.*, 2000). Interestingly, since IGF-1 can readily pass the blood brain barrier (Pan and Kastin, 2000), its effects on the brain can be achieved by direct injection in the blood circulation. Intranasal administration has been proposed in order to provide a shorter path for IGF-1 to enter the brain (Paslakis *et al.*, 2012), avoiding unwanted effects of IGF-1 in peripheral tissues. In addition, increase of peripheral IGF-1 concentration by direct injection of IGF-1 or inhibition of IGF-1 binding protein displayed anxiolytic and antidepressant effect in rodents (Duman *et al.*, 2009; Hoshaw *et al.*, 2005; Malberg *et al.*, 2007; Park *et al.*, 2011), which might be attributed in part to increased serotonin levels in the brain (Hoshaw *et al.*, 2008).

VGF: VGF nerve growth factor inducible (non-acronymic) is a small neuropeptide that plays a role in energy homeostasis, metabolism and synaptic plasticity. VGF expression is regulated by neurotrophin signalling. A recent study showed that depressed patients displayed lower expression of VGF in leukocytes, which could be reversed by antidepressant treatment (Cattaneo *et al.*, 2010). Moreover, VGF expression in the hippocampus and PFC, like the neurotrophins described above, is decreased in animal models of depression (Cattaneo *et al.*, 2010; Thakker-Varia and Alder, 2009). Interestingly, infusion of VGF in the hippocampus (Thakker-Varia *et al.*, 2007) or lateral ventricle (Hunsberger *et al.*, 2007) had an antidepressant behavioural effect in rats. This effect may be related to increased neurogenesis, which occurs after only 7 days of VGF administration to the hippocampus (Thakker-Varia and Alder, 2009). VGF expression can be increased by exogenous BDNF administration (Alder *et al.*, 2003) and BDNF might even be a requirement for regional VGF expression (Eagleson *et al.*, 2001). VGF is important in synaptic plasticity (Alder *et al.*, 2003) and it is indicated that it increases synaptic plasticity in a BDNF-dependent manner (Bozdagi *et al.*, 2008). The requirement of BDNF in VGF expression and the necessity of BDNF in VGF-induced synaptic plasticity show that VGF is heavily intertwined in the BDNF pathway. In addition, VGF expression can be upregulated by exercise (Hunsberger *et al.*, 2007) and ECT (Altar *et al.*, 2004; Newton *et al.*, 2003). Antidepressant treatment also increased VGF expression in the hippocampus and PFC of rats (Cattaneo *et al.*, 2010).

NGF: Nerve growth factor (NGF) is a growth factor first described as a neurite outgrowth factor (Olson, 1967). Later, NGF proved to be involved in neuron repair and survival (Kromer, 1987; Shigeno *et al.*, 1991; Sofroniew *et al.*, 2001; Zhao *et al.*, 2004). More recently, NGF has been implicated in neurogenesis in the striatum (Frielingsdorf *et al.*, 2007; Zhu *et al.*, 2011), hippocampal plasticity (Conner *et al.*, 2009) and proliferation and differentiation of neuronal stem cells (Cattaneo and McKay, 1990). However, only

few studies have investigated the role of this trophic factor in depression. Serum levels of NGF are lowered in elderly MDD patients (Diniz *et al.*, 2012a). Interestingly, antidepressant treatment using duloxetine, a serotonin-norepinephrine reuptake inhibitor (SNRI), could restore altered levels of NGF in major depressed patients (Martino *et al.*, 2013). However, this effect was not found in a clinical study using selective serotonin reuptake inhibitors (SSRI) antidepressant (Hellweg *et al.*, 2008). In the Flinders Sensitive Line (FSL) rat model of depression, it was found that ECT increased NGF levels in the hippocampus (Angelucci *et al.*, 2003a), and that NGF injections had antidepressant effects (Overstreet *et al.*, 2010).

IV – Intracellular signalling cascades

MAPK/PI3K: The mitogen-activated protein kinase (MAPK) cascade is activated by a G-protein called Ras (Schaeffer and Weber, 1999). Ras is activated following ligand binding to tyrosine kinase receptors and neurotrophins may exert their proliferative and neurogenic action via the Trk/Ras/MAPK pathway (Chao, 2003; Guan, 1994; Kaplan and Miller, 2000). MAPK signalling, via the induction of extracellular signal-regulated kinase (ERK), has been implicated in neuronal survival (Cavanaugh, 2004; Hetman *et al.*, 1999; Klesse *et al.*, 1999; Xia *et al.*, 1995) and neurotrophin induced survival (Creedon *et al.*, 1996; Wang *et al.*, 2007), in a way that most likely requires the involvement and activation of myocyte-enhancer factor 2C (MEF2C) (Wang *et al.*, 2007). Multiple lines of evidence point towards MAPK signalling as an important pathway in the action of neurotrophins in the brain (Kaplan and Miller, 2000). Several growth factors, including BDNF (Cavanaugh *et al.*, 2001; Obrietan *et al.*, 2002), NFG (Learish *et al.*, 2000) and EGF (Learish *et al.*, 2000) are known to increase the activity of MAPK. For example, MAPK activation was required for VEGF, EGF or FGF induced hippocampal proliferation (Abe and Saito, 2000a, b; Learish *et al.*, 2000; Lu *et al.*, 2011). In the PC12 cell line, p38 MAPK activation was required for the induction of neuronal differentiation by both NGF and epidermal growth factor EGF (Morooka and Nishida, 1998). BDNF promoted survival in neural cell cultures via a MAPK dependant activation of CREB (Bonni *et al.*, 1999; Hetman *et al.*, 2002). Decreased levels of ERK were found in the post-mortem brain of patients suffering from mood disorders (Yuan *et al.*, 2010). Administration of mitogen-activated protein kinase kinase (MEK) inhibitors was associated with increased depressive-like behaviour, suggesting the participation of the MAPK signalling cascade in the pathophysiology of depression (Qi *et al.*, 2009). Interestingly, a decrease of MAPK phosphorylation were found in the hippocampus and PFC of rats following chronic stress (First *et al.*, 2011; Gourley *et al.*, 2008; Qi *et al.*, 2006), and could be normalized successfully by chronic antidepressant treatment (First *et al.*, 2011; Gourley *et al.*, 2008; Hisaoka *et al.*, 2007). Furthermore, the Ras/MAPK pathway is known to be tightly intertwined with the phosphatidylinositide 3-kinase (PI3K) and its target kinase Akt (a serine/threonine kinase, also known as protein kinase B) in order to regulate neuronal survival (Klesse *et al.*, 1999). The activation of the PI3K/Akt pathway seems to play a role in growth factor response, including NGF, BDNF and FGF (Chen *et al.*, 2007; Peltier *et al.*, 2007; Yao and Cooper, 1995), and is implicated in LTP

and neurogenesis in the hippocampus (Bruehl-Jungerman *et al.*, 2009). The mammalian target of rapamycin (mTor) has also been described as a key-role mediator of the PI3K pathway, and many studies reported that the fast-acting and robust antidepressant-like properties of N-Methyl-D-aspartate (NMDA) antagonist might be assigned to the plasticity-related signalling underlying mTor activation (Autry *et al.*, 2011; Li *et al.*, 2010).

Wnt/ β -catenin/GSK3: The main course of action of Wnt is executed via the canonical pathway involving Frizzled receptors and dishevelled (Dsh) proteins, leading to the activation of β -catenin, possibly via the inhibition of glycogen synthase kinase 3 (GSK3) by phosphorylation (Miller *et al.*, 1999). Wnt/ β -catenin signalling is involved in cerebral development and stimulates proliferation and neurogenesis in the telencephalon (Gulacsi and Anderson, 2008). Wnt2 has been shown to be a key-role regulator of dopaminergic neuron proliferation in the developing ventral midbrain (Sousa *et al.*, 2010) and a conditional β -catenin knockout in the ventral midbrain of mice significantly reduced dopaminergic neurogenesis (Tang *et al.*, 2009). The overexpression of Wnt3 was sufficient to increase hippocampal neurogenesis of adult hippocampal progenitor cells both *in vitro* and *in vivo* (Lie *et al.*, 2005). Pharmacological blockade and genetic invalidation of Wnt pathway could attenuate adult hippocampal neurogenesis in rodents (Jessberger *et al.*, 2009). Moreover, Dickkopf-1 (Dkk-1), an inhibitor of the Wnt canonical pathway, caused neuronal cell death when injected in rat brains (Scali *et al.*, 2006), and a Dkk-1 knockout abolished the stress-related hippocampal atrophy in mice (Matrisciano *et al.*, 2011). It is interesting to note that Wnt2, Wnt5a and Wnt7a are involved in the β -catenin dependant synaptic plasticity by increasing synaptogenesis and dendritic arborisation (Ciani *et al.*, 2011; Sahores *et al.*, 2010; Varela-Nallar *et al.*, 2010; Varela-Nallar *et al.*, 2012; Wayman *et al.*, 2006; Yu and Malenka, 2003). Interestingly, post-mortem β -catenin levels were decreased in the ventral PFC of depressed patients as compared to healthy subjects (Karege *et al.*, 2012) and conditional knockout of β -catenin resulted in an increase in depressive-like behaviour in mice (Gould *et al.*, 2008). Further, BDNF proved to specifically increase the expression of Wnt1 and β -catenin in mouse neural stem cell cultures (Chen *et al.*, 2013). Chronic ECT and antidepressant treatment increased β -catenin and Wnt2 expression in the hippocampus of rats (Madsen *et al.*, 2003; Okamoto *et al.*, 2010). In addition, viral-mediated overexpression of Wnt2 in the hippocampus of rat caused antidepressant-like effects (Okamoto *et al.*, 2010). Viral-vector-induced knockdown of frizzled-6 resulted in increased depressive-like behaviour in rats (Voleti *et al.*, 2012). On the other hand, chronic antidepressant treatment and ECT decreased frizzled-3 in the rat frontal cortex (Yamada *et al.*, 2005). Furthermore, the inhibition of GSK3, an essential player in Wnt signalling, has been implicated as a potential therapeutic target for diabetes, neurodegenerative disease (Cohen and Goedert, 2004) and mood disorders such as depression (Li and Jope, 2010). Stimulation of neuroblastoma and cerebellar granule cells with BDNF led to increased phosphorylation of GSK3 β , resulting in the inhibition of its function (Foulstone *et al.*, 1999; Mai *et al.*, 2002). Similarly, NGF induced hippocampal axon growth required the inactivation of GSK3 β (Kim *et al.*, 2006; Zhou *et al.*, 2004). A post-mortem

study of depressed patients showed an increase in GSK3 kinase activity in the PFC of MDD patients, as compared to healthy controls (Karege *et al.*, 2007). It is interesting to note that inhibitors of GSK3 augmented neurogenesis of neural progenitor cells via the Wnt/ β catenin pathway *in vitro* (Lange *et al.*, 2011; Valvezan and Klein, 2012), and deletion of GSK3 α and β resulted in hyperproliferation of neural cells (Kim *et al.*, 2009). On the other hand, overexpression of GSK3 β induced neuronal death and atrophy of the DG (Sirerol-Piquer *et al.*, 2011). In rats, intraperitoneal administration of GSK3 inhibitor produced antidepressant-like effects (Gould *et al.*, 2004). Hence, the inhibition of GSK3 has been hypothesized as the mechanism by which lithium may exert influence on mood disorders such as depression (Wexler *et al.*, 2007). Accordingly, administration of antidepressant drugs like fluoxetine and imipramine that increase extracellular serotonin concentrations resulted in increased phosphorylation on GSK3 β in rodents (Beaulieu, 2007; Li *et al.*, 2004).

CREB: The cyclic AMP response element binding protein (CREB) is activated by various kinase signalling pathways (Adams *et al.*, 2000), and has been evidenced to participate in neural survival, as well as axon and dendrite outgrowth (Lonze *et al.*, 2002; Redmond *et al.*, 2002; Walton *et al.*, 1999). Post-mortem brain analysis of depressed suicide patients showed that CREB was decreased in the temporal cortex, hippocampus and PFC (Dowlatshahi *et al.*, 1998; Dwivedi *et al.*, 2003a). In addition, a decreased CRE-DNA binding activity in the hippocampus and PFC in suicide victims has been observed (Dwivedi *et al.*, 2003a). Similarly, a reduction in CREB and a significantly larger decrease in phospho-CREB were reported in the orbitofrontal cortex of MDD patients (Yamada *et al.*, 2003). In contrast, other studies indicated a higher expression of phospho-CREB in the PFC (Odagaki *et al.*, 2001) and in the amygdala of patients suffering from mood disorders (Young *et al.*, 2004). In animal models, mice modified to express constitutively active CREB showed increased BDNF expression and facilitated LTP in the hippocampus (Barco *et al.*, 2002). Further, viral-mediated overexpression of CREB in the hippocampus had an antidepressant effect (Chen *et al.*, 2001a), whereas overexpression in the basolateral amygdala increased depression and anxiety related behaviour (Wallace *et al.*, 2004). In a similar manner, viral-mediated expression of a dominant-negative mutant of CREB (mCREB) effectively blocked CREB activity in the NAc and produced an antidepressant effect (Newton *et al.*, 2002; Pliakas *et al.*, 2001), whereas overexpression in the NAc increased depressive behaviour (Pliakas *et al.*, 2001). Furthermore, a growing body of literature indicate a critical role for CREB in the mechanism of action of antidepressants. Chronic administration of SSRI and SNRI antidepressants and ECT in rats increased the transcription of CREB mRNA, as well as CRE-DNA binding activity, in the CA1 and CA3 pyramidal layer and granule layer of the DG after 10 days of treatment (Blom *et al.*, 2002; Nibuya *et al.*, 1996). An increase of phospho-CREB but not CREB was found in the PFC after chronic antidepressant treatment (Laifenfeld *et al.*, 2005). On the other hand, CREB deficient mice treated with desipramine and fluoxetine showed the same antidepressant-like effects as wild type mice, indicating that CREB is not required for the antidepressant actions of SNRIs and SSRIs (Conti *et al.*, 2002). Administration of desipramine in NAc tissue dissected

from rat brains were reported to decrease pCREB (Chartoff *et al.*, 2009). In addition, in CREB deficient mice, treatment with desipramine neither increased neurogenesis (Gur *et al.*, 2007), nor induced the BDNF upregulation normally seen after desipramine administration (Conti *et al.*, 2002) indicating that CREB may be necessary for the increase in BDNF following antidepressant treatment. Altogether, the current literature indicates that CREB has a central role in depression, but its exact involvement in the aetiology and treatment of depression remains to be further elucidated.

V – Concluding remarks

Growth factors and associated neuroplasticity signalling play an essential role in the development and maintenance of the central nervous system (Anlar *et al.*, 1999; Ford-Perriss *et al.*, 2001; Greenberg *et al.*, 2009). Accordingly, there is growing evidence that abnormal trophic support in cortico-limbic regions regulating mood and emotions might account in the pathophysiology of depression (Krishnan and Nestler, 2008). In addition, clean-cut evidence also showed that antidepressants require neuroplasticity pathways to rescue the deficits in synapse density, dendritic arborization and neuronal functioning often associated with mood disorders (Pittenger and Duman, 2008). However, several limitations about the neurotrophic hypothesis of depression remain. Current knowledge makes it difficult to conclude whether neuroplasticity and neurogenesis represent a cause or only a consequence of the pathological processes associated with depression. Hence, future research should focus on elucidating the exact involvement of neurotrophic signalling in the onset and aetiology of major depression. Furthermore, mounting evidence seems to indicate that neurogenesis might not be required for the therapeutic action of antidepressants (Bessa *et al.*, 2009). In line with this hypothesis, the usage of N-methyl-D-Aspartate (NMDA) receptor antagonists showed that rapid induction of neuroplasticity pathways such as BDNF and mTor signalling pathways was sufficient to produce a robust and prolonged antidepressant effect (Autry *et al.*, 2011; Li *et al.*, 2010). Hence, the rapid enhancement of hippocampal neuroplasticity – involving dendritic growth, spine density and synaptic transmission - represent an original strategy to circumvent the delayed efficacy of current antidepressant drugs. Finally, the use of drugs that specifically target neurotrophic signalling should provide more insights about the involvement of neuroplasticity pathways in the mediation of antidepressant response. Nevertheless, growth factors and neurotrophins have considerable limitations, including a poor blood-brain barrier penetration and a short half-life in plasma, making these small proteins difficult to use in animal models or in the clinic (Nave *et al.*, 1985; Ochs *et al.*, 2000; Poduslo and Curran, 1996). Hence, identification of small non-peptidic neurotrophin mimetics remains a current challenge in neuroscience research, and may represent an interesting target for the development of a new class of therapeutic agents for mood-related disorders.

VI – References

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CHAPTER IV

TrkB inhibition as a therapeutic target for CNS-related disorders

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Abstract

The interaction of brain-derived neurotrophic factor (BDNF) with its tropomyosin-related kinase receptor B (TrkB) is involved in fundamental cellular processes including neuronal proliferation, differentiation and survival as well as neurotransmitter release and synaptic plasticity. TrkB signaling has been widely associated with beneficial, trophic effects and many commonly used psychotropic drugs aim to increase BDNF levels in the brain. However, it is likely that a prolonged increased TrkB activation is observed in many pathological conditions, which may underlie the development and course of clinical symptoms. Interestingly, genetic and pharmacological studies aiming at decreasing TrkB activation in rodent models mimicking human pathology have demonstrated a promising therapeutic landscape for TrkB inhibitors in the treatment of various diseases, e.g. central nervous system (CNS) disorders and several types of cancer. Up to date, only a few selective and potent TrkB inhibitors have been developed. As such, the use of crystallography and *in silico* approaches to model BDNF-TrkB interaction and to generate relevant pharmacophores represents powerful tools to develop novel compounds targeting the TrkB receptor.

Key words: BDNF, Cancer, CNS disorders, TrkB inhibitors, Treatments

CHAPTER V

Blockade of the BDNF receptor TrkB by cyclotraxin-B downregulates plasticity genes concomitant with epigenetic changes in differentiated human neuroblastoma cells

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Abstract

Brain-derived neurotrophic factor (BDNF) is a key-role player in structural and functional plasticity, especially within the midbrain dopamine neurons that project to the ventral striatum. Recent evidence showed that drug abuse and social defeat in rodents could produce lasting epigenetic remodeling as well as BDNF upregulation, associated with major reorganizations within the neuronal network of the mesolimbic dopaminergic system. In the present study, we wanted to explore to which extent the inhibition of the BDNF tropomyosin-related kinase receptor B (TrkB) with the novel antagonist cyclotraxin-B could induce epigenetic remodeling at downstream TrkB target genes involved in neuroplasticity. As such, we used SH-SY5Y human neuroblastoma cells, known for their ability to differentiate into mature dopaminergic cells expressing high levels of activated TrkB. Our data indicate that cyclotraxin-B produced enduring downregulation of AKT1 and BCL2 expression, two major players in dendritic arborization and neuronal survival. Moreover, we showed that cyclotraxin-B-induced persistent changes in gene expression were associated with post-translational modifications at histone tails and changes in the machinery controlling DNA methylation. Hence, our findings provide further insight into the neuroplastic and epigenetic effects of cyclotraxin-B, and point out that the ability of TrkB antagonists to reverse abnormal BDNF-mediated epigenetic remodeling in the dopaminergic system could have therapeutic potentialities.

Key words: TrkB inhibitor, gene expression, chromatin, methylation, acetylation, epigenetics, neuronal plasticity

I – Introduction

The interaction of brain-derived neurotrophic factor (BDNF) with its tropomyosin-related kinase receptor B (TrkB) plays an essential role in the establishment and maintenance of neuronal and synaptic plasticity (Chao, 2003). It is well established that chronic stress can lead to reduced BDNF expression resulting into neuronal atrophy and cell loss in the hippocampus, which might play an important role in the pathophysiology of stress-related diseases including post-traumatic stress disorders (PTSD), anxiety disorders and depression (Autry and Monteggia, 2012). In contrast, repeated administration of psychostimulants has been associated with increased levels of BDNF in the mesolimbic dopaminergic system, a process that is required for both sensitization and dependence to drugs (Graham et al., 2007; Russo et al., 2009). In addition, the increased plasticity induced by elevation of BDNF was also shown to be important in epileptogenesis, as well as in the induction and maintenance of neuropathic pain (Isackson et al., 1991; Wang et al., 2009). Mounting evidence suggests that the variations observed in BDNF expression and downstream signaling may rely on epigenetic regulation, conferring a stable imprint on gene transcription and susceptibility for disease (Bouille et al., 2012). BDNF has a complex gene structure that has been reported to be highly sensitive to environmental variations and concomitant epigenetic modifications (Bouille et al., 2012) underlying changes in gene expression. For instance, the methyl-CpG binding protein 2 (MeCP2) binds selectively to methylated DNA at BDNF promoter IV to repress its transcription (Chen et al., 2003). In addition, stable post-translational modifications at histone tails (i.e. H3K9 acetylation and H3K27 methylation) have been reported at the BDNF gene in ventral tegmental area (VTA) dopaminergic neurons projecting into the nucleus accumbens (NAc) in response to drug administration or withdrawal (Kumar et al., 2005; Sadri-Vakili et al., 2010; Schmidt et al., 2011). Hence, the identification and further characterization of drugs that directly target TrkB and its downstream neuroplastic effectors represent a promising avenue in neuroscience research. To date, only few drugs with TrkB inhibitory properties have been described (Bouille et al., 2012). In our experiments, we used a recently developed and innovative TrkB inhibitor, known as cyclotraxin-B. This small peptidic compound acts as a selective and potent TrkB antagonist that can block both BDNF-dependent and BDNF-independent activation of TrkB (Cazorla et al., 2010). In addition, cyclotraxin-B was shown to cross the blood-brain-barrier (BBB), reduce neuropathic pain in rats (Constandil et al., 2011), and produce anxiolytic effects in mice (Cazorla et al., 2010). In the present study, we wanted to explore to which extent the blockade of TrkB with cyclotraxin-B could involve lasting epigenetic changes at downstream plasticity-related genes in an *in vitro* model of differentiated dopaminergic cells.

II – Experimental procedures

Cell culture and treatment: SH-SY5Y cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and had undergone between 20 and 30 passages before use in our experiments. The cells were cultured in a 1:1 mixture of F-12K Kaighn's modification nutrient medium and Dulbecco's Modified Eagle Medium (ATCC, Molsheim, France) supplemented with 10% fetal calf serum, 10 U/ml penicillin G, 10 mg/ml streptomycin and 2 nM of L-Glutamine. To induce differentiation, the cells were treated with 10 μ M of retinoic acid (RA) in culture medium for 5 days, and the medium was refreshed every 2 days. Pharmacological treatment was performed on 5-days-RA-differentiated SH-SY5Y cells using the TrkB antagonist cyclotraxin-B (Cazorla et al., 2010). Based on previous reports, a dose of 1 μ M cyclotraxin-B was used (Cazorla et al., 2010). Cyclotraxin-B was added to the culture medium for 6 or 24 hours, while chronic 96 h treatments consisted of 4 successive cyclotraxin-B exposures of 24 h each.

Cell survival and differentiation: Cell viability (mitochondrial activity) was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. SH-SY5Y cells were incubated for 2 h at 37°C in the presence of MTT (0.5 mg/mL) diluted in culture medium. Incubation with isopropanol was performed for one additional hour. The absorbance was then measured at 540 nm (Victor X3, Perkin Elmer). Cells treated with 10% DMSO 1 h prior to the MTT addition were used as blank and the corresponding value was subtracted as background for each sample. For neurite outgrowth measurements, images of the cells were taken using an Olympus IX81 phase-contrast microscope (Olympus, Hamburg, Germany), and the neurite extensions were traced and quantified using the Neuron J plug-in of the Image J software (ImageJ 1.42, NIH, USA).

Western blot: SH-SY5Y cells were lysed in ice-cold lysis buffer (1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0, 10 mM NaF, 1 mM sodium orthovanadate and protease inhibitors cocktail) and protein content was loaded on a SDS-PAGE gel for electrophoretic migration and then transferred to a nitrocellulose membrane for 1 h at 4°C. Non-specific binding was blocked by incubating the membranes in an Odyssey blocking buffer (LI-COR Biosciences, NE, USA) for 1 h at room temperature. Rabbit anti-TrkB (Merck Millipore, MA, USA) and rabbit anti-phospho-TrkB Y817 (Epitomics Inc, CA, USA) were used as primary antibodies for the detection of endogenous TrkB and its activated form. The membranes were incubated with the primary antibodies overnight at 4°C in the Odyssey blocking buffer. Then, after extensive washing, the membranes were incubated with the appropriate fluorescent secondary antibodies (Alexa Fluor 488 goat anti-rabbit; Molecular Probes, OR, USA) for 1 h at room temperature. Finally, the membranes were washed, dried and scanned using the Odyssey[®] infrared imaging system (Westburg, Leusden, The Netherlands).

Reverse transcription and real-time quantitative PCR (RT-qPCR): Total mRNA extraction was performed using Trizol® (Invitrogen, CA, USA), after which, cDNA synthesis was performed using a First Strand cDNA synthesis kit (Fermentas International Inc. Canada) according to the manufacturer's protocol. Amplification was made with SYBR green I Master Mix (Roche Diagnostic, IN, USA). Primer sequences are indicated in Table S1. Gene expression was normalized using HPRT and RS27A as reference genes. Gene expression analysis was performed with the LightCycler 480 Real-Time PCR System (Roche Applied Science, IN, USA) and the conditions for the reaction involved 35 cycles in a fixed sequence of 95°C for 30 s, 62°C for 15 s, and 72°C for 15 s.

Chromatin immunoprecipitation (ChIP): Briefly, cells were fixed in a 1% formaldehyde solution, after which they were lysed in ice-cold SDS lysis buffer containing protease inhibitors (Roche, Meylan, France). The chromatin was sheared in order to obtain DNA fragments that were approximately between 400 and 800 base pairs. The solution of chromatin was pre-cleared with a mixture of protein A-agarose (Pierce, Thermo-Fischer, Illkirch, France) and salmon sperm (Agilent technologies, CA, USA), and then immunoprecipitated overnight at 4°C with 2 µg of rabbit anti-H3K9ac, mouse anti-H3K27me3 (Millipore, Molsheim, France) or rabbit anti-MeCP2 (Sigma Aldrich, MO, USA) antibodies. After intensive washes, the immunoprecipitate was eluted from the beads using a 1 % SDS elution buffer and the protein-DNA cross-links were reversed by incubating the samples overnight at 65°C in the presence of 5 M NaCl. DNA fragments were then treated with proteinase K in order to digest the proteins, and DNA was isolated using phenol/chloroform/isoamyl alcohol and then precipitated with 100% ethanol and 20 µg of glycogen. Fragments of promoter regions of target genes were quantified by quantitative PCR (see Table S2 for primer sequences and design). Ct values of immunoprecipitated samples were normalized to Ct values obtained from Input DNA. The specificity of the antibodies used in the experiments was determined by immunoprecipitation of the sample with non-specific HA-probe (Y-11) antibody (Santa Cruz Biotechnology Inc, CA, USA).

Statistical analysis: Data are presented as mean + SEM. Statistical significance was determined using a two-tailed Student's t test for comparison between two groups and, when necessary, a Welch's correction was used (Prism 5.0, GraphPad software, USA). The level of significance was set as $P < 0.05$.

III – Results

Effects of RA treatment on cell differentiation and BDNF/TrkB signaling: To induce differentiation, we treated SH-SY5Y cells with 10 µM of RA for 5 days. Tracing and quantification of neurite length indicated that RA treatment increased neurite outgrowth as compared to non-treated cells ($p=0,0019$, after 5 days of treatment; Figure 1A). In a similar manner, RA-treated SH-SY5Y cells showed increased

viability as compared to non-treated cells ($p=0.0007$; Figure 1A). Then, we wanted to investigate the potential implication of BDNF/TrkB signaling in RA-induced SH-SY5Y cell differentiation. We thus performed different kinetics of stimulation with 10 μM of RA in order to visualize the time-dependent implication of BDNF/TrkB signaling during SH-SY5Y cell differentiation. As shown by western blot analysis, the expression of full-length TrkB (≈ 145 kDa) increased gradually over the 5 days of RA treatment (Figure 1B). In a similar manner, the phosphorylation of TrkB at tyrosine residue 817 progressively increased with RA treatment, indicating an overall increase of TrkB activation during RA-induced SH-SY5Y cell differentiation (Figure 1B). Parallel RT-PCR quantifications showed that TrkB mRNA levels were increased by more than 200 times after a 5-day RA treatment ($p=0.0087$; Figure 1B), while those of BDNF mRNA did not significantly change (Figure 1B). As such, we also measured the expression of diverse genes implicated at different levels of neuronal and synaptic plasticity, and known to be involved in BDNF/TrkB downstream signaling. Accordingly, the transcripts of AKT1, BCL2, cFOS, HOMER1 and VGF were significantly upregulated ($p=0.033$, $p=0.0008$, $p<0.0001$, $p=0.004$ and $p=0.03$ respectively; Figure 2), while those of CREB, ERK1 and JNK genes did not change significantly after a 5-day RA treatment ($p>0.05$; Figure 2).

Effects of cyclotraxin-B on gene expression and epigenetic regulation: Because differentiated SH-SY5Y cells were found to express high levels of TrkB and phospho-TrkB, experiments were performed in order to assess whether they could represent a model of choice to explore the cellular and molecular changes evoked by TrkB blockade. Differentiated SH-SY5Y cells were exposed for various times to the TrkB antagonist cyclotraxin-B (1 μM), and the resulting effects on the expression of downstream target genes were evaluated by real-time RT-qPCR. Quantitative determination of gene expression profiles at various times (see Figure 3) revealed that the AKT1, BCL2, TRKB and VGF genes were significantly downregulated after a 6 h exposure to cyclotraxin-B ($p=0.011$, $p=0.0003$, $p=0.0062$ and $p=0.042$ respectively), whereas mRNA expression of the BDNF and cFOS genes remained unchanged. After a 24 h treatment with cyclotraxin-B, only the AKT1 and BCL2 gene expression was still downregulated ($p=0.0002$ and $p=0.0021$ respectively; Figure 3). Finally, a tendency for a downregulation of AKT1 expression and a significant decreased expression of BCL2 were observed after the 96 h exposure to cyclotraxin-B ($p=0.10$ and $p=0.014$ respectively; Figure 3). Thus, the gene expression profiles pointed towards a lasting downregulation of BCL2 and AKT1 during exposure to cyclotraxin-B.

We then examined whether these changes observed at the gene expression level could be attributed to epigenetic modifications. As such, we performed chromatin immunoprecipitation after 4 successive stimulations of 24 hours each with 1 μM of cyclotraxin-B and measured H3K9ac, H3K27me3 and MeCP2 binding at the AKT1, BCL2, TRKB and VGF genes. A t-test comparison between control and cyclotraxin-B groups showed a specific enrichment of H3K27me3 (generally known as a repressive

mark) at the AKT1 gene promoter ($p=0.036$; Figure 4). Similarly, MeCP2, known as a repressor of gene transcription, was significantly increased at the TRKB and VGF gene promoters ($p=0.0057$ and $p=0.030$ respectively; Figure 4). In contrast, a tendency ($p=0.083$; Figure 4) for decreased H3K9ac, known as a permissive mark, was observed at the BCL2 gene promoter. Of note, a general tendency towards a decreased H3K9ac and increased H3K27me3 at TrkB target genes was observed. Hence, cyclotraxin-B appears to mediate its effects on gene expression via modification at histone tails and MeCP2 binding.

IV – Discussion

In the present study, we explored to which extent the inhibition of BDNF/TrkB signaling by cyclotraxin-B underlies epigenetic remodeling at downstream target genes. Our results showed that cyclotraxin-B induced a lasting downregulation of the expression of a subset of genes involved in neuroplasticity pathways, concomitant with post-translational modifications at histone tails as well as altered MeCP2 binding at those genes.

A recent study demonstrated that RA treatment of SH-SY5Y cells induced their differentiation into a predominantly mature dopaminergic-like neuronal phenotype, characterized by increased dopamine synthesis and reuptake, together with a substantial suppression of other neurotransmitter phenotypes (Korecka et al., 2013). In the present study, we showed that RA treatment induced the expression and activation of TrkB receptor as previously reported (Encinas et al., 2000). Hence, the RA-treated SH-SY5Y cells constitute a unique *in vitro* approach to explore BDNF/TrkB signaling in human dopaminergic neuron-like cells, and the high levels of TrkB phosphorylation represent a valuable model to study the action of TrkB inhibitors. An increase of TrkB signaling in the mesolimbic dopaminergic system has been reported following repetitive administration of psychostimulants, as well as after social defeat stress in rodents (Krishnan et al., 2007; Russo et al., 2009). In SH-SY5Y cells, we showed that BDNF levels were not significantly modulated by RA treatment indicating that the increased TrkB activation observed in differentiated SH-SY5Y cells might be triggered by BDNF-independent mechanisms. Such kind of phenomenon, known as transactivation, has been described for the TrkB receptor, and might be responsible for the maintenance of an excessive TrkB activation and a disturbed cellular homeostasis (Bouille et al., 2012). Interestingly, cyclotraxin-B was previously shown to inhibit both BDNF-dependent and -independent activation of TrkB (Cazorla et al., 2010). The administration of cyclotraxin-B on RA-treated cells resulted in the lasting downregulation of the AKT1 and the BCL2 genes. Activation of the PI3K/Akt pathway through TrkB stimulation, involving AKT1 gene, is known to lead to structural changes in neuronal plasticity such as dendritic arborization and cellular morphology (Kumar et al., 2005), whereas BCL2 has been described as an important regulator of cell survival (Davies, 1995). Some studies found that the activation of Akt signaling in the striatal dopaminergic

system mediated excessive alcohol drinking behavior in rats, as well as nicotine and cocaine-induced plasticity (Neasta et al., 2011; Collo et al., 2012, 2013). Similarly, repetitive administration of cocaine was shown to induce BCL2 activation in the rat dorsal striatum (Ahn et al., 2010).

On the other hand, an expanding body of evidence indicates that the maintenance of neuronal plasticity and integrity is controlled by epigenetic regulation involving DNA methylation and histones modifications (Maze et al., 2012; Sultan and Day, 2011). In particular, BDNF appears to be a primary target for epigenetic regulation, and persistent epigenetic modification of BDNF in the striatum has been observed after cocaine administration or prolonged stressors of high intensity (Boulle et al., 2012). Hence, the lasting upregulation of BDNF might subsequently impact on TrkB signaling and the epigenetic control of downstream gene expression. In the present study, we provide evidence that cyclotraxin-B, via the downregulation of TrkB signaling, could involve persistent changes in gene expression, mediated by epigenetic regulations. We found that cyclotraxin-B impacts on post-translational modifications at histone residues as observed by the changes in H3K9 acetylation and H3K27 tri-methylation. Similarly, cyclotraxin-B increased MeCP2 binding at some specific TrkB target genes, suggesting higher levels of methylation at the corresponding promoters. These latter observations might explain, at least in part, the persistent downregulation of TrkB target gene expression and indicate that epigenetic remodeling is part of the pharmacological action of cyclotraxin-B.

Altogether, our data support the notion that TrkB signaling can underlie epigenetic remodeling at plasticity-related genes, and that its selective blockade by cyclotraxin-B is able to induce epigenetic remodeling in order to significantly impact on neuroplasticity. The usage of such compounds might be relevant to investigate the therapeutic value of TrkB inhibitors in models of drug addiction, where epigenetic alterations at BDNF/TrkB signaling seem to be critically implicated in underlying pathophysiological mechanisms.

V – Acknowledgements

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Chapter V

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Figures and legends.

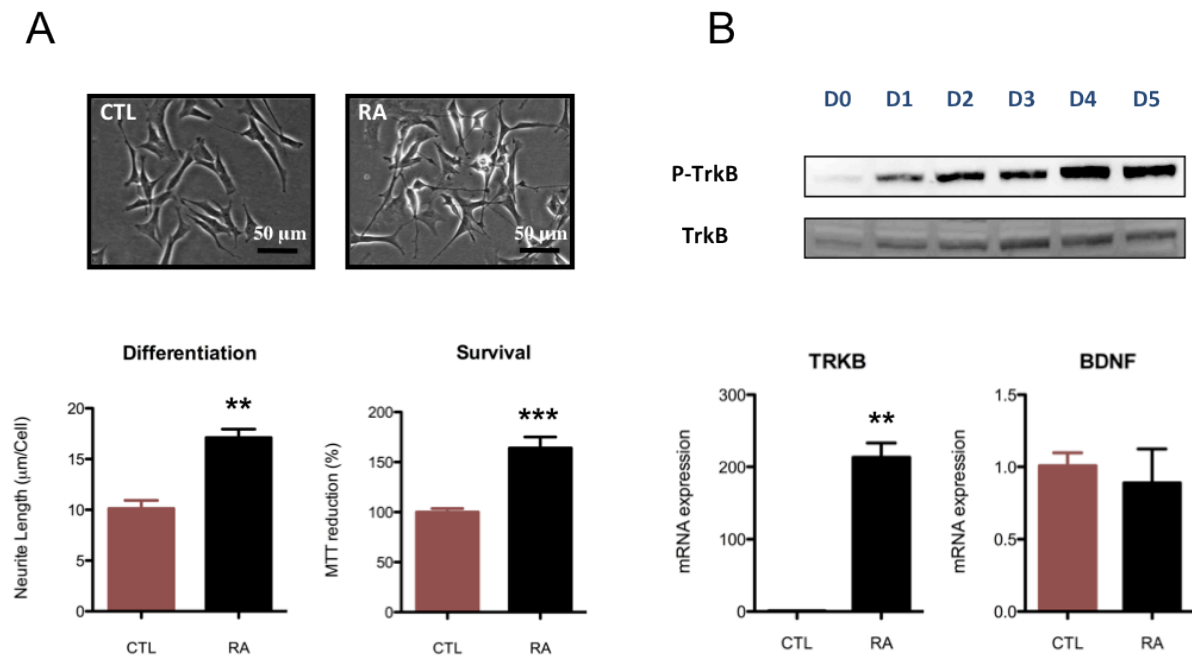


Figure 1. TrkB receptor is essential for SH-SY5Y cells differentiation and survival.

(A) SH-SY5Y cells were treated with RA (10 μM) or kept in normal culture medium for 5 days. Representative micrographs of 3 independent experiments are shown in the top panel (scale bar = 50 μm). Neurite extensions, measured using the Neuron J plug-in of Image J software, are expressed as mean length (μm) per cell (bottom panel left). SH-SY5Y cell viability after 5 days of RA was determined by a MTT assay (bottom panel right). (B) Western Blot analysis of SH-SY5Y cells treated for 1, 2, 3, 4 or 5 days (D1, D2, D3, D4 and D5) with 10 μM of RA. A representative picture from 3 independent experiments is shown. TRKB and BDNF mRNA expressions after 5 days of RA treatment are depicted in the bottom panel. Data are expressed as mean + SEM of triplicate determinations in 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs. CTL; Student's t test.

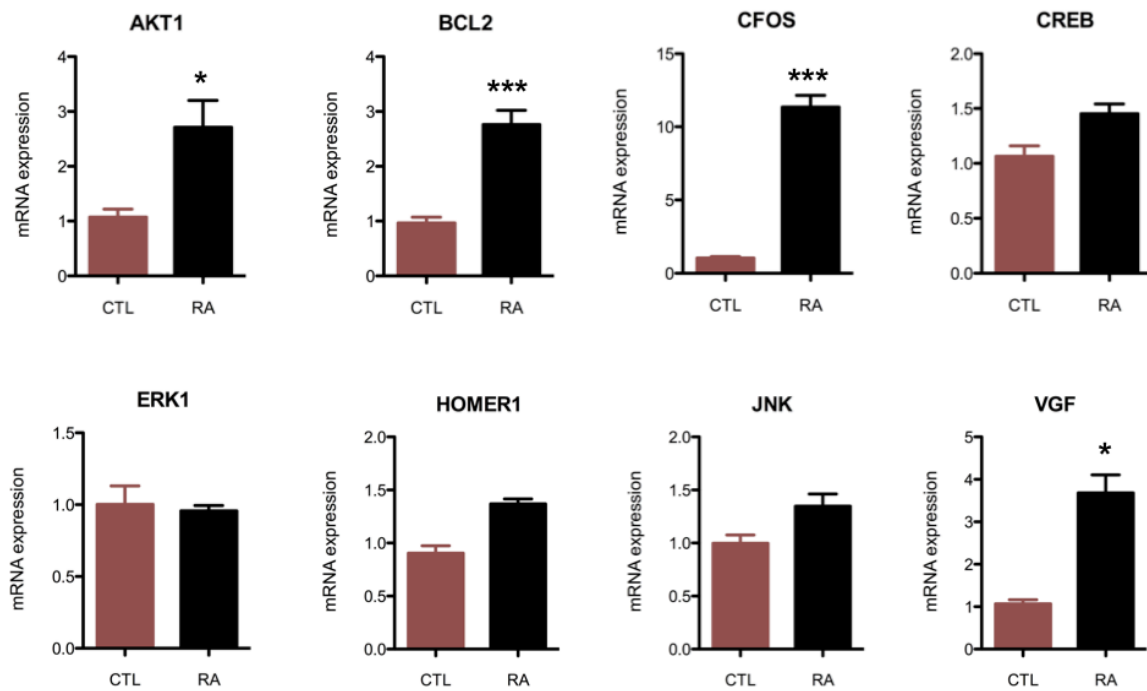


Figure 2. Effects of RA treatment on the expression of genes involved in TrkB-mediated neuroplasticity.

SH-SY5Y cells were treated with RA (10 μ M) or maintained under normal control conditions for 5 days before RT-qPCR determinations of mRNAs. The fold changes evoked by RA are expressed with respect to non-treated cells (CTL). Each bar is the mean + SEM of two representative experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CTL; Student's t test.

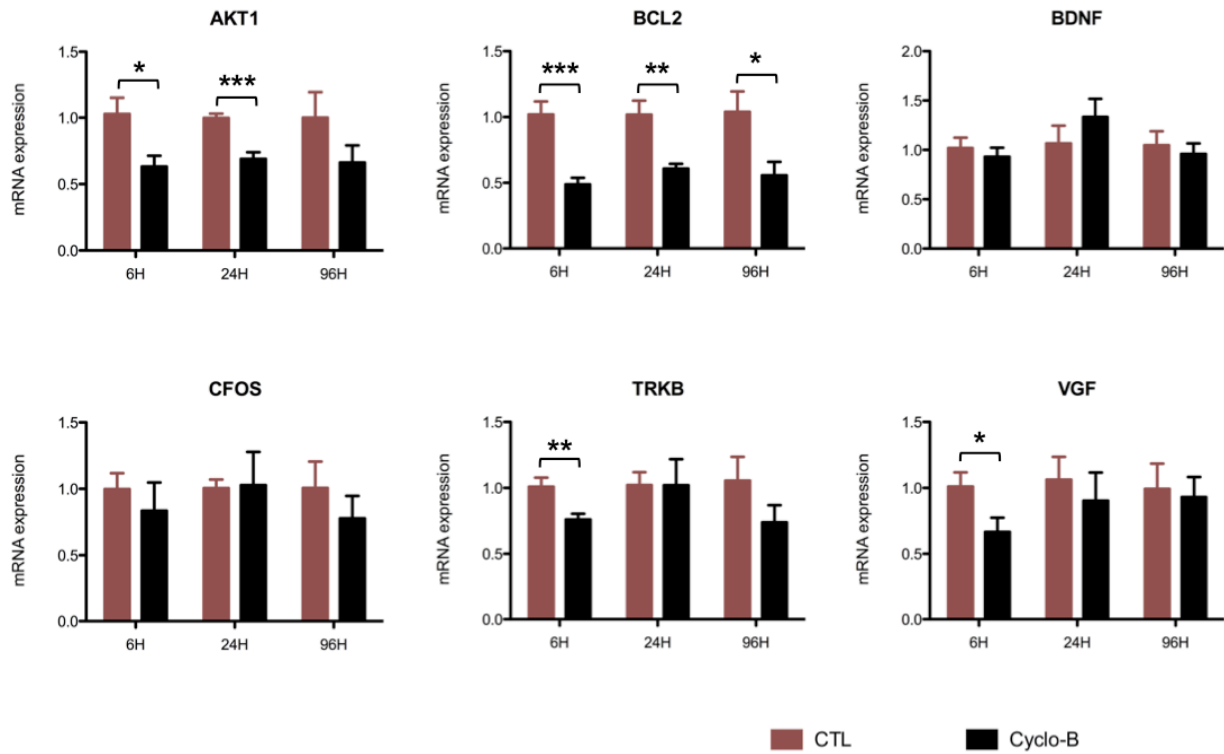


Figure 3. Gene expression profiles after TrkB inhibition in differentiated cells.

Differentiated SH-SY5Y cells were exposed to 1 μ M of cyclotraxin-B (Cyclo-B) for 6, 24 or 96 hours. Exposure for 96 hours corresponded to 4 successive administrations, every 24 hours. Cyclotraxin-B-induced effects on mRNA expression are expressed as fold changes in respective mRNA levels compared to the non-treated conditions (CTL). Each bar is the mean + SEM of two representative experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. CTL; Student's t test.

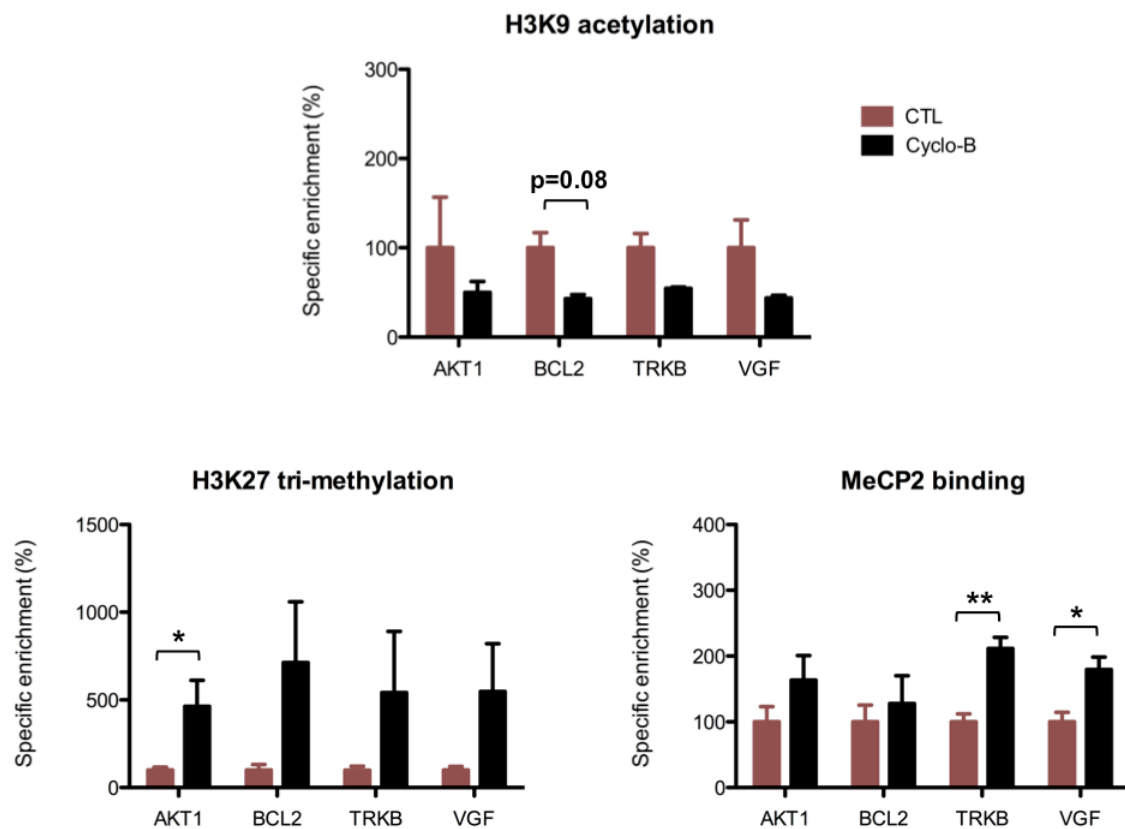


Figure 4. Epigenetic expression profiles after TrkB inhibition in differentiated cells.

Differentiated SH-SY5Y cells were exposed to 1 μ M of cycloheximide (Cyclo-B) for 96 hours, corresponding to 4 successive administrations for 24 hours each. The specific enrichment of H3K9ac, H3K27me3 and MeCP2 at corresponding genes is expressed as percentage of non-treated conditions (CTL). Each bar is the mean + SEM of 3 independent determinations. * $p < 0.05$, ** $p < 0.01$ vs. CTL; Student's t test.

Supplementary Material.

Gene Abbreviation	Forward primer	Reverse primer
AKT1	5'-ACCATGAAGACCTTTTGCGG-3'	5'-CATCTCGTACATGACCACGC-3'
BCL2	5'-ACTGAGTACCTGAACCGGC-3'	5'-CCGTACAGTTCCACAAAGGC-3'
BDNF	5'-AAACATCCGAGGACAAGGTG-3'	5'-AGAAGAGGAGGCTCCAAAGG-3'
CREB1	5'-GAGTTGTTATGGCATCCTCCC-3'	5'-CTTCCCTGTTCTTCATTAGACGG-3'
CFOS	5'-GAGAAAAGGAGAATCCGAAGGG-3'	5'-TCTAGTTGGTCTGTCTCCGC-3'
HOMER1	5'-TCAGTTTAGATGGCTCAAAGGC-3'	5'-TCCATAAACGGTGTGTTGCC-3'
HPRT	5'-CTTGGTCAGGCAGTATAATCCAA-3'	5'-CTTATATCCAACACTTCGTGGGG-3'
MAPK3	5'-TTCTCCTGGCAACTCCCC-3'	5'-TTCTGCCACTCTGAAAAGGC-3'
RS27A	5'-GGTTAAGCTGGCTGTCTCGAA-3'	5'-AGAAGGGCACTCTCGACGAA-3'
TRKB	5'-ACTTGCTGGTGAAAATCGGG-3'	5'-ATGGGCAGCATTGTGTGG-3'

Table S1. Sequences of primers used for real time quantitative RT-PCR.

Gene Abbreviation	Forward primer	Reverse primer
AKT1	5'-CTTTTAGGTCCTCTTGGCCG-3'	5'-GGCTTAGGTTGACTTTCAGGC-3'
BCL2	5'-TGTTCAAGGTGGACACGGG-3'	5'-TTTAAGCAATGTCTAGGGACGC-3'
TRKB	5'-GGTGCTGTCCAAGAATTCCC-3'	5'-TCGGATCTAGGCCAAGCG-3'
VGF	5'-CACCCCTCCATTATTCTCCC-3'	5'-TGAAATGCTGAAGCGGGC-3'

Table S2. Sequences of primers used for chromatin immunoprecipitation.

The promoter regions and transcription start sites (TSS) were predicted using the “TRED Promoter Database”. Genomic regions of 2000 bases upstream of the first TSS of each candidate gene were used to design the different primer pairs. Further, CpG islands in the promoter regions were determined with Methprimer software and used as a putative indicator of MeCP2 binding sites.

CHAPTER VI

Long-term behavioral and neurobiological consequences of developmental exposure to fluoxetine in a rodent model of maternal adversity

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In preparation

Abstract

There is a growing use of selective serotonin reuptake inhibitor (SSRIs) medications for treatment of depression during pregnancy and the postpartum period. However, very little research has investigated the long-term effects of perinatal SSRI exposure on offspring development. The aim of the present study was to determine the role of developmental fluoxetine exposure on affect-related behaviors and associated molecular mechanisms in adult male and female offspring using a rodent model of maternal adversity. For this purpose, gestationally stressed and non-stressed Sprague-Dawley rat dams were treated with either fluoxetine (5mg/kg/day) or its vehicle from postnatal day 1 (P1) to postnatal day 21 (P21). At adulthood, male and female offspring were assessed for anxiety- and depressive-like behaviors, and after sacrifice, epigenetic regulation of hippocampal brain-derived neurotrophic factor (BDNF) signaling was investigated. Results show that among male offspring, maternal fluoxetine exposure increased anxiety in naïve animals, while it decreased anxiety in prenatally stressed offspring. Moreover, among male offspring, exposure to prenatal stress increased depressive-like behavior, whereas among females, fluoxetine exposure, independent of maternal adversity, profoundly increased depressive-like behavior in adulthood. In addition, developmental exposure to fluoxetine decreased BDNF exon IV mRNA levels, which was associated with increased levels of the repressive histone 3 lysine 27 tri-methylated (H3K27me3) mark at the corresponding promoter in female offspring. Altogether, these data provide important evidence on the long-term programming effects of early-life exposure to SSRIs on the regulation of adult mood-related behaviors and their underlying molecular mechanisms.

Keywords: Antidepressant, Prenatal stress, Mood, Serotonin, Epigenetics, BDNF

I – Introduction

Selective serotonin reuptake inhibitor (SSRIs) medications are commonly used for the treatment of maternal mood disorders with up to 10% of pregnant women being prescribed these medications (Cooper et al., 2007; Oberlander et al., 2006; Ververs et al., 2006). SSRIs cross the placental barrier and are present in breast milk and, therefore, can affect development (Homberg et al., 2010; Kristensen et al., 1999; Rampono et al., 2004). Recent clinical studies reported that neonates exposed to SSRI medications during gestation have an increased risk for complications at birth such as low birth weight, younger gestational age, and reduced heart rate variability (Moses-Kolko et al., 2005; Nulman et al., 2002; Oberlander et al., 2009). In addition, clinical reports in infants also showed that prenatal exposure to SSRI-medications may alter neurodevelopment as evidenced by alterations in S100B levels (Pawluski et al., 2009), alter serotonergic system functioning (Laine et al., 2003), and the actions of the hypothalamic-pituitary-adrenal (HPA) system (Oberlander et al., 2009; Oberlander et al., 2008; Pawluski et al., 2011; Pawluski et al., 2012b). Although there is limited clinical evidence on the long-term outcomes of developmental exposure to SSRI medications on mood-related outcomes in children, preclinical research is showing that perinatal exposure to SSRIs can impact upon measures of mood and HPA physiology in juvenile and adult offspring (Noorlander et al., 2008; Pawluski et al., 2012b; Rayen et al., 2011). Studies in rodent models have shown that there is a well-documented increase in anxiety-like behavior in adult male rodents perinatally exposed to fluoxetine (Ansorge et al., 2004; Noorlander et al., 2008; Karpova et al., 2009; Smit-Rigter et al., 2012; Lee et al., 2009). Interestingly, the reverse is true in female offspring, with a recent report showing that perinatal exposure to fluoxetine decreases anxiety- and depressive-like behaviors in adult female offspring (McAllister et al., 2012). Nevertheless, the molecular pathways underlying neurodevelopmental changes and long-term behavioral effects of early-life SSRIs exposure are still poorly understood. Serotonin plays an important role in the development of the brain modulating neuronal differentiation, axon guidance or dendritic pruning (Gaspar et al., 2003; Homberg et al., 2010). Conversely, administration of SSRIs, possibly by increasing the levels of serotonin at the synapse, is known to enhance brain-derived neurotrophic factor (BDNF) levels and promote neuroplasticity (Castren et al., 2008).

Brain-derived neurotrophic factor (BDNF) is a small-secreted protein known to be critically involved in the regulation of neuronal plasticity, and alterations of BDNF signaling have been extensively implicated in the pathophysiology and treatment of mood disorders (Castren et al., 2010). Accordingly, a recent study showed that early postnatal administration of fluoxetine could induce long-lasting behavioral impairment, accompanied with changes in hippocampal BDNF mRNA levels in adult mice (Karpova et al., 2009). Similarly, epigenetic changes at the BDNF gene in adulthood have been associated with mood disorders (Boulle et al., 2012). Furthermore, serotonin transporter (5-HTT) knockout rats displayed decreased BDNF levels in the hippocampus and frontal cortex, concomitant with epigenetic dysregulation at the BDNF gene – i.e. increased DNA methylation at BDNF promoters IV and VI (Molteni et al., 2010).

Interestingly, mounting evidence suggest that early-life experience, including environmental adversity, reduced maternal care and exposure to toxins, can induce epigenetic reprogramming and increase the susceptibility for the development of mood disorders in adulthood (Onishchenko et al., 2008; Roth et al., 2009; Weaver et al., 2004).

The aim of the present study was to investigate the long-term molecular and behavioral consequences of developmental administration of fluoxetine, a popular SSRI regularly used during pregnancy and the post-partum period. Since perinatal maternal stress and depression itself can have marked, sex-dependent effects on the development of mood disorders and stress regulation (Morley-Fletcher et al., 2003; Maccari et al., 2007; Van den Hove et al., 2013), it is also important to investigate the effects of SSRIs using a model of maternal adversity (Ishiwata et al., 2005; Van den Hove et al., 2008; Pawluski., 2011). Hence, we explored the effects of maternal fluoxetine using a model of prenatal stress in rat, and focused particularly on measures related to affective behavior, HPA axis responsivity as well as epigenetic regulation of hippocampal BDNF signaling in adult male and female offspring.

II – Material and methods

Animals and procedures: All experiments were approved by the Animal Ethics Board of Maastricht University in accordance with Dutch governmental regulations (DEC 2008-157 and 2008-158). All efforts were made to minimize the pain and stress levels experienced by the animals. Thirty-eight adult female Sprague-Dawley rats (250–300 g; Charles River Laboratories, France) were used in the present study. Females were initially housed in pairs in opaque polyurethane bins (48 cm × 27 cm × 20 cm) with ad libitum access to rat chow (Sniff, The Netherlands) and tap water. Rats were kept under standard laboratory conditions in a 12 h:12 h light/dark schedule (lights on at 07:00 h). Pregnancy was determined by observation of vaginal plugs (embryonic day 0 – E0). Restraint stress was performed daily during the last week of pregnancy (E14-E21). Pregnant female rats (n=18) were individually restrained 3 times a day (at approximately 9.00, 13.00, and 17.00 h) for 45 minutes in transparent plastic cylinders, whilst being exposed to bright light (Van den Hove et al., 2005; Ward and Weisz, 1984). Control pregnant females (n=20) were left undisturbed in their home cages. Only litters of 10 or more pups were included in this study. Litters were culled to 10 pups if necessary (5 males and 5 females). Dams were randomly assigned to one of two treatment groups: fluoxetine (5 mg/kg/day) or vehicle, for a total of four groups of dams: (1) No stress + Vehicle (NS VEH), (2) No stress + Fluoxetine (NS FLX), (3) Prenatal Stress + Vehicle (PNS VEH), (4) Prenatal Stress + Fluoxetine (PNS FLX). A maximum of 2 male and female pups per litter were examined to prevent litter effects (Chapman and Stern, 1978). At postnatal day 21 (P21), pups were weaned and group-housed for further examination (2 male or 2 female rats/cage; n=12-16 rats per experimental condition per sex). Rats were kept at a reversed day-night cycle from this point onwards

(lights on from 17.00 - 5.00 h). Anxiety- and depression-related behavior of the rats was analyzed from P140 onwards (in the order as discussed below). At P196, the animals were killed by quick decapitation.

Fluoxetine treatments: Fluoxetine and its active metabolite, norfluoxetine, can pass to offspring through lactation (Gentile et al., 2007), therefore fluoxetine treatment was administered via osmotic minipumps (Alzet Osmotic pumps, 2ML4) to the dams as previously described (Rayen et al., 2011). Implants were filled with either fluoxetine-HCl (Fagron, Belgium) dissolved in vehicle (50% propylenediol in saline; 5mg/kg/day), or with vehicle as previously described (Pawluski et al., 2012a). Minipumps were implanted subcutaneously in the dorsal region of the dams while the dams were under mild isofluorene anaesthesia on post-partum day 1 (P1). Implants took a maximum of 20 min and therefore dams were separated from their litters for a maximum of 20 min. Fluoxetine exposure to offspring occurred during a stage of neural development in rodents analogous to that of the third trimester in humans (Romijn et al., 1991).

Behavioral testing:

Open field test (OFT)

The open field test (OFT) was conducted in a square Plexiglas base (100 x 100 cm) with a black floor and transparent plexiglas walls (40 cm high; Van den Hove et al., 2013). The arena was subdivided into a 60 x 60 cm central zone, 20 x 20 cm corners and 60 x 20 cm walls. Immediately after the rat had been placed in the centre of the open field, the movements of the animal were scored automatically under low light conditions using a camera connected to a computerized system (Ethovision Pro, Noldus, The Netherlands). Both the time spent in the different zones and total distance moved were scored. After five minutes, the rat was removed from the apparatus and the maze was cleaned with ethanol (70%) and water and dried thoroughly to prevent transmission of olfactory cues.

Forced swim test (FST)

In the forced swimming test (FST), originally designed by Porsolt and colleagues (2006), four cylindrical glass tanks (50 cm tall, 20 cm in diameter) were filled to a height of 30 cm with 25°C water. The movements of the rat were scored automatically with a computerized system (Ethovision Pro, Noldus, The Netherlands) during a 10 min session under low light conditions. Scored were 'immobility', which reflects no movement at all and/or minor movements necessary to keep the nose above the water, and 'strong mobility', reflecting 'escape behavior' (e.g. climbing against the walls and diving). Settings within Ethovision were adjusted based on manually recorded sessions and were attuned for each sex separately (immobility/mobility threshold: 12 and 20; mobility/strong mobility threshold: 16.5 and 23.9 for males and females, respectively (Strackx et al., 2009; Van den Hove et al., 2013).

Elevated zero maze (EZM)

The elevated zero maze (EZM) introduced by Shepherd and colleagues (1994) consisted of a circular alley (diameter of 100 cm; path width 10 cm) made from black plastic material that was transparent for infrared light and elevated 20 cm above the floor. The maze was divided into four parts, i.e., two opposite open parts and two opposite closed parts with sidewalls 30 cm in height. The open parts (open arms; OA) had borders with a height of 5 mm to prevent the rat from stepping down from the apparatus. For the test, the rat was placed into one of the open parts facing a closed part (closed arms; CA) of the apparatus. After five minutes the rat was removed from the apparatus and the maze was cleaned with ethanol (70%) and water and dried thoroughly. The movements of the rat were scored automatically under dark conditions with a computerized system using an infrared video camera (Ethovision Pro, Noldus, The Netherlands). Percentage of time spent in the open part of the maze and total distance traveled were determined as described previously (Van den Hove et al. 2013).

Corticosterone response and radioimmuno assay: To test the HPA axis responsivity, rats were individually placed in a type II (mouse) cage filled with 500 ml 25°C water. Experiments were performed in an isolated room between 09.00 and 13.00 h. Immediately after taking the rat from its home cage, a first blood sample was collected via a saphenous vein puncture representing the basal corticosterone level. Immediately after this first sample was taken, the rat was put in the cage filled with water for 20 min after which a second blood sample was taken. Afterwards, the animal was returned to its home cage and left undisturbed for 40 min after which a final blood sample was taken. Blood samples were kept on ice and centrifuged at 5000 rpm for 10 min at 4 °C, after which the plasma was frozen down to -75 °C for subsequent determination of corticosterone levels. Samples were run in duplicate using a commercially available radioimmunoassay (RIA) kit for rat corticosterone from MP Biomedicals (Corticosterone I-125 for rats and mice, MP Biomedicals), as explained previously (Van den Hove et al., 2013). The average intra- and inter-assay coefficients of variation for all assays were below 10%. The assay had a sensitivity of 7.7 ng/mL.

Quantitative reverse transcriptase PCR (qRT-PCR): Total mRNA extraction was performed using RNeasy lipid tissue mini kit (Qiagen, Venlo, The Netherlands), after which, cDNA synthesis was performed using a First Strand cDNA synthesis kit (Fermantas International Inc. Canada) according to the manufacturer's protocol. Amplification was performed using SYBR green I Master Mix (Roche Diagnostic, IN, USA). Primer sequences are indicated in Table S1. Gene expression analysis was performed with the LightCycler 480 Real-Time PCR System (Roche Applied Science, IN, USA) and the conditions for the reaction involved 35 cycles in a fixed sequence of 95°C for 30 s, 62°C for 15 s, and 72°C for 15 s. Gene expression was normalized using HPRT and RS27A as reference genes.

Chromatin immunoprecipitation (ChIP): Since developmental exposure to fluoxetine markedly affected behavior and BDNF IV mRNA expression in females as compared to males, we decided to investigate the epigenetic regulation at BDNF promoters only in the female gender. Hippocampal tissues were fixed in a 1% formaldehyde solution, after which they were lysed in ice-cold SDS lysis buffer containing protease inhibitors (Roche, Meylan, France). The chromatin was sheared in order to obtain DNA fragments that were approximately between 400 and 800 base pairs. The solution of chromatin was pre-cleared with a mixture of protein A-agarose (Pierce, Thermo-Fischer, Illkirch, France) and salmon sperm (Agilent technologies, CA, USA), and then immunoprecipitated overnight at 4°C with 2 µg of rabbit anti-H3K9ac, mouse anti-H3K27me3 (Millipore, Molsheim, France) or rabbit anti-MeCP2 (Sigma Aldrich, MO, USA) antibodies. After intensive washing, the immunoprecipitate was eluted from the beads using a 1 % SDS elution buffer and the protein-DNA cross-links were removed by incubating the samples overnight at 65°C in the presence of 5 M NaCl. DNA fragments were then treated with proteinase K in order to digest the proteins, after which DNA was isolated using phenol/chloroform/isoamyl alcohol and subsequently precipitated with 100% ethanol and 20 µg of glycogen. Fragments of promoter regions of target genes were quantified by quantitative PCR (see Table S2 for primer sequences and design). Ct values of immunoprecipitated samples were normalized to Ct values obtained from Input DNA. The specificity of the antibodies used in the experiments was determined by immunoprecipitation of the sample with non-specific HA-probe (Y-11) antibody (Santa Cruz Biotechnology Inc, CA, USA).

Statistical analysis: Because of profound expected sex and experimental condition interactions (e.g. Van den Hove et al., 2013; Zuena et al., 2008), parameters studied in the offspring were analyzed for each sex separately using a two-way analysis of variance (ANOVA; condition x treatment). Plasma corticosterone levels were analyzed by a repeated measures ANOVA (experimental condition x time) and also independently at the different time points using ANOVA. Statistical significance was assumed to exist at $p \leq 0.05$. All statistics were carried out using SPSS software version 17 (SPSS Inc, USA).

III – Results

Effect of prenatal stress and maternal fluoxetine on affective behaviors:

Elevated Zero Maze (EZM)

The time spent in the open arms and the distance covered in the EZM are depicted in Figure 1. In addition, the frequency to visit the open and closed arms is shown in Table S3. No differences were found in terms of the time spent in the open arms or the frequency of open arm visits in the EZM.

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Among males, a significant condition x treatment interaction was observed regarding the distance traveled within the EZM [$F(3,51)=4.038$; $p=0.05$], indicating that FLX exposure decreased the distance traveled in naïve animals, while it increased the distance moved in animals previously exposed to PNS.

Among females, there was significant condition x treatment interaction effect regarding the distance traveled within the EZM [$F(1,52)=3.99$, $p=0.05$].

Open Field Test (OFT)

Time spent in the various zones of the open field as well as the number of visits to the center and the total distance covered in the OFT are shown in Figure 2 and Figure S4.

Among males, a significant condition x treatment interaction was observed on the number of visits to the center of the open field [$F(1,51)=5.04$; $p=0.029$], with FLX decreasing the number of visits to the center in naïve animals and increasing the frequency of visits to the center in prenatally stressed offspring (NS FLX < PNS FLX; $p=0.024$). Similarly, regarding the distance traveled within the open field, a tendency towards a condition x treatment interaction was observed in adult males [$F(1,51)=3.48$; $p=0.067$], indicating that FLX exposure differentially affected distance traveled in NS versus PNS adult male offspring.

Among females, PNS exposure tended to increase time spent along the walls [$F(1,52)=3.78$; $p=0.057$] of the open field. There were no other significant effects observed in any of the other parameters.

Forced Swim Test (FST)

Results from the FST are shown in Figure 3. Among males, a significant effect of PNS on strong mobility in the FST was found [$F(3,51)=4.09$; $p=0.048$], indicating that PNS increased escape-like behavior i.e. struggling in this behavioral test.

Among females, a significant treatment effect was observed [$F(1,52)=11.48$; $p=0.001$], indicating increased immobility after FLX exposure, independent of prenatal stress experience (NS VEH < NS FLX; $p=0.016$ and PNS VEH < PNS FLX; $p=0.025$).

Effects of prenatal stress and maternal fluoxetine on biological parameters:

Weight and corticosterone response

Plasma corticosterone levels prior to restraint, immediately after 20 min of restraint stress, and 40 min after being placed back into the home cage are depicted in Figure S2. As expected, there was a significant main effect of time in both males ($F(2,36)=71.215$, $p<0.001$) and females ($F(2,38)=72.284$, $p<0.001$) showing that 20 min after restraint stress plasma corticosterone levels were elevated. There were no other significant differences with treatment in males or females in corticosterone levels ($0.2 < p < 0.9$).

In addition, the weight of the animals was modified neither by developmental fluoxetine exposure nor than maternal adversity in male and female offspring (Figure S3).

Gene expression in the hippocampus

Hippocampal gene expression profiles are shown in Figure 4 and Table S4. Among males, a significant effect of FLX treatment on BDNF IV and tropomyosin-related kinase receptor B (TrkB) mRNA levels was observed [$F(1,51)=5.57$; $p=0.022$ and $F(1,51)=7.21$; $p=0.0098$ respectively], indicating that FLX decreased both BDNF IV and TrkB mRNA levels. In addition, BDNF IV mRNA levels were decreased in animals exposed to PNS (PNS VEH < NS VEH; $p=0.041$). Finally, there was a tendency towards a stress x treatment interaction for BDNF IX mRNA levels [$F(1,51)=4.30$; $p=0.043$].

Among females, there was a significant effect of PNS exposure on BDNF IV mRNA levels [$F(1,47)=8.34$; $p=0.0058$]. Accordingly, BDNF IV mRNA expression was increased in the hippocampus of female rats exposed to PNS (NS VEH < PNS VEH; $p=0.018$). Finally, there was a significant effect of FLX exposure on BDNF IV mRNA levels [$F(1,47)=5.01$; $p=0.030$], pointing towards a global decrease of BDNF IV mRNA levels independent of prenatal stress experience.

Epigenetic regulation of the BDNF gene

The specific enrichments of methyl CpG binding protein 2 (MeCP2), histone 3 lysine 27 tri-methylated (H3K27me3) and histone 3 lysine 9 acetylated (H3K9ac) at the BDNF promoter IV and the BDNF promoter IX in female offspring are represented in Figure 5. There was a significant stress x treatment interaction for H3K27me3 enrichment at the BDNF promoter IV [$F(1,32)=6.35$; $p=0.017$]. In addition, FLX treatment significantly increased the specific enrichment of H3K27me3 at the BDNF promoter IV (PNS VEH < PNS FLX; $p=0.016$). Of note, a trend for PNS exposure to decrease the specific enrichment of H3K27me3 at the BDNF promoter IV (PNS VEH < PNS FLX; $p=0.056$) was observed. Furthermore, a tendency for a treatment effect on MeCP2 specific enrichment at the BDNF promoter IV was observed [$F(1,30)=3.00$; $p=0.093$], indicating that FLX tended to increase MeCP2 occupancy in this promoter region. It is interesting to note that MeCP2 binding at the BDNF promoter IV correlated with immobility score in the FST (Figure S7). Finally, a tendency for a stress x treatment effect on H3K9ac specific enrichment was also reported [$F(1,30)=3.00$; $p=0.073$], indicating that FLX tended to increase H3K9ac at the BDNF promoter IX, while decreasing H3K9ac at this same promoter.

IV – Discussion

Fluoxetine, an antidepressant belonging to the class of SSRIs, is widely used in the clinic for the treatment of mood disorders during pregnancy and the post-partum period (Cooper et al., 2007; Oberlander et al., 2006; Ververs et al., 2006). However, the long-term effects of early-life treatment with this medication are still poorly understood. In this context, the aim of the present study was to investigate

the long-term effects of developmental fluoxetine exposure using a model of maternal adversity, at the level of anxiety and depression-related behavior, HPA axis responsivity and epigenetic regulation of BDNF/TrkB signaling in adult male and female offspring.

Effect of prenatal stress and maternal fluoxetine on affective behavior and HPA response: Our data showed that maternal adversity tended to increase depressive-like behavior in male offspring, whereas, among females, no effects of prenatal stress on depressive-like or anxiety-like behaviors were observed. Those results are in accordance with previous studies that reported similar observation in male and females offspring prenatally exposed to maternal stress, and further support a sex-dependent effect of perinatal maternal stress on mood-related behaviors (Van den Hove et al., 2013).

Further, among male offspring, maternal fluoxetine exposure increased anxiety in naïve animals, while it decreased anxiety in prenatally stressed offspring. Increase in anxiety behavior following perinatal exposure to fluoxetine has been well documented in male rodents (Ansorge et al., 2004; Ansorge et al., 2008; Noorlander et al., 2008; Karpova et al., 2009; Smit-Rigter et al., 2012; Lee et al., 2009). However, the present study was one of the first to investigate the long-term effects of fluoxetine using a model of maternal adversity. Accordingly, this work shows that the effects of developmental exposure to SSRI medications may profoundly differ when using healthy dams and offspring in comparison to using a model of maternal adversity. This notion may have important implications when examining e.g. the long-term behavioral effects (and its neurobiological underpinnings) of early life SSRI exposure.

On the other hand, among females, fluoxetine exposure profoundly increased depressive-like behavior in adulthood, independent of maternal adversity. This finding is of particular interest because it demonstrates that the effects of developmental SSRI exposure are strongly sex- and age-dependent, as we have previously shown, using the exact “fluoxetine-maternal adversity model” that developmental fluoxetine exposure was able to reverse the effects of prenatal maternal stress on depressive-like behavior in *adolescent* male and female offspring (Rayen et al., 2011), i.e. pointing towards overall beneficial effects of perinatal SSRI exposure. Here, we provide clean-cut evidence that early-life exposure to SSRIs negatively impacts upon mood-related behaviors in *adult* female offspring.

Nevertheless, previous findings using the exact prenatal stress procedures found an alteration in HPA axis responsivity, showing that male offspring prenatally exposed to maternal restraint stress displayed a significant alteration in basal and stress-induced corticosterone secretion (Van den Hove et al., 2013). The discordance between this study and previous reports may be attributed to the implantation mini-pump. Such type of surgery might represent additional stress for the mothers and result in differential outcome in the neuroendocrine measures.

Effect of prenatal stress and maternal fluoxetine on BDNF/TrkB signaling: To further understand the molecular pathways underlying behavioral dysfunction in male and female offspring prenatally exposed to fluoxetine, we examined the hippocampal expression of BDNF/TrkB downstream target genes. BDNF

IV mRNA levels were decreased in males prenatally exposed to stress, whereas the opposite was observed in females. The literature on prenatal stress and BDNF expression is not very consistent, and the timing, duration and intensity of stress as well as the species or strain of animals used seems to meticulously interfere with this biological parameter (Roceri et al., 2002; Karpova et al., 2009; Neeley et al., 2011). The present findings indicate that distinct mechanisms are implicated in the adaptation and response to stress in male versus female offspring, leading to differential BDNF expression. Moreover, maternal fluoxetine decreased BDNF IV mRNA levels only in naïve male offspring. Similarly, the mRNA levels of TrkB, the high-affinity receptor for BDNF, were decreased in naïve male offspring exposed to maternal fluoxetine. These findings, together with the observation that maternal fluoxetine increased anxiety-like behavior in naïve male offspring, support the notion that BDNF/TrkB signaling plays an important role in anxiety regulation (Chen et al 2006; Martinowich et al., 2007).

Moreover, maternal fluoxetine decreased BDNF IV mRNA levels independently of exposure to prenatal stress in female offspring, an observation that correlated with the increased despair-like behavior found in the FST. A decrease of BDNF in the hippocampus has been reported in post-mortem brain analysis of suicide victims (Dwivedi et al., 2003; Pandey et al., 2008), and rodent models of stress and depression also showed similar decrease of BDNF expression, including reduced levels of BDNF exon IV and exon IX (Tsankova et al., 2006; Onishchenko et al., 2008; Fuchikami et al., 2009). BDNF expression is tightly regulated at the level of transcription with differential exon usage, suggesting a crucial role for the various transcripts in different functions and related processes (Pruunsild et al., 2007; Baj et al., 2011). More specifically, the BDNF IV transcript was shown to be involved in the regulation of the stress response, and in the mechanism of action of antidepressants (Boulle et al., 2012). Interestingly, fluoxetine treatment in adulthood has been shown to increase BDNF levels (including BDNF IV mRNA levels) in cortico-limbic structures to induce antidepressant and anxiolytic effects in rodents (Martinowich et al., 2007). Data from the current study suggest that the molecular mechanisms involved in early-life versus adulthood intervention with fluoxetine are distinct. This can partly be explained by the fact that during postnatal development, certain populations of neurons express a transient serotonergic phenotype that no longer exists at adulthood, and the modulation of this partial serotonin system might interfere with the fine-tuning process of neuronal networks (Cases et al., 1998; Gaspar et al., 2003). Moreover, fluoxetine was shown to interact with several targets including serotonin receptors (2A, 2C and 2B) and ion channels (Mostert et al., 2008), next to the serotonin transporter. Hence, the differential patterns of expression of these systems in early-life might be determinant in the long-term biological action of fluoxetine.

Epigenetic regulation of BDNF/TrkB signaling in female offspring: Since maternal fluoxetine mostly affected long-lasting behavioral and molecular measures in female offspring, we conducted additional investigations in this sex to test whether the observed alterations could be attributed to epigenetic programming. Developmental experiences have been shown to modulate post-translational histone

modifications and DNA methylation, thereby altering the epigenetic program and leading to long-lasting neurochemical changes causally related to adult psychopathology (Onishchenko et al., 2008; Weaver et al., 2004). In addition, increasing evidences suggest that epigenetic regulations are involved in the molecular mechanisms of action of antidepressant drugs, including SSRIs (Vialou et al., 2013). In the present study, maternal fluoxetine tended to increase MeCP2 binding at BDNF promoter IV in female offspring, independent of prenatal stress exposure. MeCP2 has been shown to selectively bind to BDNF promoter IV to repress its transcription in post-mitotic neurons (Chen et al., 2003; Martinowich et al., 2003). In addition, this epigenetic regulator is essential in neurodevelopment (Gonzales et al., 2010), and increased MeCP2 binding and upregulation in the brain promoted anxiety in mice (Samaco et al., 2012). Furthermore, developmental fluoxetine increased H3K27me3 at the BDNF promoter IV in female offspring prenatally exposed to maternal adversity. H3K27me3 is a repressive histone mark. Hence, this observation is in accordance with the decreased expression of BDNF IV mRNA in the hippocampus of female offspring. This finding is of particular interest because it indicates that the long-term neurochemical effects of developmental fluoxetine might reside in long-lasting epigenetic repression of gene transcription. Of note, this effect was observed only in female offspring prenatally exposed to stress, indicating that maternal adversity increased the vulnerability for fluoxetine-induced epigenetic reprogramming in the hippocampus.

Conclusion and perspectives: The number of children exposed to SSRI medications during development is constantly increasing (Oberlander et al., 2006; Cooper et al., 2007), and the long-term effects of such exposure are still to be determined. In this context, we showed that the developmental exposure to fluoxetine, a popular SSRI for post-partum depression, had negative impact on mood-related behaviors, which appeared to be strongly age- and sex- dependent. In addition, the effect on developmental fluoxetine may profoundly differ when using healthy dams and offspring in comparison to using a model of maternal adversity in rodents. Further, the present study brings novel insights into the long-term molecular regulations underlying developmental exposure to fluoxetine. To our knowledge, this study is the first one to demonstrate that maternal fluoxetine exposure could be associated with persistent epigenetic remodeling at BDNF/TrkB signaling in adulthood. Hence, our data support the idea that exposure to SSRI medications during development might have negative consequences on neurodevelopment and mood-related behavior in adulthood, which may be attributed, at least in part, to enduring epigenetic reprogramming in signaling pathways implicated in neuronal plasticity.

V – References

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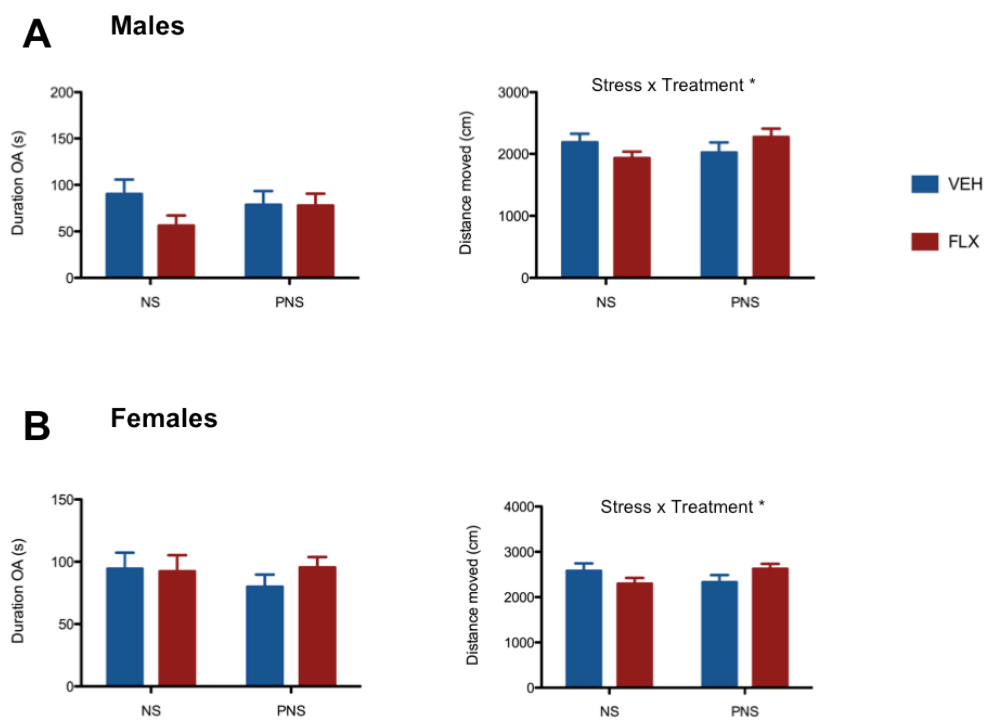
Figures and legends.

Figure 1. Effect of prenatal stress and maternal fluoxetine in the elevated zero maze (EZM)

Males (A) and females (B) offspring were exposed to an EZM, and the time spent in open arms (OA) and the total distance travelled are represented. In both male and female offspring, there was a significant stress x treatment interaction in the distance moved in the EZM, suggesting a differential effect of fluoxetine in naïve versus prenatally stressed animals. Data are expressed as mean + SEM of n=12-16 rats per group. * $p < 0.05$; Two-way ANOVA. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

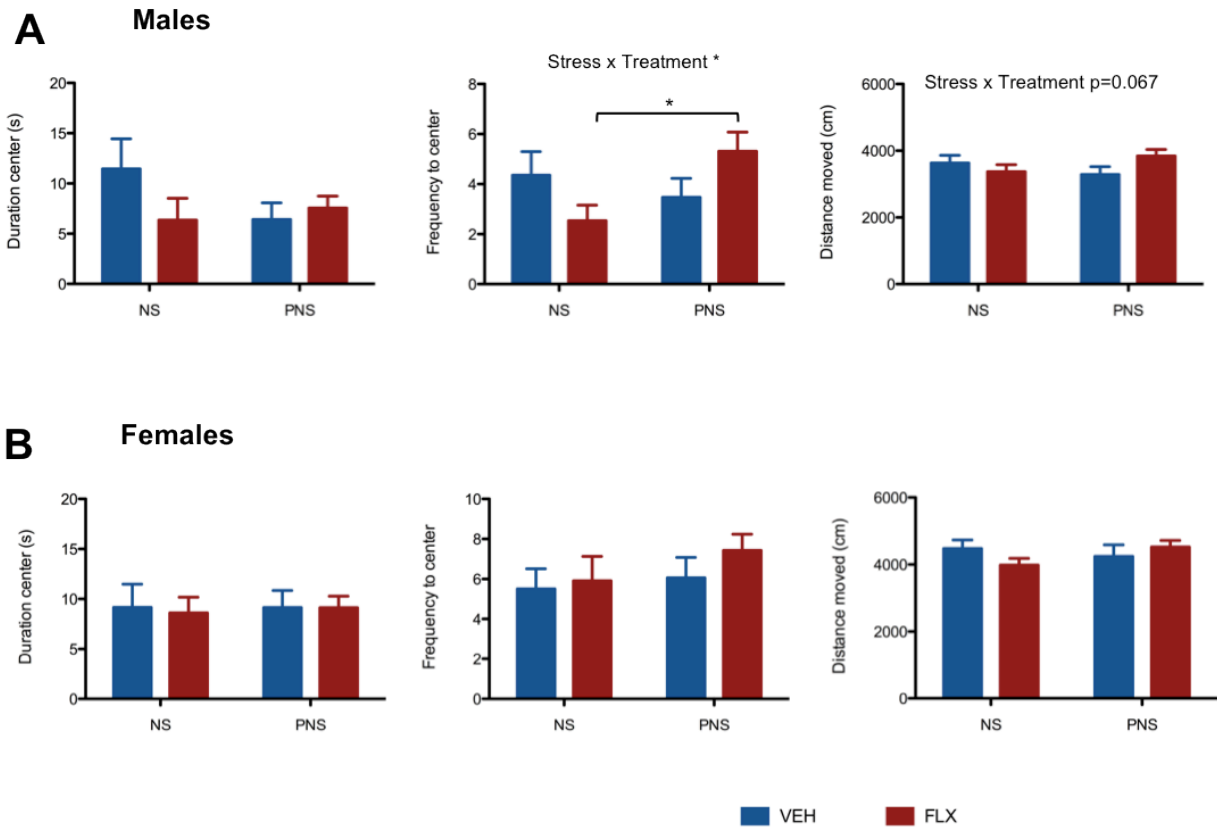
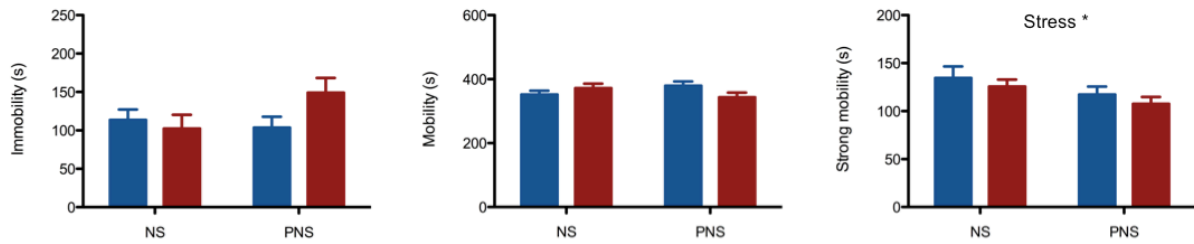


Figure 2. Effect of prenatal stress and maternal fluoxetine in the open field test (OFT)

Males (A) and females (B) offspring were exposed to an OFT, and the time spent in the center, the frequency to the center and the total distance travelled are represented. Maternal fluoxetine treatment (FLX) had opposite effects on the frequency to the center in naïve animals (NS) as compared to prenatally stressed animals (PNS) in male offspring. Of note, a trend towards a similar effect was observed in the distance moved in males. No differences in any parameter were observed in females. Data are expressed as mean + SEM of n=12-16 rats per group. * $p < 0.05$; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

A Males



B Females

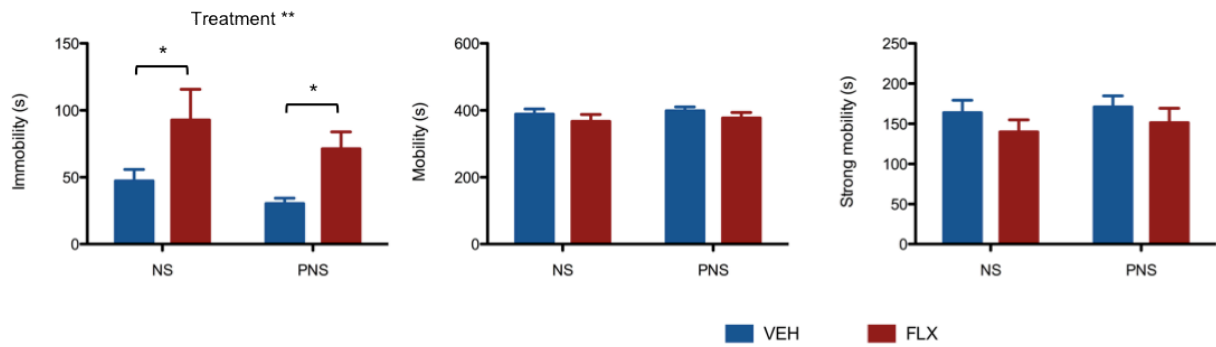


Figure 3. Effect of prenatal stress and maternal fluoxetine in the forced swim test (FST)

Males (A) and females (B) offspring were exposed to a FST, and the time spent in immobility, mobility and strong mobility is represented. There was a significant stress x treatment interaction in the mobility time in males. A trend toward a similar effect in the immobility time was also observed. In addition, male rats exposed to prenatal stress (PNS) showed a decrease time of strong mobility as compared to non-stress animals (NS). In females, maternal fluoxetine treatment (FLX) significantly increased the time spent in immobility in naïve (NS) and prenatally stressed (PNS) animals. No differences were observed in the mobility and strong mobility time in female offspring. Data are expressed as mean + SEM of n=12-16 rats per group. *p < 0.05; **p < 0.01; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

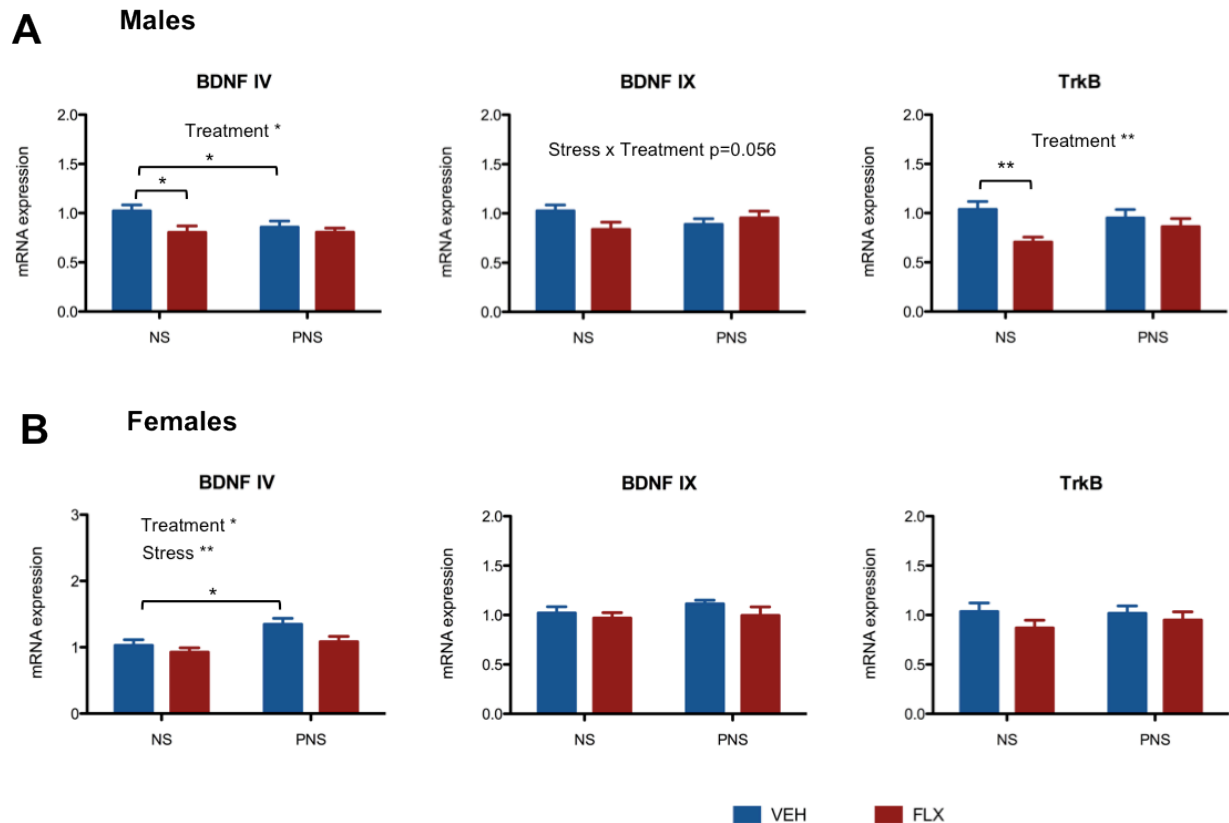


Figure 4. Effect of prenatal stress and maternal fluoxetine on BDNF/TrkB signaling in the hippocampus

mRNA levels of BDNF and TrkB in the hippocampus of males (A) and females (B) offspring were measured by quantitative real-time RT-PCR, and are expressed as fold changes as compared to naïve animals (NS SAL). There was a significant effect of maternal fluoxetine treatment (FLX) on BDNF IV and TrkB mRNA levels in males. In addition, male rats exposed to prenatal stress (PNS) showed decreased BDNF IV mRNA expression as compared to non-stressed animals. In females, there was a significant effect of maternal fluoxetine on BDNF IV mRNA levels. In contrast to what observed in males, prenatal stress increased BDNF IV mRNA levels in female offspring. No differences were observed in BDNF IX and TrkB mRNA levels in females. Data are expressed as mean + SEM of n=12-16 rats per group. * $p < 0.05$; ** $p < 0.01$; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

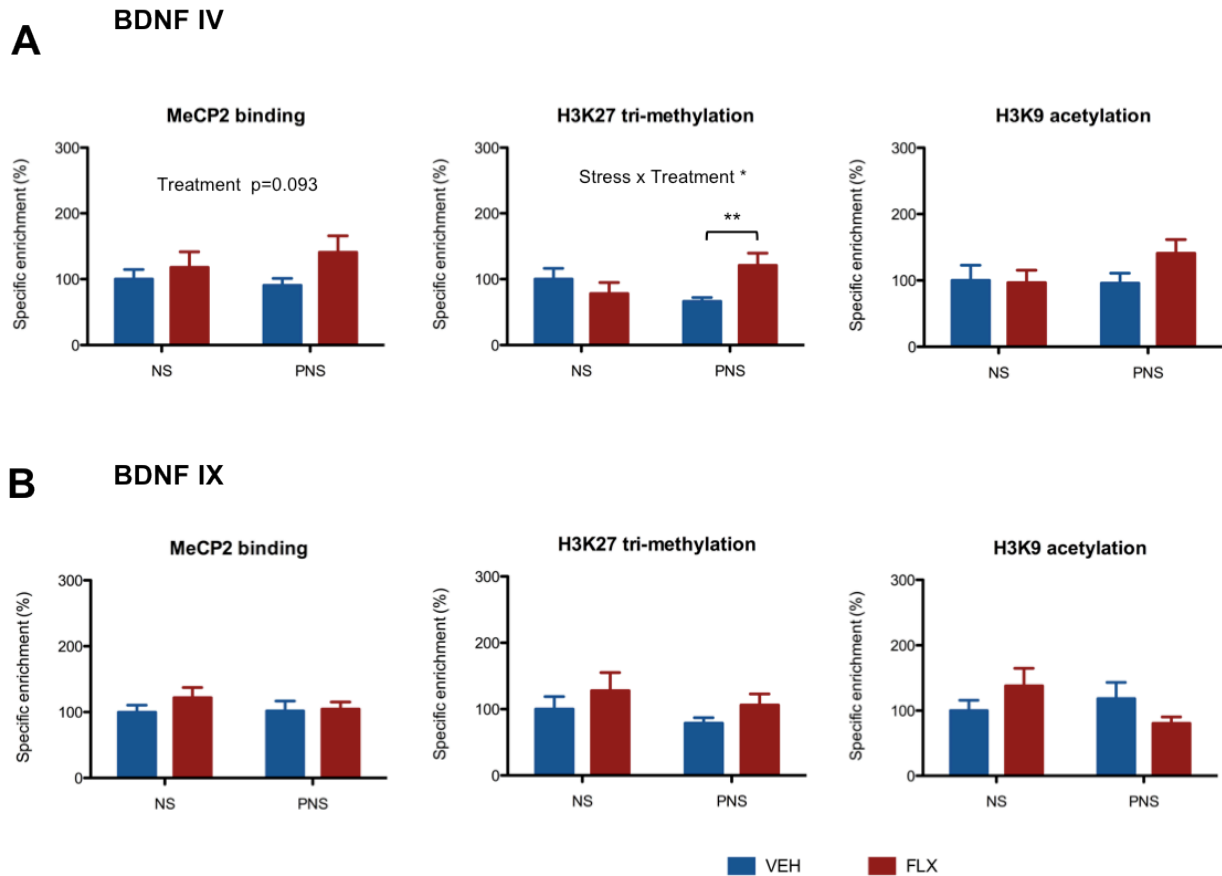
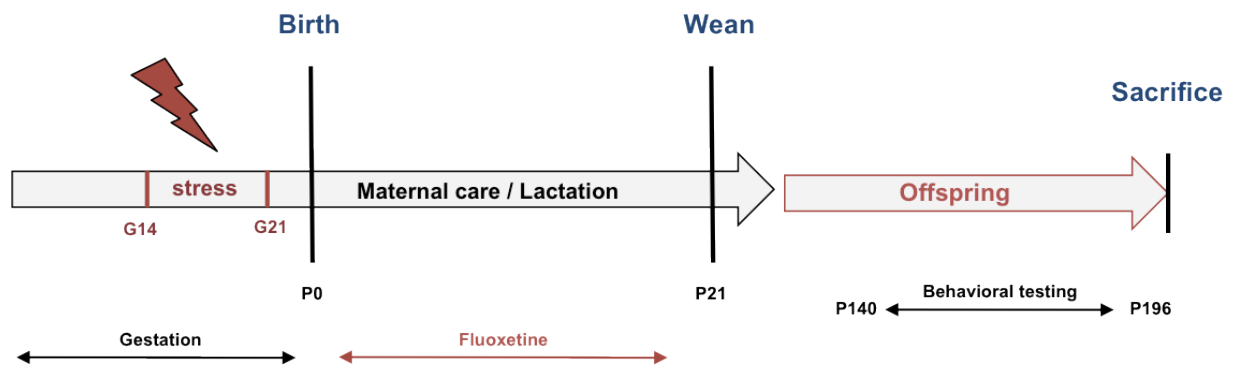


Figure 5. Effect of prenatal stress and maternal fluoxetine on epigenetic regulation of BDNF gene in female offspring

The specific enrichment of MeCP2, H3K27me3 and H3K9ac at the BDNF promoter IV (A) and the BDNF promoter IX (B) was measured by chromatin immunoprecipitation (ChIP) followed by quantitative real-time PCR, and is expressed as percentage of control animals (NS SAL). A trend towards an increase of MeCP2 binding at the BDNF promoter IV was observed after maternal fluoxetine treatment (FLX). In addition, maternal fluoxetine treatment significantly increased the enrichment of H3K27me3 at the BDNF promoter IV in female rats exposed to prenatal stress (PNS). Further, a tendency for a stress x treatment interaction for H3K9ac enrichment at the BDNF promoter IX was observed, but did not reach statistical significance. Data are expressed as mean + SEM of n=7-9 rats per group. **p < 0.01; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

Supplementary data.**Figure S1. Schematic representation of experimental timeline**

Restraint stress was performed daily during the last week of gestation (G14-G21). Fluoxetine or with vehicle was administered via osmotic minipumps (Alzet Osmotic pumps, 2ML4) to the dams during the postpartum period (P1-P21). At postnatal day 21 (P21), pups were weaned and group-housed for further examination (2 male or 2 female rats/cage; n=12-16 rats per experimental condition per sex). Anxiety- and depression-related behavior of the rats was analyzed from P140 onwards (in the order as discussed below). At P196, the animals were killed by quick decapitation.

Behavioral measure	NS VEH	NS FLX	PNS VEH	PNS FLX	ANOVA	LSD post hoc
<u><i>A - Elevated zero maze (EZM)</i></u>						
Duration open arms (s)	90.2 ± 15.5	56.2 ± 10.8	78.5 ± 14.9	77.75 ± 12.9	-	-
Frequency open arms	20.3 ± 8.3	9.7 ± 2.0	12.2 ± 2.0	15.21 ± 2.6	-	-
Frequency closed arms	20.5 ± 8.3	9.6 ± 2.1	12.4 ± 2.0	15.71 ± 2.5	-	-
Distance moved (cm)	2165 ± 130	1932 ± 104	1956 ± 157	2274 ± 135	Stress x Treatment *	-
<u><i>B - Open field test (OFT)</i></u>						
Duration center (s)	11.4 ± 2.9	6.3 ± 2.2	6.4 ± 1.6	7.5 ± 1.1	-	-
Duration walls (s)	119.8 ± 4.9	107.4 ± 8.2	122.7 ± 5.2	119.3 ± 5.4	-	-
Duration corners (s)	165.3 ± 5.8	183.1 ± 8.4	167.9 ± 5.8	169.8 ± 6.1	-	-
Frequency center	4.3 ± 0.9	2.5 ± 0.6	3.4 ± 0.7	5.1 ± 0.7	Stress x Treatment *	NS FLX < PNS FLX *
Distance moved (cm)	3632 ± 228	3372 ± 210	3289 ± 235	3845 ± 190	-	-
<u><i>C - Forced swim test (FST)</i></u>						
Immobility (s)	113.7 ± 13.2	102.5 ± 17.9	103.7 ± 14.1	149.0 ± 19.3	-	-
Mobility (s)	351.8 ± 12.5	371.9 ± 13.9	379.1 ± 13.4	343.4 ± 14.2	-	-
Strong mobility (s)	134.6 ± 12.1	125.6 ± 7.3	117.2 ± 8.3	107.6 ± 7.2	Stress *	-

Table S1. Summary table of behavioral analysis in male offspring

Data are represent mean ± SEM of n=12-16 rats per group. *p < 0.05; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

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Behavioral measure	NS VEH	NS FLX	PNS VEH	PNS FLX	ANOVA	LSD post hoc
<u>A - Elevated zero maze (EZM)</u>						
Duration open arms (s)	94.4 ± 12.8	92.2 ± 12.9	79.9 ± 9.8	95.4 ± 8.3	-	-
Frequency open arms	25.5 ± 10.4	16.6 ± 2.4	13.5 ± 1.6	18.5 ± 1.6	-	-
Frequency closed arms	25.9 ± 10.4	17.0 ± 2.3	13.8 ± 1.6	18.6 ± 1.7	-	-
Distance moved (cm)	2581 ± 162	2300 ± 125	2333 ± 156	2611 ± 126	Stress x Treatment *	-
<u>B - Open field test (OFT)</u>						
Duration center (s)	9.1 ± 2.3	8.6 ± 1.56	9.1 ± 1.7	9.1 ± 1.1	-	-
Duration walls (s)	100.5 ± 5.6	97.7 ± 5.8	105.9 ± 7.3	116.6 ± 4.3	-	-
Duration corners (s)	187.1 ± 7.3	190.6 ± 6.8	182.1 ± 7.8	170.8 ± 5.0	-	-
Frequency center	5.5 ± 1.0	5.9 ± 1.2	6.0 ± 1.0	8.0 ± 0.8	-	-
Distance moved (cm)	4475 ± 255	3980 ± 203	4244 ± 342	4522 ± 193	-	-
<u>C - Forced swim test (FST)</u>						
Immobility (s)	47.3 ± 8.5	92.7 ± 22.9	30.4 ± 4.0	71.3 ± 12.6	Treatment **	NS VEH < NS FLX * PNS VEH < PNS FLX *
Mobility (s)	388.9 ± 15.2	367 ± 20.3	398.6 ± 11.7	377.3 ± 16.31	-	-
Strong mobility (s)	163.8 ± 15.4	139.8 ± 15.0	170.9 ± 13.7	151.3 ± 18.0	-	-

Table S2. Summary table of behavioral analysis in female offspring

Data are represent mean ± SEM of n=12-16 rats per group. *p < 0.05; **p < 0.01; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

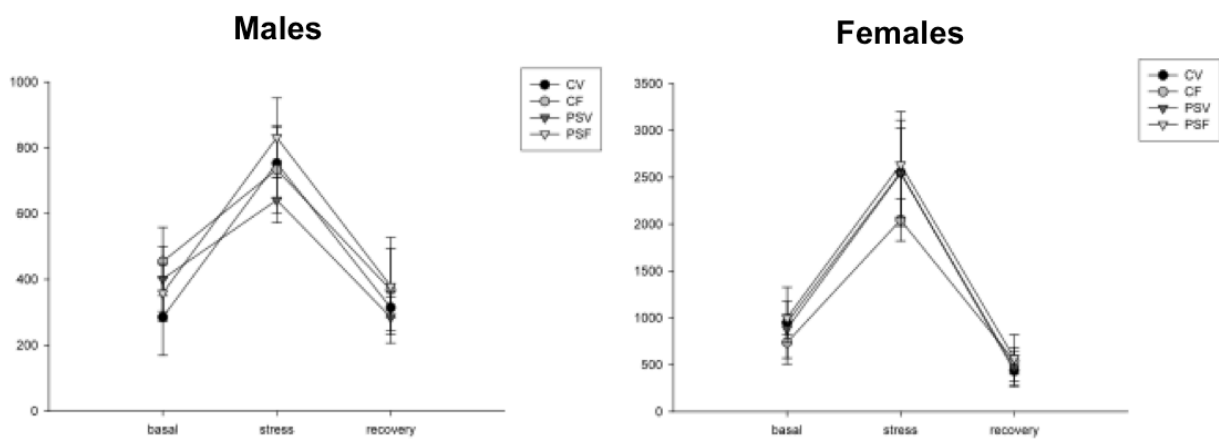


Figure S2. Effect of prenatal stress and maternal fluoxetine on corticosterone response

The plasma corticosterone levels were measured by radioimmuno assay in basal condition, stress condition and after recovery from stress. Corticosterone levels were increased after 20 min of stress exposure in both males and females, and returned to baseline after 40 min after the stress exposure. Maternal fluoxetine treatment and prenatal stress did not affect corticosterone response in basal, stress or recovery condition. Data are expressed as mean \pm SEM of n=12-16 rats per group. CV = control vehicle, CF = control fluoxetine, PSV = prenatal stress vehicle, PSF = prenatal stress fluoxetine

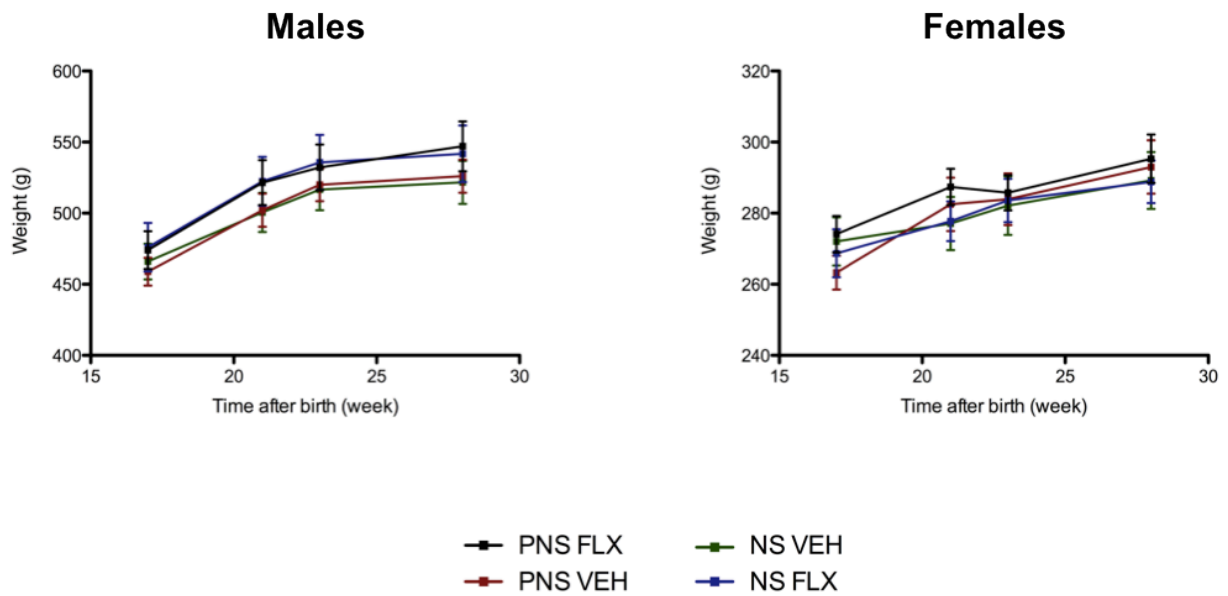


Figure S3. Effect of prenatal stress and maternal fluoxetine on body weight

The weight of rats was measured on week 17, week 21, week 23 and week 28 after birth. No differences between groups were found at any time point. Data are expressed as mean \pm SEM of n=12-16 rats per group. Two-way ANOVA. NS = no stress, PNS = prenatal stress, SAL = saline, FLX = fluoxetine.

Gene	Definition	Forward	Reverse
Akt1	RAC-alpha serine/threonine-protein kinase	GAAGGAGAAGGCCACAGGTC	TTCTGCAGGACACGGTTCTC
Bcl2	B cell leukemia/lymphoma 2	CTTCTCTCGTCGCTACCGTC	CAATCCTCCCCAGTTCACC
Bdnf4	Brain-derived neurotrophic factor exon 4	AGCAGCTGCCTTGATGTTTAC	ACCATAGTAAGGAAAAGGATGGTC
Bdnf9	Brain-derived neurotrophic factor exon 9	TCACAGCGGCAGATAAAAAG	TGGGATTACACTTGGTCTCG
Cdh13	Cadherin 13	GGAAATCCTGGGCAGAGCTT	CCAAGACCGTGATGTGGACA
cFos	FBJ osteosarcoma oncogene	TACTACCATTCCCAGCCGA	GCTGTCACCGTGGGGATAAA
Egr1	Early growth response 1	AACAACCCTACGAGCACCTG	AAAGGGGTTCCAGGCCACAAA
Mapk3	Mitogen-activated protein kinase 3	GGCATCCGAGACATCCTCAG	TATGTACTTGAGGCCCCGGA
NeuroD1	Neurogenic differentiation 1	CCCTACTCCTACCAGTCCCC	GTGCAATCAGTTAGGGGGCT
Rpl13a	Ribosomal protein L13A	CCCTCCACCCTATGACAAG	AGGTAAGCAAACCTTCTGGTAG
Slc2a3	Solute carrier family 2	TGGAAGAGCGGTTGGAAGAC	CCCAAGGATGGCAATCAGGT
TrkB	Neurotrophic tyrosine kinase, receptor, type 2	GCTGACGAGTTTGTCCAGGA	TGCTGTCTCATTGAGGCAA

Table S3. Primer sequences used in q RT-PCR experiments

Gene	Promoter	Forward	Reverse
Bdnf	IV	TCTATTTTCGAGGCAGAGGAGGTATC	AATGGGAAAGTGGGTGGGAG
Bdnf	IX	GGCTTTACGGTCCCCTTCA	CTCAGAACTGCTTGGACGGT

Table S4. Primer sequences used in ChIP experiments

The primers for the BDNF IV promoter were obtained from previous studies (Gomez-Pinilla et al., 2011). The promoter region of BDNF IX and its transcription start sites (TSS) were predicted using the “TRED Promoter Database”. Genomic regions of 2000 bases upstream of the first TSS of each candidate gene were used to design the different primer pairs. Further, CpG islands in the promoter regions were determined with Methprimer software and used as a putative indicator of MeCP2 binding sites.

Genes	NS VEH	NS FLX	PNS VEH	PNS FLX	ANOVA	LSD post hoc
Akt1	1.00 ± 0.14	0.75 ± 0.10	0.94 ± 0.11	0.83 ± 0.09	-	-
Bcl2	1.00 ± 0.07	0.92 ± 0.10	1.07 ± 0.08	1.01 ± 0.09	-	-
Bdnf IV	1.00 ± 0.05	0.80 ± 0.06	0.85 ± 0.06	0.80 ± 0.04	Treatment *	NS VEH > NS FLX * NS VEH > PNS VEH *
Bdnf IX	1.00 ± 0.06	0.83 ± 0.07	0.88 ± 0.05	0.95 ± 0.06	-	-
Cdh13	1.00 ± 0.08	1.20 ± 0.35	1.10 ± 0.16	0.96 ± 0.10	-	-
cFos	1.00 ± 0.08	0.86 ± 0.10	1.02 ± 0.21	0.95 ± 0.08	-	-
Egr1	1.00 ± 0.08	0.71 ± 0.09	0.98 ± 0.14	0.86 ± 0.06	-	-
Mapk3	1.00 ± 0.12	0.79 ± 0.08	0.96 ± 0.09	0.83 ± 0.07	-	-
NeuroD1	1.00 ± 0.06	0.83 ± 0.07	0.86 ± 0.07	0.94 ± 0.09	-	-
Slc2a3	1.00 ± 0.10	0.76 ± 0.10	1.11 ± 0.19	0.98 ± 0.12	-	-
TrkB	1.00 ± 0.07	0.68 ± 0.04	0.91 ± 0.08	0.83 ± 0.07	Treatment *	NS VEH > NS FLX *

Table S5. Effect of prenatal stress and maternal fluoxetine on hippocampal gene expression in male offspring

mRNA levels of BDNF/TrkB downstream target genes were measured in the hippocampus of males offspring by quantitative real-time RT-PCR, and are expressed as fold changes as compared to naïve animals (NS SAL). Data are represent mean ± SEM of n=12-16 rats per group. *p < 0.05; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

Genes	NS VEH	NS FLX	PNS VEH	PNS FLX	ANOVA	LSD post hoc
Akt1	1.00 ± 0.14	1.02 ± 0.13	0.80 ± 0.10	0.61 ± 0.08	Stress *	NS FLX > PNS FLX *
Bcl2	1.00 ± 0.05	0.99 ± 0.13	1.27 ± 0.08	0.91 ± 0.08	Treatment *	PNS VEH > PNS FLX *
Bdnf IV	1.00 ± 0.08	0.92 ± 0.06	1.34 ± 0.08	1.08 ± 0.07	Treatment * Stress **	-
Bdnf IX	1.02 ± 0.06	0.96 ± 0.05	1.11 ± 0.03	0.99 ± 0.08	-	-
Cdh13	1.00 ± 0.09	0.95 ± 0.13	1.10 ± 0.09	0.89 ± 0.11	-	-
cFos	1.00 ± 0.14	1.04 ± 0.12	1.08 ± 0.11	0.86 ± 0.06	-	-
Egr1	1.00 ± 0.16	0.80 ± 0.15	0.51 ± 0.12	0.41 ± 0.06	Stress **	NS VEH > PNS VEH *
Mapk3	1.00 ± 0.10	1.01 ± 0.13	1.02 ± 0.13	0.59 ± 0.07	-	-
NeuroD1	1.00 ± 0.20	0.90 ± 0.15	1.19 ± 0.12	0.93 ± 0.11	-	-
Slc2a3	1.00 ± 0.20	0.77 ± 0.13	0.86 ± 0.12	0.67 ± 0.13	-	-
TrkB	1.00 ± 0.08	0.86 ± 0.07	1.01 ± 0.07	0.94 ± 0.08	-	-

Table S6. Effect of prenatal stress and maternal fluoxetine on hippocampal gene expression in female offspring

mRNA levels of BDNF/TrkB downstream target genes were measured in the hippocampus of females offspring by quantitative real-time RT-PCR, and are expressed as fold changes as compared to naïve animals (NS SAL). Data are represent mean ± SEM of n=12-16 rats per group. *p < 0.05; Two-way ANOVA, Fisher LSD *post hoc* test. NS = no stress, PNS = prenatal stress, VEH = vehicle, FLX = fluoxetine.

CHAPTER VII

Behavioral and neurochemical characterization of TrkB-dependent mechanisms of agomelatine in glucocorticoid receptor-impaired mice

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Abstract

Growing evidences indicate that impairment of the hypothalamo-pituitary-adrenal (HPA) axis, a key regulator of the stress response, might be responsible for the hippocampal atrophy and dysfunction often observed in mood disorders. Antidepressants, possibly through the activation of BDNF signaling, may be able to enhance neuroplasticity and restore a normal hippocampal function. In this context, glucocorticoid receptor-impaired (GRi) mice – a transgenic mouse model for mood disorders – were used to investigate the role of BDNF/TrkB signaling in the behavioral and neurochemical effects of the new generation antidepressant agomelatine. GRi mice and their wild-type littermates were treated for 21 days with either agomelatine (50mg/kg/day; i.p) or the TrkB inhibitor Ana12 (0.5mg/kg/day, i.p) alone, or in combination. GRi mice exhibited a marked alteration in depressive and anxiety-like behaviors as well as fear response, together with a decreased cell proliferation and altered neuroplastic and epigenetic regulation in the hippocampus. Chronic treatment with agomelatine resulted in antidepressant and anxiolytic effects in GRi mice, and reversed the deficit in hippocampal cell proliferation and alteration of plasticity-related gene expression. The dual administration with Ana12 could block the anxiolytic effect of agomelatine as well as its ability to restore a normal expression of *Bcl2* and *cFos* mRNA. Altogether, our findings indicate that agomelatine represents an efficient alternative to reverse the neuroplastic and behavioral alterations caused by HPA axis impairment. In addition, our findings provide further insights into the role of BDNF/TrkB signaling in the molecular and behavioral actions of antidepressants, as well as in the pathophysiology of mood disorders.

Key words: Plasticity, hippocampus, antidepressants, BDNF, glucocorticoid, epigenetic

I – Introduction

The impairment of the hypothalamo-pituitary-adrenal (HPA) axis, a major component of the neuroendocrine system regulating the stress response, has been implicated in the pathophysiology of mood disorders (Pariante et al., 2008; Vreeburg et al., 2009). Among several growth factors, brain-derived neurotrophic factor (BDNF) has been postulated as a key mediator of stress-induced synaptic dysfunction and neuronal atrophy often observed in the prefrontal cortex and hippocampus of mood disorder patients (Duman et al., 2012; Berton et al., 2006). The decrease of BDNF and its tropomyosin-related kinase receptor (TrkB) were both observed in the hippocampus of suicide completers and depressed patients (Autry et al., 2012; Dwivedi et al., 2003), and the functional val66met polymorphism in the gene encoding BDNF has been associated with a wide variety of psychiatric phenotypes (Hong et al., 2009). In addition, animal studies showed that prolonged exposure to stress could be associated with decreased BDNF signaling, as well as impaired neurogenesis in the hippocampus (Mirescu et al., 2006; Taliatz et al., 2011; Tsankova et al., 2006). Accordingly, various classes of antidepressants were shown to activate BDNF-mediated signaling and reverse neuronal atrophy and cell loss induced by chronic stress in rodents (Castren et al., 2010; Schmidt et al., 2007), and infusion of BDNF directly into the hippocampus resulted in antidepressant effect in naïve mice (Shirayama et al., 2002; Ye et al., 2011). BDNF heterozygous knockout mice (BDNF^{+/-}), displaying a reduction by half of BDNF levels, failed to show any depressive-like behavior in basal condition (Korte et al., 1995; MacQueen et al., 2001), whereas knockdown of BDNF in specific brain sites could impair neurogenesis and precipitate depressive-like behavior (Taliatz et al., 2010). In addition, genetic mice models lacking BDNF showed an increased vulnerability to stress as compared to their wild-type littermates (Yu et al., 2012), as well as an inability to respond to antidepressant treatment (Adachi et al., 2008; Yu et al., 2012), indicating a critical role for BDNF in the behavioral and neurochemical mediation of antidepressant response. Increasing evidences also suggest that the elevation of BDNF levels observed after antidepressant treatment might be attributed to epigenetic regulation (Boulle et al., 2012). Interestingly, diverse classes of antidepressants were evidenced to target the epigenetic machinery, which appeared to be necessary in mediating their therapeutic effects (Vialou et al., 2013). For instance, chronic treatment with imipramine was sufficient to increase histone acetylation specifically at the BDNF gene, which causally correlate with the reversal of behavioral impairments induced by social defeat stress in mice (Tsankova et al., 2006). Additionally, in this same model, the downregulation of histone deacetylase 5 (HDAC5) was necessary to correct for histone H3K9 hypoacetylation in the hippocampus and mediate the antidepressant action of imipramine (Tsankova et al., 2006). Moreover, DNA methyltransferase (DNMT) inhibitors and HDAC inhibitors produced antidepressant-like effects when administered in naïve mice (Sales et al., 2011; Covington et al., 2011), and the local administration of HDAC inhibitors in the nucleus accumbens exhibited similar gene expression profiles than the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine

(Covington et al., 2009). Among antidepressants, agomelatine has emerged as an innovative and atypical treatment, mediating its effects via the activation of melatonin receptor 1 (MT1) and 2 (MT2), and the inhibition of the serotonin 2C receptor (5-HT_{2C}). Agomelatine was shown to normalize altered BDNF regulation and impaired neurogenesis in rodent models of depression (Paizanis et al., 2010). Hence in the present study, we wanted to determine whether TrkB signaling was required for the neurobiological and behavioral actions of agomelatine. We used a recently developed TrkB inhibitor, Ana-12, which has been described as a potent and selective TrkB inhibitor that could lead to a decreased TrkB activity in mouse brain after peripheral administration (Cazorla et al., 2011). In our experiments, we performed dual administration of agomelatine, together with Ana-12, in a transgenic mouse model of affective disorders known as the glucocorticoid-receptor impaired (GR-i) mice. We previously showed that GR-i mice displayed a deficit in the hypothalamo-pituitary-adrenal (HPA) axis feedback under stressful conditions as well as neuroplastic impairment and despair-like behavior (Froger et al., 2004; Paizanis et al., 2010). Hence, in this transgenic mouse model we wanted to test the ability of agomelatine to rescue the behavioral and neurochemical alterations, and explore the involvement of TrkB in the mechanism of action of this new generation antidepressant.

II – Material and methods

Animals: Transgenic mice (strain B6C3F1; line 1.3) bearing a transgene for the glucocorticoid receptor (GR-i) and their paired wild-type (WT) were used in the experiments. Mice issued from the same gestation were housed in the same cage, under standard conditions (22± 21 C, 60% humidity, 12-h light–dark cycle with lights on at 7.00 am, food and water ad libitum). The mice were bred at INSERM UMR894 / UPMC animal facility. Experiments were performed using 12-week-old mice (25–30 gm body weight). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive 87-848, October 19, 1987, Ministère de l’Agriculture et de la Forêt, Service Veterinaire de la Sante et de la Protection Animale; permissions 75-116 to M.H. and 006269 to L.L.).

Drug treatments: Administration of drugs was performed everyday at 6.00 pm (i.e. 1 hour before the onset of dark period), for a total period of 21 days. The doses used in the experiments have been chosen on the basis of previous studies performed in validated mice models of depression and anxiety (Cazorla et al., 2011; Paizanis et al., 2010). First, Ana-12 (0.5 mg/kg, Maybridge, Illkirch, France) or its vehicle (NaCl 0,9%, tween80 1% and dimethylsulphoxyde 1%) was administered via intraperitoneal injections. After a 30-minute delay, agomelatine (50 mg/kg, Servier, Suresnes, France) or its vehicle hydroxyethylcellulose (HEC 1%, Servier, Suresnes, France) were administered via the same injection mode. The animals were sacrificed 18 hours after the last injection.

Behavioral testing:*Tail suspension test (TST)*

The apparatus consists of three suspension units divided by walls (ID-Tech-Bioseb, Chaville, France). The mice were suspended by the tail with an adhesive tape, and the duration of immobility was measured automatically by the software over a 6-minute period.

Splash test

The splash test, previously described by Surget et al., 2008, consists into spraying a 10% sucrose solution on the back of mice in their home cage. Because of its viscosity, the sucrose solution dirties the coat and induces grooming behavior. The total time of grooming over a 5-minute period was recorded in our experiments.

Open field test

The apparatus consisted of four open boxes of 40 x 40 cm separated by white wood walls and equipped with an infrared floor for the measurement of motor activity. The arena was subdivided into a central and a peripheral zone. Mice were placed in the open field boxes for 20 min under normal light conditions, and the motor activity of the animals were automatically scored with a camera connected to a computerized system (Viewpoint, Lyon, France). The time spent in the different zones and the distance moved were scored.

Forced swim test (FST)

In the FST, originally designed by Porsolt and colleagues (1977), mice were placed into plastic buckets (19 cm diameter, 23 cm deep, filled with 23°C–25°C water) under low light conditions and videotaped for 6 min (Viewpoint, Lyon, France). The different outcome measures were “immobility” reflecting no movement at all and/or minor movements necessary to keep the nose above the water; “mobility” reflecting medium activity such as swimming; and “bursts” reflecting strong mobility and struggling behaviors.

Fear conditioning

The fear conditioning paradigm consisted into 3 days of behavioral testing, and was conducted in an operant chamber (26 cm long × 18 cm wide × 22 cm high) possessing aluminum sidewalls and Plexiglas rear and front walls, and a stainless steel grid floor. On the first day, all mice were allowed to acclimate to the training chamber for 3 min, and then a tone (CS; conditioned stimulus) of 2500 Hz frequency and 85 dB intensity was presented for 30 s and terminated in the last 2 s with a mild foot shock (0.75 mA) (US; unconditioned stimulus). After a period of two minutes (ITI; inter-trial interval), another CS-US pairing was presented. For the contextual fear conditioning test, rats were returned to the same chamber used for

the conditioning trial 24 h after conditioning and allowed to explore for 20 min without presentation of the auditory CS or foot shock. Twenty-four hours after the contextual fear conditioning test, the mice were tested for cued fear memory by returning them to the same chamber, which was modified by adding various hatch designs to the walls as well as a vanilla scent. Stimulus presentation and data acquisition were controlled by a computerized system interface. Freezing behavior, defined as complete absence of voluntary movements except for respiratory movements, was measured every 1 s.

Social interaction

Mice were placed in an open boxes of 40 x 40 cm² separated by white wood walls for 20 minutes the day preceding the experiment in order to habituate the animal to the novel arena, and to maximize the duration of social interactions. The following day, mice of same genotype and unfamiliar to each other were placed in the same arena for 10 minutes, and videotaped using a fixed camera. Total time spent in active social interactions (including sniffing, chasing and grooming) was scored by an observer unaware of groups.

Quantitative reverse transcriptase PCR (qRT-PCR): Total mRNA extraction was performed using NucleoSpin RNA II kit (Macherey-Nagel, Hoerdt, France), after which, the cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. The amplification was made with Absolute SYBR Green ROX Mix (Thermo Scientific, Illkirch, France) using the 7300 Real Time PCR System (Applied Biosystems, Courtaboeuf, France) and the sequences of primers used are indicated in Supplementary Table 1. Gene expressions were normalized using Hprt as a reference gene and the conditions for the reaction included 35 cycles in a fixed sequence of 95°C for 20 s, 62°C for 30 s, and 72°C for 30 s. The $2^{\Delta\Delta CT}$ (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions.

Immunohistochemistry: Coronal sections of 25 μ m between bregma levels -1.340 and -2.300 were collected at the cryostat (Leica CM 3050, Leica Microsystems, Wetzlar, Germany), after which sections were directly mounted on double dipped gelatin coated glass slides and stored at -80°C until use. The sections were then fixed with 4% paraformaldehyde at 4°C for 10 min. After permeabilizing the membranes with TBS-triton 0.05 %, the sections were incubated overnight at 4°C with the Ki-67 antibody (1:1000, Vector Laboratories, Burlingame, Calif., USA). The sections were then incubated for 2 hour at room temperature in presence of appropriated biotinylated secondary antibodies, and the reaction was amplified using the ABC staining system (Vector Laboratories, Burlingame, Calif., USA) for one additional hour. Finally, the sections were treated with 3,3'-diaminobenzidine for 4 minutes. After dehydrating the sections and mounting with cover slip, the positive cells were counted under the microscope (Nikon Eclipse E400, Nikon Instruments Inc., Shanghai, China).

Statistical analysis: Data are represented as mean \pm SEM and were analyzed using Student's t test for comparison between two groups (Prism 5.0, GraphPad software, USA). When more than two groups of data, a one-way analysis of variance (ANOVA) was used followed by Fisher's LSD *post hoc* test in order to compare all the groups between them. In addition, a two-way ANOVA (agomelatine treatment x Ana12 treatment) was sometimes used to investigate the effect of the drug treatments in the GRi phenotype. The level of significance was set as $P < 0.05$.

III – Results

Effects of impaired GR function on behavior: The battery of test routinely used to characterize depressive-like, anxiety-like and fear-related behaviors in rodents showed that GRi mice exhibited robust alterations in mood and emotions as compared to their wild-type littermates. As shown by figure S1, GRi mice showed an increased immobility in the TST (+ 27 %, Figure S1) and decreased grooming behavior in the splash test (– 28 %, Figure S1). As previously reported by Barden and colleagues, GRi mice showed increased escape-like behavior in the FST (Barden et al., 2005). GRi mice had an increase of swimming behavior (+ 59 %, Figure S1), and a decrease immobility time (– 20 %, Figure S1) as compared to wild type mice. GRi mice also showed increased anxiety behavior and exaggerated fear responses. GRi mice had a decreased motor activity (– 23 % in small movement duration) and exploration (– 31 % in distance moved) in the open field test (Figure S2). In addition, these transgenic mice displayed a high score of inactivity in the open field arena (+ 15 %, Figure S2), which might involve freezing behaviors (Figure S2). Accordingly, GRi mice displayed an increased freezing behavior when subjected to the fear conditioning paradigm (Figure S3). GRi mice showed higher rates of freezing in the fear acquisition test, the contextual fear conditioning test and the cued fear memory test. In addition, GRi mice showed elevated freezing behavior at baseline in the cued fear memory test, possibly indicating a generalization of fear (data not shown). Hence, the phenotype characterization of GRi mice pointed towards a marked dysregulation in mood and emotion responses, making this transgenic mouse a suitable model to explore different aspects of antidepressant action.

Effects of impaired GR function on hippocampal cell proliferation and gene expression: To further elucidate the cellular and molecular mechanisms underlying the reduced glucocorticoid receptor function, the expression of Ki67 (a marker of cell proliferation), as well as the expression of various genes implicated in neuronal plasticity and in transcription and epigenetic regulations, were measured. Immunohistochemical analysis revealed that cell proliferation in the subventricular zone (SVZ) of the dentate gyrus was decreased in GRi mice (– 34 %, Figure 4). Similar results using BrdU and DCX stainings in this region were reported in GRi mice (Paizanis et al., 2011). qRT-PCR analysis revealed a complex regulation of genes involved in neuronal plasticity. There was a decreased expression of the

Bdnf4 transcript (– 24 %, Figure 5). Similarly, GRi mice exhibited a decreased expression of the B-cell lymphoma 2 (*Bcl2*) gene (– 26 %, Figure 5), a critical mediator of cell survival, as well as decreased expression of the FBJ osteosarcoma oncogene (*cFos*) gene (– 50 %, Figure 6), known as a marker of neuronal activity. In contrast, the expression of glial cell line-derived neurotrophic factor (*Gdnf*) and nerve growth factor inducible (*Vgf*), two genes involved in neurotrophic signaling, were increased in GRi mice (+ 58 % and + 37 % respectively, Figure 5). The expression of the corticotropin releasing factor (*Crf*) gene was also increased in GRi mice. The GR usually exert a negative feedback on *Crf* expression, so it is not surprising to observe such variation. Similarly, the gene expression of various transcription factors, including the cAMP responsive element binding protein (*Creb*) gene and the early growth response 1 (*Egr1*) gene was increased (+ 31 % and + 22 % respectively, Figure 5 and 6). Finally, expression of the epigenetic regulators was investigated. The expression of the DNA methyltransferase 1 (*Dnmt1*) gene and the DNA methyltransferase 3a (*Dnmt3a*) gene, both involved in active DNA methylation, was decreased in GRi mice (- 43 % and – 46 % respectively, Figure 6). In addition, the expression of the histone-lysine N-methyltransferase HRX 3 (*Mll3*) gene and the histone-lysine N-methyltransferase HRX 4 (*Mll4*) gene, involved in histone methylation activity and transcription co-activation, was decreased in GRi mice (- 35 % and – 28 % respectively, Figure 6), but did not reach statistical significance.

Effects of agomelatine and TrkB antagonist on grooming and despair-like behavior: In a second step of the work, the ability of agomelatine to reverse the behavioral alterations was investigated. We combined the administration of agomelatine with a newly developed TrkB antagonist, Ana12, in order to investigate the involvement of TrkB in the mechanism of action of agomelatine. Agomelatine could significantly increase the time spent in grooming behavior in GRi mice (Figure 1). However, this effect could not be blocked when co-administered with Ana12 (Figure 1). In the TST, chronic administration of agomelatine to GRi mice could restore the immobility time to the level observed in wild type (GRi HEC SAL vs. GRi AGO SAL: – 42 %, Figure 1). Chronic treatment with the TrkB inhibitor Ana12 could reverse the increased immobility observed in GRi mice receiving only the vehicle (GRi HEC SAL v.s. GRi HEC ANA: – 44 %, Figure 1). This effect has already been described after acute exposure of 4h with Ana12 in naïve mice (Cazorla et al., 2011). However, the co-administration of agomelatine and Ana12 did not show such kind of antidepressant effects. In the FST, GRi mice treated for 3 weeks with the vehicle of agomelatine and Ana12 (GRi HEC SAL) showed increased immobility as compared to WT mice treated with the 2 vehicles (WT HEC SAL), which appeared to be in opposition to what have been reported in basal conditions. This contrasting observation might be attributed to the effects of manipulation and stress of chronic injections on innate flight response and reactivity. However, chronic treatment with agomelatine significantly decreased the immobility time in the FST in the GRi phenotype (Two-way ANOVA, Figure 1), an effect usually observed after acute treatment with selective serotonin reuptake inhibitor (SSRI) antidepressants in this behavioral test. Similarly, agomelatine could increase the mobility

of GRi mice, as shown by higher swimming and burst duration (Figure 1). Of note, the antidepressant-like effect of agomelatine could not be abolished by Ana12 treatment (Figure 1).

Effects of agomelatine and TrkB antagonist on fear and anxiety: Chronic treatment with agomelatine significantly reversed the anxious phenotype of GRi mice in the open field test (Figure 2). Accordingly, agomelatine increased the distance covered and the small movement activity, and decreased the inactivity duration. In contrast, Ana12 had an anxiogenic effect in the open field test by decreasing the distance covered and duration in small movement activity, and increasing the inactivity duration. Interestingly, the effect of agomelatine on the distance covered and activity duration could be blocked when this antidepressant was administered together with Ana12. No effect was observed for the inactivity duration. Further, the effects of agomelatine and Ana12 on fear response were investigated. GRi mice displayed robust freezing behavior in response to fearful stimuli, as well as impairment to erase fear memories (Figure S3 and Figure 3). However, neither agomelatine nor Ana12 could prevent the exaggerated fear response in the fear conditioning test.

Effects of agomelatine and TrkB antagonist on hippocampal gene expression and cell proliferation: As previously reported, GRi mice have an impaired hippocampal neurogenesis including a decrease proliferation in the SGZ of the dentate gyrus (Paizanis et al., 2011). In this same study, it was evidenced by BrdU labeling that chronic treatment of 21 days with agomelatine (50 mg/kg/day) could significantly restore a normal cell proliferation in the SGZ. Here, a similar effect of agomelatine on cell proliferation in the SGZ was evidenced by Ki67 immunolabelling (Figure 4). Agomelatine increase the number of proliferative cells by approximately 74 % in GRi mice. In addition, clear-cut evidences demonstrate that this effect could be abolished when agomelatine was administered together with the TrkB antagonist Ana12 (- 44 % between GRi AGO SAL and GRi AGO ANA, Figure 4). Further, the levels of *Bcl2*, *Bdnf4* and *cFos* mRNAs, which was decreased in GRi mice, could be significantly upregulated after chronic treatment with agomelatine (+ 66 %, + 50 % and + 61 % respectively, Figure 5 and 6). In contrast, chronic administration of agomelatine significantly decreased the levels of *Vgf* mRNAs (- 37 %, Figure 5). Furthermore, chronic treatment with Ana12 significantly decreased the expression of *Gdnf* and *cFos* in GRi mice (two-way ANOVA, Figure 5 and 6), whereas an upregulation of *Bdnf4*, *Crf* and *Egr1* mRNA levels was observed (GRi HEC SAL vs. GRi HEC ANA: + 116 %, + 31 % and + 30 % respectively, Figure 5 and 6). However, no modulations of *Creb*, *Dnmt1*, *Dnmt3a*, *Mll3* and *Mll4* mRNA levels were observed after either agomelatine or Ana12 treatments. Finally, there was a significant difference between GRi mice treated with agomelatine alone (GRi AGO SAL) and GRi mice treated with agomelatine + Ana12 (GRi AGO ANA) regarding the expression of *Bcl2* and *cFos* genes (GRi AGO SAL vs. GRi AGO ANA: - 39 % and - 49 % respectively, Figure 5 and 6), suggesting that Ana12 could block the ability of agomelatine to restore a normal expression at those specific genes.

IV – Discussion

Mounting evidences suggest that HPA axis impairment may play an important part in the pathophysiology of major depression, and that the neuronal loss and synaptic dysfunction induced by prolonged stress exposure might involve alterations in neurotrophic signaling (Pariante et al., 2008; Vreeburg et al., 2009). Furthermore, many studies have reported a critical involvement of BDNF/TrkB signaling in the mechanism of action of conventional antidepressants (Castren et al., 2010). In this context, the aim of the present study was to investigate the implication of TrkB signaling in the behavioral and neuroplastic effect of the new generation antidepressant agomelatine in the GRi transgenic mouse, a rodent model of glucocorticoid-induced mood disorders.

Effect of impaired GR function on neuroplasticity and behavior: As previously reported, GRi mice displayed a reduced expression of GR mRNA in the brain and a disrupted HPA axis (Pepin et al., 1992; Froger et al., 2004). In addition, the present study showed an elevation of *Crf* mRNA in the hippocampus of GRi mice. Under normal conditions, the HPA axis exerts a negative feedback on *Crf* expression via GRs. Hence, it is not surprising that a reduced function of GR resulted in increasing *Crf* mRNA levels in the brain. Further, an impairment of hippocampal cell proliferation and decreased *Bdnf* mRNA levels were observed in previous investigations (Paizanis et al., 2011). Here, we confirmed the deficit in hippocampal cell proliferation, and bring more insight into the exon-specific regulation of the *Bdnf* gene and in GRi mice. The expression of *Bdnf4* transcript was decreased in the hippocampus of GRi mice. BDNF expression is tightly regulated at the level of transcription with differential exon usage, suggesting a crucial role for the various transcripts in different functions and related processes (Pruunsild et al., 2007; Baj et al., 2011). The *Bdnf4* transcript has been particularly reported in rodent models of stress, and in the mechanism of action of antidepressants (Tsankova et al., 2006; Dwivedi et al., 2006; Boulle et al., 2012). Next to that, the mRNA levels of *Bcl2* and *cFos* were decreased in GRi mice. *Bcl2* plays a critical role in cell survival, and *cFos* is a marker of neuronal activation (Bullitt et al., 1990; Davies et al., 1995; Zhang et al., 2002). Hence, the decrease of these two factors, together with *Bdnf4*, is in accordance with the impaired hippocampal neurogenesis in GRi mice. In contrast, the mRNA levels of *Gdnf*, *Vgf*, *Creb* and *Egr1* were increased in the hippocampus of GRi mice. These genes are also implicated, at different levels, in the mediation of neuronal plasticity (Benito et al., 2010; Bozdagi et al., 2008; Li et al., 2005; Sariola et al., 2003). Accordingly, it is possible that compensatory mechanisms involving other types of neuroplastic adaptations might be activated in response to the deficit in neurogenesis. Finally, the investigation of epigenetic markers revealed a strong alteration in the machinery controlling histone post-translational modifications and DNA methylation. Such kind of epigenetic regulations in the central nervous system has been described as a key-role mechanism in the maintenance of neuronal plasticity (Maze et al., 2012; Sultan and Day, 2011). In particular, stress and environmental adversity has been

shown to impact on chromatin and histone regulations to generate an epigenetic reprogramming, possibly increasing the susceptibility for mood disorders (Tsankova et al., 2007; Boulle et al., 2012).

At the behavioral level, we found that GRi mice exhibited an anxious phenotype as well as a marked impairment in fear-related behaviors. GRi mice also showed a decrease in grooming behavior, an indicator of a self-neglecting and motivational deficit that has been linked to altered ACTH function (Argiolas et al., 2000). As reported by previous findings, GRi mice displayed an increase in immobility time in the TST and increased activity in the FST in basal conditions (Barden et al., 2005; Paizanis et al., 2011). These results could be confirmed in the present study. However, the observed phenotype in the FST tended to disappear after 21 days of chronic drug injections. This effect can be attributed to the manipulation of the animals that might interfere with the innate mechanisms involved in the stress response. Altogether, the behavioral analysis indicated a marked alteration in mood-related behaviors in the transgenic GRi mice. Accordingly, the impairment of neurogenesis, together with the alterations in gene expression and epigenetic regulations, might be causally related to the alterations observed at the behavioral level.

TrkB signaling in the mechanism of action of agomelatine: Chronic treatment with agomelatine could produce an antidepressant-like effect in GRi mice, as observed by the decrease of despair-like behavior in the TST and FST, and increase of self-grooming behavior in the splash test. Interestingly, the effect of agomelatine in the splash test and the FST was not dependent on TrkB signaling. On the other hand, the effects of agomelatine in the TST were blocked by Ana12, suggesting a critical role of TrkB signaling in the neurochemical pathways underlying this behavioral performance. Similarly, the inhibition of TrkB could abolish the anxiolytic effect of agomelatine in the open field test. This observation is especially interesting since other studies, using genetic BDNF knockdown approaches in mice, also showed an inability for diverse classes of antidepressants to exert their effects in the above-mentioned behavioral tasks (Adachi et al., 2008; Yu et al., 2012). In addition, post-mortem studies showed that the expression of BDNF was significantly higher in patients treated with antidepressants (Chen et al., 2001), and some subpopulation of individuals carrying the functional val66met polymorphism in the gene encoding for BDNF showed an impaired antidepressant response (Niitsu et al., 2013). Further, agomelatine could restore the deficit in hippocampal cell proliferation observed in GRi mice. This effect was entirely dependent on TrkB, since the co-administration of agomelatine with Ana12 completely abolished the increased hippocampal cell proliferation observed with agomelatine treatment alone. This finding is of particular relevance because BDNF/TrkB signaling has been frequently associated with neurogenesis, and alteration of such physiological mechanisms might be causally linked to mood disorders (Snyder et al., 2011). Accordingly, the decrease of *Bcl2*, *Bdnf4* and *cFos* in GRi mice could be restored by chronic agomelatine treatment, which appeared to be dependent on TrkB signaling. This latter finding confirmed the importance of BDNF/TrkB signaling and downstream neuroplastic changes in the mediation of

antidepressant actions. Interestingly, agomelatine could normalize the expression of other genes such as *Egr1* and *Vgf* further supporting that this antidepressant is efficient to restore a normal balance in gene expression profiles in pathological models. However, chronic treatment with agomelatine could not restore normal epigenetic patterns in the hippocampus of GRi mice. These latter findings do not support the notion that the reversal of epigenetic alterations by antidepressant drugs is required for their therapeutic response.

Effect of TrkB antagonist on mood-related behaviors: Moreover, the present findings also bring some novel insights into the behavioral and neuroplastic properties of TrkB inhibitors. As already shown by Cazorla and colleagues in a recent study, the newly developed TrkB inhibitor Ana12 displayed antidepressant properties in the TST when injected acutely (Cazorla et al., 2011). Using this same dose of 0.5 mg/kg, we found a comparable effect after chronic injections of 21 days. These findings are rather provocative because a growing body of literature indicated similar results after administration of BDNF directly in the hippocampus, or peripheral administration of TrkB agonists (Liu et al., 2010; Shirayama et al., 2002). However, clean-cut evidences in both rodents and human studies demonstrated that stress and/or major depression could be associated with increased BDNF levels in the nucleus accumbens (Krishnan et al., 2007). Hence, the pathophysiology of depression appears to be complex, and might involve a region-specific regulation of BDNF/TrkB signaling. Further, chronic administration of Ana12 resulted in anxiogenic effects in GRi mice, and might causally correlate with the reduced expression of *cFos* and *Gdnf* observed in the hippocampus. In line with the present results, previous studies showed the val66met polymorphism in the gene encoding BDNF has been linked to anxiety disorders (Chen et al 2006), and reduced levels of BDNF in the hippocampus have been associated with an increased anxiety in rodents (Martinowich et al., 2007).

Conclusion and perspectives: In conclusion, our data provide a deeper understanding about the involvement of TrkB signaling in the mechanism of action of agomelatine, and in the treatment of mood disorders. In accordance with other studies that proposed a critical role for BDNF in the therapeutic action of classical antidepressants (Castren et al., 2010), our findings provide further evidences that novel antidepressant displaying an atypical mechanism of action could also target BDNF/TrkB signaling to exert its behavioral and neuroplastic effects in a transgenic mouse model of mood disorders. Our data support the idea that therapeutic actions of antidepressants involve various neurocircuits, and that some of these neurochemical pathways may require BDNF/TrkB and downstream neuroplastic signaling to regulate mood.

V – References

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Chapter VII

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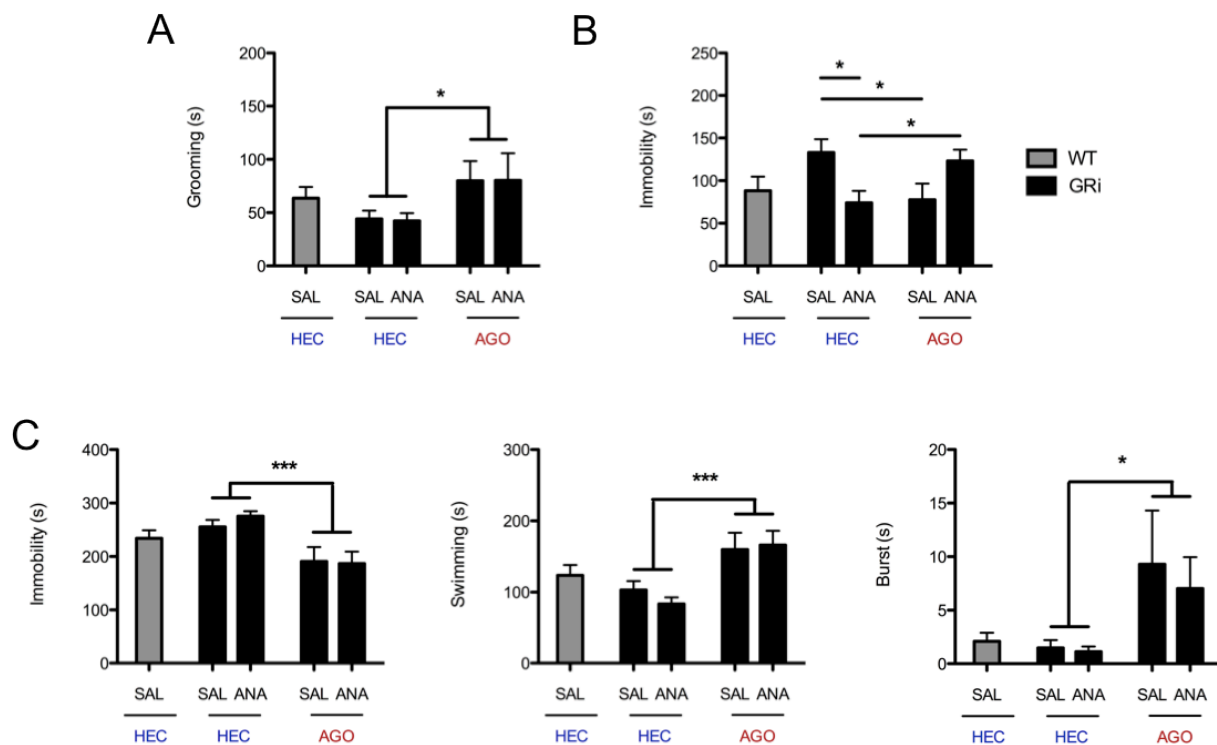
Figures and legends.

Figure 1. Effects of chronic agomelatine treatment in combination with a TrkB inhibitor on depressive-like behavior

(A) The time spent in grooming behavior over a total period of 5 minutes was measured in mice. The two-way ANOVA between agomelatine and Ana12 factors in the GRI genotype indicated a significant effect of agomelatine [$F(1,21)=5.71$; $p<0.01$], but no effect of Ana12 [$F(1,21)=0.002$; $p>0.05$] on the time spent in grooming behavior. (B) The immobility time in the tail suspension test paradigm was measured after chronic mild stress. The one-way ANOVA indicated a significant interaction $F(4,28)=3.17$; $p<0.05$]. A LSD post hoc test was used to determine the significant difference between groups ($*p < 0.05$). (C) Mice were subjected to a forced swim test, and the time spent in immobility, swimming and high mobility (burst), over a 6-minute period, is represented. The two-way ANOVA between agomelatine and Ana12 factors in the GRI genotype indicated a significant effect of agomelatine on immobility [$F(1,22)=17.65$; $p<0.001$], swimming [$F(1,22)=17.94$; $p<0.001$] and high mobility [$F(1,22)=6.39$; $p<0.05$]. No significant effects were observed in the LSD *post hoc* test. Data are expressed as mean + S.E.M of $n=5-8$ mice per group. $*p < 0.05$; $***p < 0.001$.

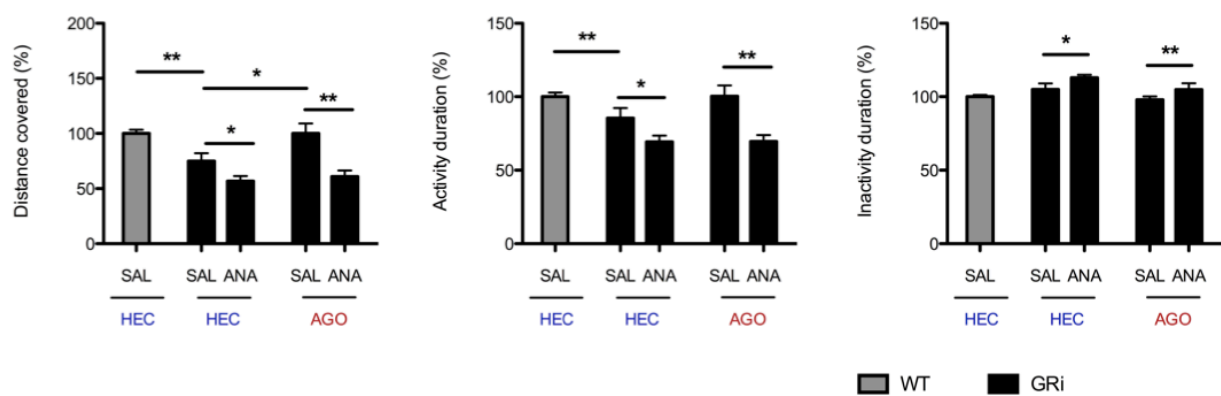


Figure 2. Effects of chronic agomelatine treatment in combination with a TrkB inhibitor on anxiety behavior

Mice were subjected to an open field test, and the distance covered, the time spent in small movement activity and the time spent in inactivity were measured over 20 minutes. A one-way ANOVA indicated a significant interaction in the distance covered [F(4,32)=10.72; $p < 0.001$], the time spent in small movement activity [F(4,32)=9.20; $p < 0.001$] and the time spent in inactivity [F(4,32)=6.91; $p < 0.01$]. A LSD *post hoc* test was used to determine the significant difference between groups (* $p < 0.05$; ** $p < 0.01$). Data are expressed as mean + S.E.M of $n=5-8$ mice per group.

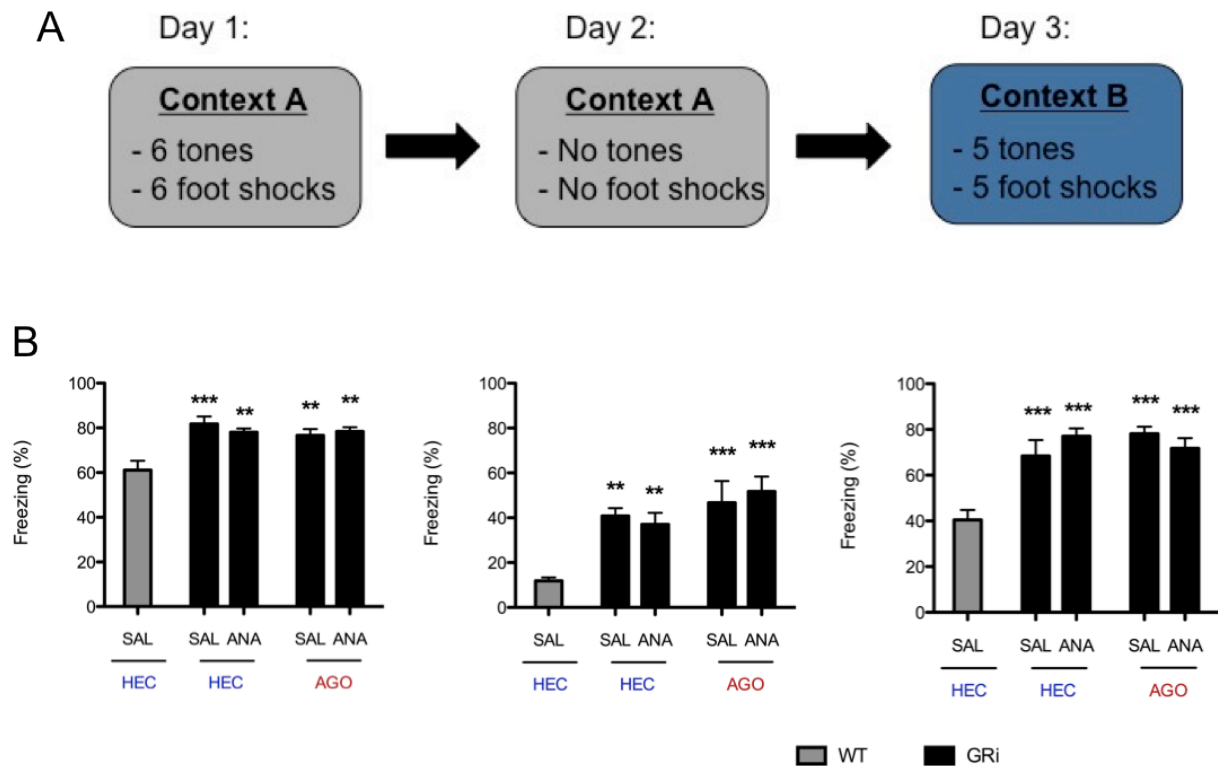


Figure 3. Effects of chronic agomelatine treatment in combination with a TrkB inhibitor on fear behavior

(A) A schematic representation of the fear conditioning procedure is represented. For more details see the material and methods section. (B) The freezing behavior during the fear acquisition (Day 1; left panel), the contextual fear conditioning (Day 2; center panel) and the cued fear memory (Day 3; right panel) test was measured during the tone periods, and the average freezing is represented. A one-way ANOVA indicated a significant interaction in the fear acquisition test [$F(4,32)=6.76$; $p<0.001$], the contextual fear conditioning test [$F(4,32)=9.23$; $p<0.001$] and the cued fear memory test [$F(4,32)=13.75$; $p<0.001$]. The LSD *post hoc* test revealed a significant difference in freezing between WT mice treated with vehicles (WT HEC SAL) and GRi mice treated with all the different combinations of treatments (GRi HEC SAL, GRi HEC ANA, GRi AGO SAL and GRi AGO ANA). Data are expressed as mean + S.E.M of $n=5-9$ mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

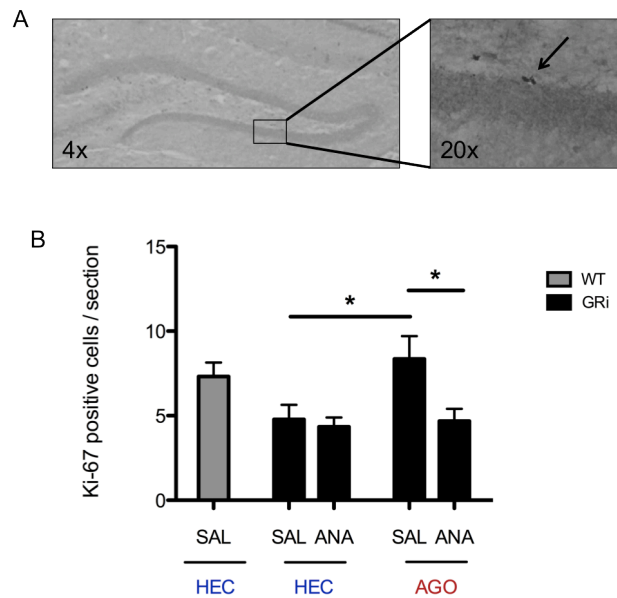


Figure 4. Effects of chronic agomelatine treatment on hippocampal cell proliferation

(A) An example of proliferative Ki67 immunoreactive cells in the dentate gyrus (DG) of the hippocampus is illustrated. (B) Mice were treated with agomelatine with or without TrkB inhibitor, and the number of proliferative Ki67 immunoreactive cells per section in the SGZ of the DG was investigated. A one-way ANOVA indicated a significant interaction [$F(4,29)=3.07$; $p<0.05$], and the significant differences between groups were determined with a LSD *post hoc* test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Data are expressed as mean + S.E.M of $n=5-9$ mice per group.

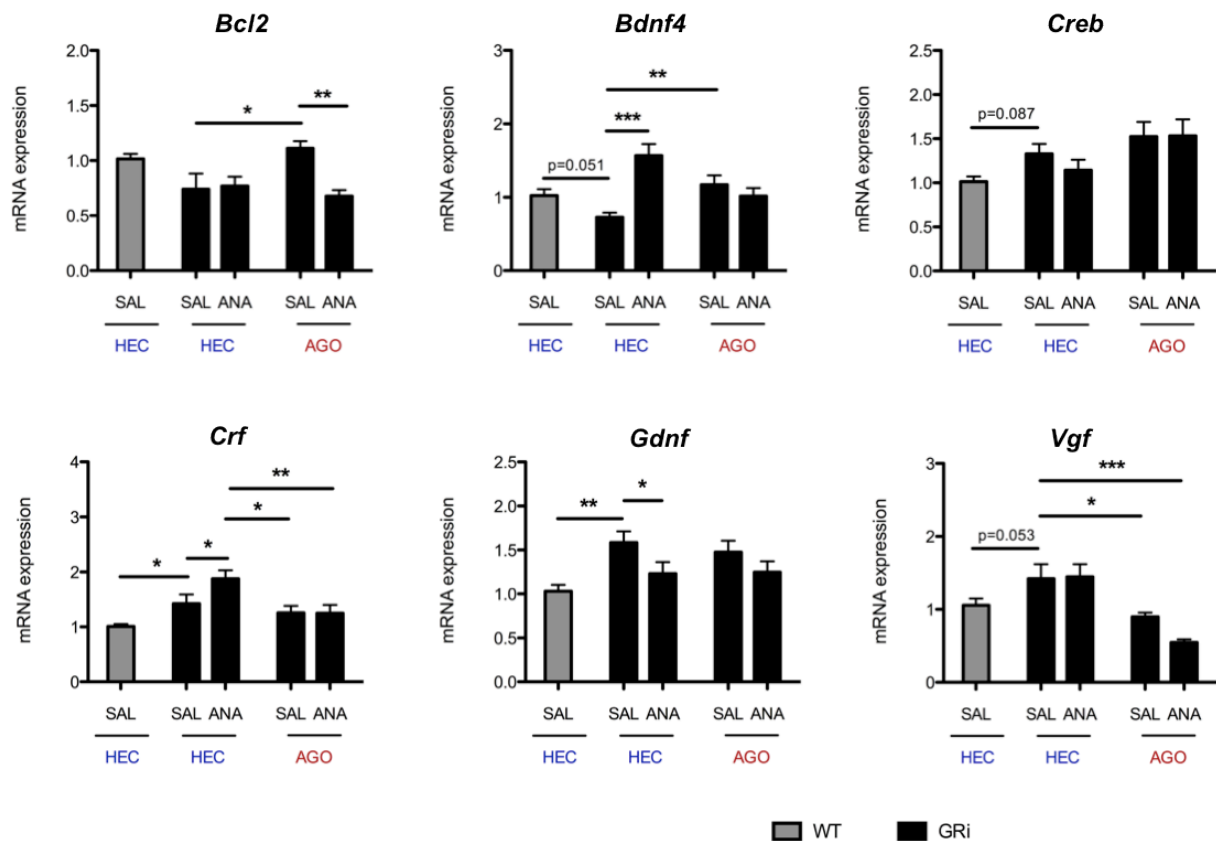


Figure 5. Effects of chronic agomelatine treatment in combination with a TrkB inhibitor on plasticity-related gene expression in the hippocampus

mRNA levels of plasticity-related genes in the hippocampus of mice were measured by quantitative real-time RT-PCR, and are expressed as fold changes as compared to the WT mice treated with the two vehicles (WT HEC SAL). A one-way ANOVA indicated a significant interaction for *Bcl2* [F(4,65)=4.16; $p < 0.01$], *Bdnf4* [F(1,29)=7.12; $p < 0.01$], *Creb* [F(4,34)=3.86; $p < 0.05$], *Crf* [F(4,34)=7.61; $p < 0.001$], *Gdnf* [F(4,66)=7.44; $p < 0.001$] and *Vgf* [F(4,66)=7.56; $p < 0.001$]. In addition, the significant differences between each groups revealed by the LSD *post hoc* test are represented (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Of note, the two-way ANOVA between Agomelatine and Ana12 factors in the GRI genotype indicated a significant effect of Ana12 on *Gdnf* [F(1,45)=12.64; $p < 0.001$]. Data are expressed as mean + S.E.M of $n=5-9$ mice per group.

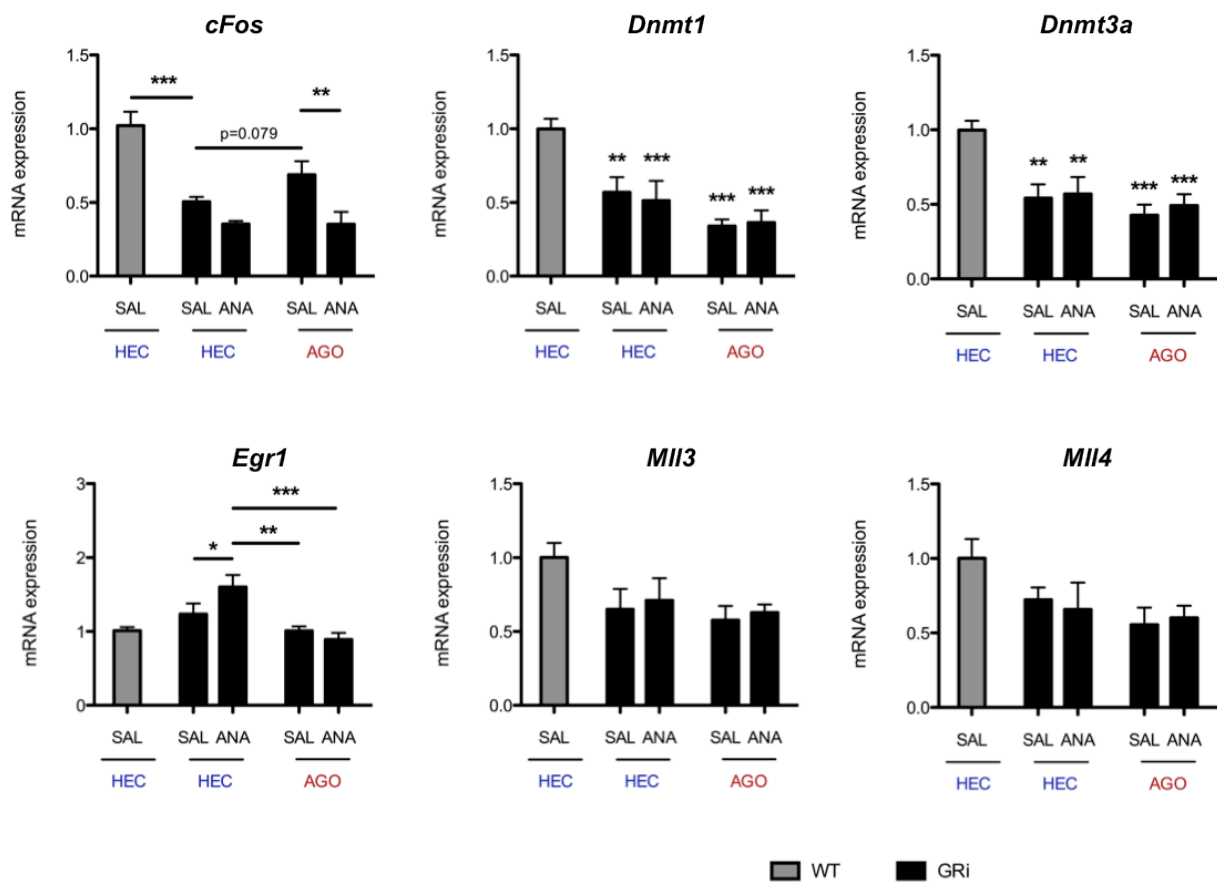
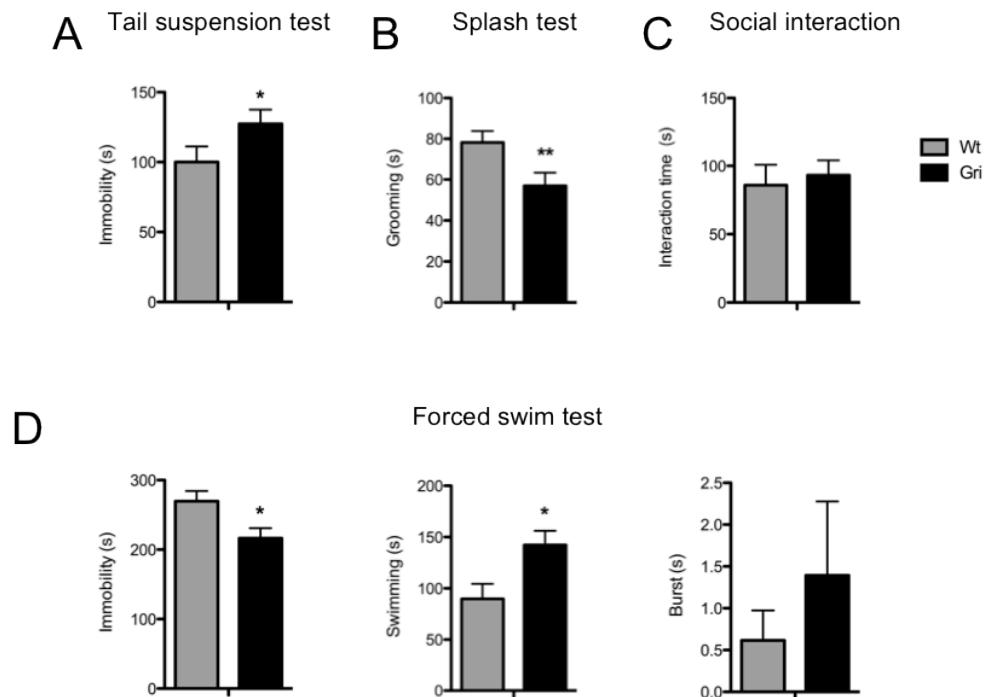


Figure 6. Effects of chronic agomelatine treatment in combination with a TrkB inhibitor on the expression of transcription factors in the hippocampus

mRNA levels of genes encoding for transcription factors were measured in the hippocampus of mice by quantitative real-time RT-PCR, and are expressed as fold changes as compared to the WT mice treated with the two vehicles (WT HEC SAL). A one-way ANOVA indicated a significant interaction for *cFos* [F(4,30)=16.20; $p < 0.001$], *Dnmt1* [F(4,29)=12.88; $p < 0.001$], *Dnmt3a* [F(4,29)=8.26; $p < 0.001$], *Egr1* [F(4,34)=6.81; $p < 0.001$], *MII3* [F(4,29)=2.39; $p = 0.076$] and *MII4* [F(1,29)=1.42; $p > 0.05$]. In addition, the significant differences between each groups revealed by the LSD *post hoc* test are represented (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Of note, the two-way ANOVA between agomelatine and Ana12 factors in the GRI genotype indicated a significant effect of Ana12 on *cFos* [F(1,21)=15.02; $p < 0.001$], and a significant effect of agomelatine on *Egr1* [F(1,22)=12.70; $p < 0.01$]. Data are expressed as mean + S.E.M of $n = 5-9$ mice per group.

Supplementary data.**Figure S1. Characterization of mood-related behaviors in GRi mice**

Mice were subjected to a battery of test routinely use to measure depressive-like behavior and response to antidepressant drugs. The difference between wild type (WT) and glucocorticoid receptor-impaired (GRi) mice in basal conditions was monitored. (A) The immobility time in the tail suspension test paradigm was measured, and a Student's t-test revealed a significant increase of immobility in GRi mice. (B) The time spent in grooming behavior over a total period of 5 minutes was measured in mice, and a Student's t-test revealed a significant decrease of the time spent in grooming behavior in GRi mice. (C) The time spent in active social interaction was measure over 10 minutes, and no significant difference was found in the Student's t-test analysis. (D) Mice were subjected to a forced swim test, and the time spent in immobility, swimming and high mobility (burst), over a 6-minute period, is represented. The Student's t-test revealed a significant increase in mobility, together with a decrease of immobility, in GRi mice as compared to their wild type littermates. Data are expressed as mean \pm S.E.M of n=8-12 mice per group. *p < 0.05; **p < 0.01; ***p < 0.001.

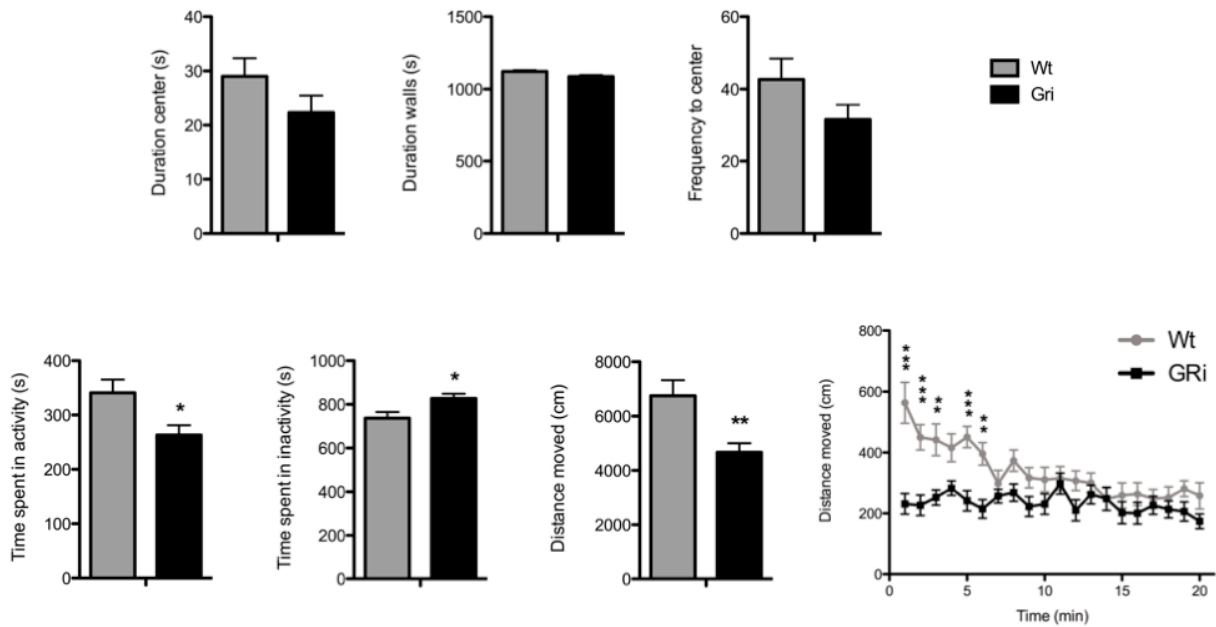


Figure S2. Characterization of anxiety-like behavior using the open field test in GRi mice

Mice were subjected to an open field test for 20 minutes. The difference between wild type (WT) and glucocorticoid receptor-impaired (GRi) mice in basal conditions was investigated. There was no difference in the time spent in the periphery between WT and GRi mice. However, a trend was observed for the time spent in the center, as well as the number of entries in the central compartment, but did not reach statistical significance. Further, there was a significant difference in the motor behavior and exploration of GRi mice. The Student's t-test revealed that GRi mice spent more time in inactivity, and less time in small movement activity. Similarly, the distance covered by GRi mice was significantly decreased, especially during the first 6 minutes of the test, where a lower exploration was observed in GRi mice. Data are expressed as mean \pm S.E.M of n=8-12 mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

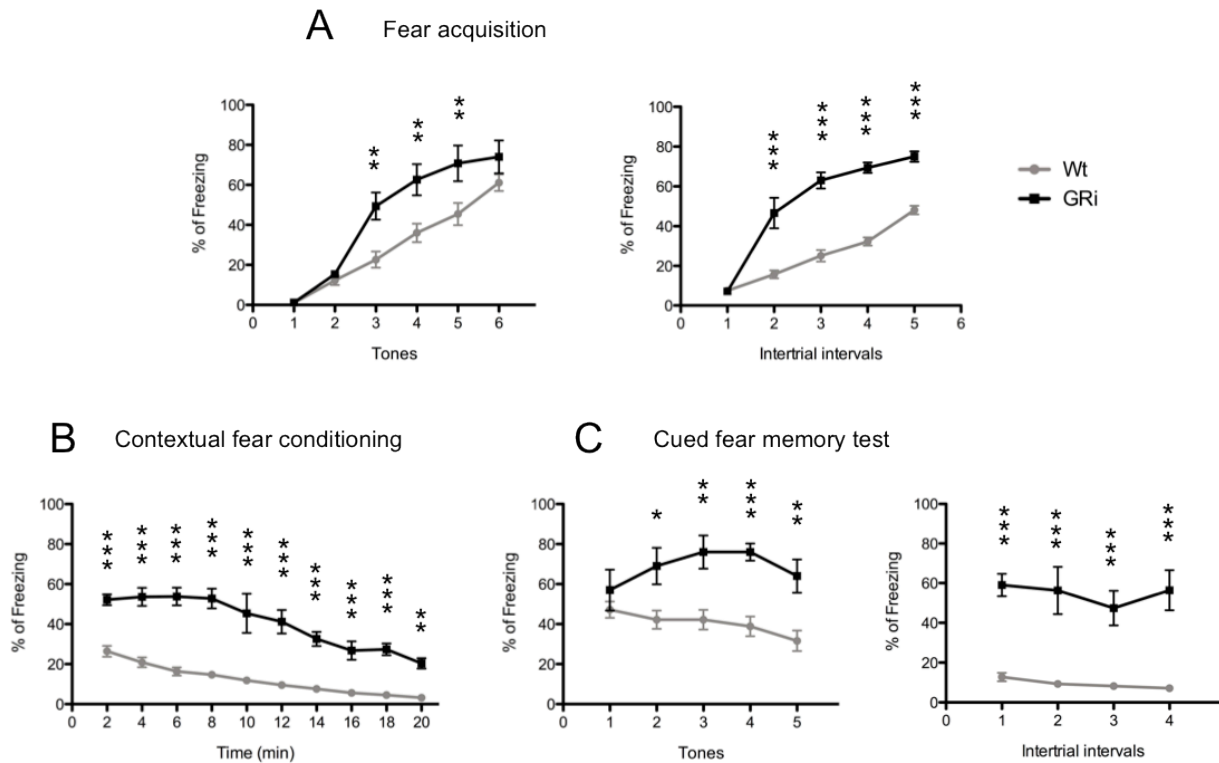


Figure S3. Characterization of fear response using the fear conditioning paradigm in GRi mice

Mice were tested in the fear conditioning paradigm, and the average freezing during the fear acquisition (A), the contextual fear conditioning (B) and the cued fear memory (C) is represented. A two-way ANOVA followed by a Bonferroni *post hoc* test revealed that GRi mice displayed significantly more freezing behaviors than WT. Data are expressed as mean \pm S.E.M of n=5-9 mice per group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Gene	Definition	Primer sense	Primer antisense
Bcl2	B-cell CLL/lymphoma 2	ATGACTGAGTACCTGAACCG	ATGCTGGGGCCATATAGTTC
Bdnf4	Brain derived neurotrophic factor exon 4	AATCTCCCAGCCTCTGCCTA	CGAGTCTTGGTGGCCGATA
cFos	FBJ murine osteosarcoma viral oncogene homolog	AGGGAACGGAATAAGATGGC	TGCAACGCAGACTTCTCATC
Creb	cAMP responsive element binding protein 1	GTTGTTATGGCGTCTCCC	TACGACATTCTCTTGCTGCC
Crf	Corticotropin releasing factor	CCTTGAATTTCTTGACGCCG	GGACTTCTGTTGAGATTCCCC
Dnmt1	DNA methyltransferase (cytosine-5) 1	AGCCGCTCAAAGCAAAGTG	TGGGGTTCATCCACAGCATC
Dnmt3b	DNA methyltransferase (cytosine-5) 3b	GATGAGGAGAGCCGAGAACG	CAGAGCCCACCCTCAAAGAG
Egr1	Early growth response 1	TCAATCCTCAAGGGGAGCC	ACTCGTCTCCACCATCGC
Gdnf	Glial derived neurotrophic factor	TGGGTTTGGGCTATGAAACC	AACATGCCTGGCCTACTTTG
Hprt	Hypoxanthine-guanine phosphoribosyltransferase	GATGATGAACCAGGTTATGAC	GTCCTTTTACCAGCAAGCTTG
Mll3	Histone-lysine N-methyltransferase HRX 3	TCCCGATAGTTTCGTCCCCT	AGAATGGGGATGATGGGGGA
Mll4	Histone-lysine N-methyltransferase HRX 4	CTCGCCACTGAGGATACGTC	CCCTGTAGACCCTGTAGGCT
Myst2	MYST histone acetyltransferase 2	GGTACTGCTCCGATACCTGC	TCTGAAGGGCTTGACAGAGTG
Ncoa6	Nuclear receptor coactivator 6	TCTACCCAGCATAGCCCCTT	AGTTTCTTCGGCTCGTCAGG
Vgf	VEGF nerve growth factor inducible	TTGACACCCTTATCCAAGGC	ACCTGAGCTAATCCTTGCTG

Table S1. Primer sequences used in q RT-PCR experiments

CHAPTER VIII

Agomelatine normalizes hippocampal and behavioral dysfunction in a mouse model of environmental stress

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In preparation

Abstract

Stress-induced alterations in neuronal plasticity and associated hippocampal dysfunctions have been reported as a major culprit in the course and development of mood disorders. Here, we investigated the activation of intracellular signaling cascades, and the expression of epigenetic markers and plasticity-related genes in the hippocampus of a mouse model of hyperactive and mixed affective disorders. Further, we analyzed the ability of agomelatine, a mixed MT1/MT2 melatonin receptor agonist/5-HT_{2c} receptor antagonist, to prevent stress-induced neurobiological and behavioral alterations. Adult male C57BL/6J mice were exposed to daily unpredictable socio-environmental stressors of mild intensity for a total period of 3 weeks. Behavioral investigations showed that stressed mice exhibited robust increase in emotional arousal, vigilance and motor activity, together with reduced anxiety as well as motivation and reward deficits. Neurobiological investigations showed an increased activation of Akt and Creb signaling pathways in the hippocampus of stressed mice. The decreased hippocampal levels of the repressive epigenetic marks HDAC2 and H3K9me2, as well as the increased levels of the permissive mark H3K9/14ac suggested that chronic mild stress could be associated with increased gene transcription, and clear-cut evidence for such changes was found for neuroplasticity-related genes, including *Arc*, *Bcl2*, *Bdnf*, *Gdnf*, *Igf1* and *Neurod1*. Altogether, the present data suggest that chronic ultra-mild stress can model the hyperactivity or psychomotor agitation, as well as mixed affective behaviors often observed in the manic state of bipolar disorders. Interestingly, agomelatine could normalize both behavioral and neurobiological alterations induced by stress, providing further molecular and behavioral evidence in the action of this new generation antidepressant.

Key words: Plasticity, hyperactivity, hippocampus, stress, mood, epigenetic

I – Introduction

Stress is a predisposing factor for the course and development of a broad range of behavioral and pathophysiological disturbances, among which mood disorders have been abundantly reported (Gunnar and Quevedo, 2007). More specifically, the hippocampus - a key region in emotional and cognitive process - is known to be highly sensitive to glucocorticoid providing to this limbic structure an important role in the integration of stress response (McEwen et al., 1992; Sala et al., 2004). However, the exact mechanism by which stress, via glucocorticoid, modulates functional and structural plasticity in the brain remains largely unknown, and the timing and intensity of the stressors seem to be critical in the determination of the neuroendocrine and behavioral outcomes (McEwen et al., 2007). It is well accepted that severe and long-lasting traumatic stress might lead to neuronal loss and synaptic dysfunction in the hippocampus, generally associated with low mood, anxiety and impaired cognition, which constitute core symptoms of major depression (Apfel et al., 2011; Snyder et al., 2011, Stockmeier et al., 2004). Nevertheless, stressful life events, especially when disrupting circadian rhythms, can also be a precipitant of manic episodes of bipolar disorders including elevated mood, hyperactivity, impulsivity and increased vigilance (Malkoff-Schwartz et al., 1998; Proudfoot et al., 2011).

At the molecular level, the sustained elevation of glucocorticoids observed in stressful situations might interfere with various intracellular signaling and transcriptional processes. In particular, chromatin remodeling via histones modifications occurs in response to diverse environmental variations and represents a key regulator of nuclear integration to promote structural and functional adaptations underlying neuronal plasticity (Maze et al., 2013). Hence, during the past decade, epigenetic mechanisms have emerged as a key process by which environmental variations could leave persistent imprints on gene expression thereby increasing the risk for mood disorders (Boulle et al., 2012). In addition, antidepressants and mood stabilizers have been shown to mediate their therapeutic effects at least in part through epigenetic remodeling (Boulle et al., 2012; Vialou et al., 2012).

More recently, Agomelatine, a mixed melatonin receptor 1 (MT1) and 2 (MT2) agonist and serotonin 5-HT_{2C} receptor antagonist, has been described as an emerging and efficient treatment for major depressive episodes (De Bodinat et al., 2010). Agomelatine is thought to exert its effects via a re-synchronization of circadian rhythms (De Bodinat et al., 2010). Moreover, disruption of circadian periodicity has been proposed as a major player in the onset of bipolar disorders, and successful antidepressant treatment is associated with normalization of disturbed biological rhythms (Harvey AG, 2008; Lanfumey et al., 2013). Hence, the modulation of melatonin receptors may provide a specific an innovative way to treat bipolar disorders.

Hence, in our experiments, we used a prolonged repetition of low-intensity stressors in adult mice, including social isolation, perturbations of circadian rhythms and daily life habits, in order to induce affective disorders. We previously reported that this chronic mild stress procedure was associated with reduced glucocorticoid receptor expression in the hippocampus and impulsive choice in the decision making task (Froger et al., 2004). In the present study, we aimed at further characterizing the manic-like phenotype of the mice, as well as exploring the intracellular signaling, epigenetic regulations and gene transcription profiles following stress exposure. In addition, we wanted to test the ability of the new-generation antidepressant agomelatine to reverse the eventual cellular and molecular alterations and to restore a normal phenotype in stressed mice.

II – Material and methods

Animals: C57BL/6J male mice (Charles River Laboratories, l'Arbresle, France) were used in the experiments. Upon arrival in the laboratory, the mice were housed in groups of four per cage under standard conditions (22± 21 C, 60% humidity, 12-h light–dark cycle with lights on at 07h00, food and water ad libitum) for 1 week before the beginning of experimental procedures. Experiments were performed using 10- to 12-week-old mice (25–30 g body weight). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (Council directive 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Veterinaire de la Sante et de la Protection Animale; permissions 75-116 to M.H. and 006269 to L.L.).

Stress and treatments: The chronic ultra mild stress procedure used in this study has been presented elsewhere (Froger et al., 2004), and is a modified version of the chronic mild stress protocol described by Willner in rats (Willner, 1992). Briefly, mice were subjected to daily socio-environmental stressors of low intensity, in an unpredictable manner (See supplementary table S1) during a 25-day period. Administration of compounds was initiated at the beginning of stress procedures, everyday at 6:00 pm (i.e. 1 hour before the onset of dark period), for a total period of 25 days. The doses used in the experiments have been chosen on the basis of previous studies performed in validated mice models of depression and anxiety (Paizanis et al., 2010). Agomelatine (50 mg/kg, Servier) or its vehicle (Hydroxyethylcellulose; HEC 1%, Servier) was administrated via intraperitoneal injections.

Behavioral tests: The animals were tested for behavioral analysis after the fourth week of stress. The tests were performed at least 18 hours after the last stressor, so as to avoid an eventual effect of acute stress. The animals were kept in the testing room for more than 2 hours prior to the test so as to enable the mice to habituate to the new environment.

The behavioral tests were adapted from existing protocols. Classical test for measuring depressed like behavior (TST, FST, splash test, saccharin intake) locomotor activity (OF, actimetry) and anxiety (EPM, OF) described in supplementary material, were performed between day 21 and day 25.

Finally, the animals were sacrificed 18 hours after the last day of both stress and drug injection, and the brains were kept at -80°C until use.

Quantitative reverse transcriptase PCR (qRT-PCR): Total mRNA extraction was performed using NucleoSpin RNA II kit (Macherey-Nagel, Hoerd, France), after which the cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. One part of the amplification was made with Absolute SYBR Green ROX Mix (Thermo Scientific, Illkirch, France) using the 7300 Real Time PCR System (Applied Biosystems, Courtaboeuf, France), and the other part of gene expression was measured using TaqMan (Applied Biosystems, Courtaboeuf, France) gene expression assays. The sequences of primers used are indicated in Table S2 and Table S3. The $2^{\Delta\Delta CT}$ (Delta-Delta Comparative Threshold) method was used to normalize the fold change in gene expressions for both qPCR technologies.

Western blots: The hippocampus were lysed in ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0, 10%, 10 mM NaF, 1 mM sodium orthovanadate and protease inhibitor cocktail) and protein extracts was loaded on a SDS-PAGE gel for electrophoresis migration and then transferred to a PVDF membrane for 7 min using the iBlot gel transfer system (Life technologies, St Aubin, France). Non-specific binding was blocked by incubating the membranes in a blocking buffer (PBS-Tween 0.01% supplemented with 5% of BSA) for 1 h at room temperature. The membranes were incubated with the primary antibody (see Table S4) overnight at 4°C in the same blocking buffer. Then, after extensive washing, the membranes were incubated with the appropriate fluorescent secondary antibodies (Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 488 donkey anti-mouse; Molecular Probes, OR, USA) for 1 h at room temperature. Finally, the membranes were washed, dried and scanned using the Odyssey infrared imaging system (Westburg, Leusden, The Netherlands).

Immunohistochemistry: Coronal sections of 20 μm were collected using a cryostat, after which sections were directly mounted on Superfrost Plus slides (Thermo Scientific, Illkirch, France) and stored at -80°C until use. The sections were then fixed with 4% paraformaldehyde at 4°C for 10 min. After blocking non-specific binding with 0.4% of BSA and 5% of appropriate serum, and permeabilizing the membranes with 0.05% of tween 20, the sections were incubated overnight at 4°C with the primary antibodies (see Table S4). The sections were then incubated for 1 hour at room temperature in presence of appropriated biotinylated secondary antibodies, and the reaction was amplified using the ABC staining system (Vectastain ABC Elite Kit, Vector Laboratories, Les Ullis, France) for one additional hour. Finally, the sections were treated with 3,3'-diaminobenzidine (Sigma FAST™, D4293, Sigma-Aldrich, Saint

Quentin-Fallavier, France). After dehydrating the sections and mounting with cover slip, pictures were taken under optic microscope (Leica, zoom 2,5) and optical density was measured and then normalized by the background using ImageJ software.

Statistical analysis: Data are represented as mean \pm SEM and were analyzed using Student's *t* test for comparison between two groups (Prism 5.0, GraphPad software, USA). When more than two groups of data were compared, two-way ANOVA (Stress x Treatment) were used followed by Bonferroni *post hoc* test, or when necessary a *planned* pairwise comparison using Dunn's *t*-test. The level of significance was set as $P < 0.05$.

III – Results

Agomelatine reverses stress-induced alterations in behavior: A battery of well-established behavioral tests usually employed to measure mood, emotions and motor performances in rodents were performed at the end of the stress period. Stressed animals displayed a pronounced hyperactivity, as shown by the increase exploration in the open field (OF) arena (Figure 1A, + 30 \pm 6.4 %), especially during the first minutes of the test (Figure 1A, upper panel). This increase in motor activity was confirmed by measuring spontaneous locomotion in actimetry boxes. The ambulation, corresponding either to the total number of square crossed (Figure S7) or the number of crossing in the center of the actimetry box (Figure 1B), was increased in stressed mice (+ 82 \pm 11.5 %). The numbers of rearings, corresponding to a vertical activity where the mouse leaves the floor with the fore feet, was also increased after exposure to stress (+ 95 \pm 12.5 %, Figure 1B). Similarly, the distance covered in the elevated plus maze (EPM) test was increased in mice subjected to chronic mild stress (+ 20 \pm 7.6 %, Figure S6). However, the performance of motor activity in the rotarod test was altered in stressed mice. Mice exposed to stress displayed a decrease in the latency to fall (– 39 \pm 9.5 %, Figure S6) indicating impairment of balance and movement coordination. Interestingly, the alterations in the distance covered in the OF, as well as the numbers of crossings and rearings in actimetry boxes could be significantly prevented by chronic administration of agomelatine (Figure 1A and 1B). Further, we assess the motivation and reward alterations, and measured the consumption of saccharin solution and the time spent in self-grooming. Data on Figure 2 indicate that chronic mild stress significantly decreased saccharin intake and grooming behavior (– 35 \pm 6.5 % and – 43 \pm 7.6 % respectively, Figure 2A and 2B). Agomelatine could normalize the alteration in both saccharin consumption and grooming behavior (Figure 2A and 2B). We also performed some tests to measure the defense mechanisms and anxiety levels such as the tail suspension test (TST), the forced swim test (FST), the OF test and the EPM test (Figure 2, Figure S5, Figure S6 and Figure S7). Chronic mild stress significantly reduced the immobility time in the tail suspension test (– 52 \pm 11.7 %, Figure 2D), which could be prevented by agomelatine treatment. There was no difference in the immobility time in the FST

(Figure S5). However, a small increase in the number of burst (especially during the first 2 minutes, see Figure S5) and mobility was observed in the FST after stress exposure ($+ 16 \pm 7.2 \%$ and $+ 9 \pm 4.8 \%$ respectively, Figure 2E and 2F). The mobility time was significantly different between stressed mice treated with vehicle or agomelatine, indicating a preventive effect of agomelatine on escape-like behaviors in this paradigm (Figure 2F). In the OF, we observed a reduced anxiety as shown by the increased numbers of entries in the center ($+ 46 \pm 12.4 \%$, Figure 2C), which could be corrected by agomelatine. Of note, the chronic mild stress procedure did not modify the water consumption and weight of the animals (Figure S4). Altogether, the behavioral analysis indicate that chronic ultra mild stress caused a marked hyperactivity, together with increase of defense mechanisms and decrease of motivation and reward. Those behavioral alterations could be prevented when mice were treated with agomelatine (50 mg/kg/day) during the whole stress procedure.

Agomelatine reverses stress-induced alterations in intracellular signaling cascades: To further elucidate the cellular mechanisms underlying chronic ultra mild stress, the activation of hippocampal intracellular signaling pathways involved in survival, proliferation and differentiation was measured. As shown by western blots analyses (Figure 3), chronic ultra mild stress significantly impacted on hippocampal intracellular signaling cascades. The phosphorylation of the protein-kinase B (Akt), the extracellular signal-regulated kinase (Erk), the p-38 mitogen-activated protein kinase (p38), the cAMP response element-binding protein (Creb), and the activating transcription factor 1 (Atf1) were increased after chronic ultra mild stress ($+ 17 \pm 6.2 \%$, $+ 42 \pm 10.5 \%$, $+ 22 \pm 8 \%$, $+ 55 \pm 14.8 \%$ and $+ 71 \pm 14.6 \%$ respectively, Figure 3), whereas no significant modulations were observed at TrkB phosphorylation. The increase of phospho-Akt, phospho-Erk, phospho-Creb and phospho-p38 induced by stress could be normalized by chronic agomelatine treatment (Figure 3). The western blot investigations suggest that specific effectors of the MAPK and PI3K pathways, involving Akt, Erk, p38, Creb and Atf1, were increased after chronic ultra mild stress, and that the administration of agomelatine could significantly prevent most of those alterations.

Agomelatine reverses stress-induced abnormal epigenetic regulation and gene expression: To further elucidate the molecular and transcriptional mechanisms underlying chronic ultra mild stress, the expression of various genes implicated in the regulation of transcription such as epigenetic regulators or transcription factors, as well as genes involved in neuronal plasticity was measured (Table S5, S6 and S7). First, qPCR screening (Table S5) revealed that chronic stress induced the hippocampal expression of many plasticity-related genes including the brain derived neurotrophic factor (*Bdnf*) gene ($+ 28 \pm 7 \%$), the neurogenic differentiation 1 (*Neurod1*) gene ($+ 69 \pm 10 \%$), the insulin growth factor 1 (*Igf1*) gene ($+ 41 \pm 7 \%$), the activity-regulated cytoskeleton-associated protein (*Arc*) gene ($+ 56 \pm 10 \%$), the expression the glial cell-derived neurotrophic factor (*Gdnf*) gene ($+ 58 \pm 15 \%$) and the b-cell lymphoma 2 (*Bcl2*) gene ($+ 46 \pm 9 \%$). In addition, the expression of the nuclear receptor subfamily 1, group D, member 2

(*Nr1d2*), known as a transcription activator was increased ($+ 40 \pm 5 \%$, Table S6), and the expression of the nuclear receptor co-repressor 1 (*Ncor1*), known as a transcription repressor, was decreased ($- 37 \pm 6 \%$, Table S6). A screening for modulations of epigenetic marks involved in synaptic plasticity including histone deacetylase, DNA methyltransferase, methyl binding domain protein and histone acetyltransferase was also investigated (Table S7). Among all the epigenetic regulators, a significant reduction of the epigenetic repressor *Hdac2* ($- 22 \pm 4 \%$, Table S7) - a key role player in hippocampal plasticity - was observed. Hence, we expressed further interest in the expression of epigenetic markers, and measured the expression of HDACs and post-translational modifications at histone tails (Figure S1, S2 and S3). As shown by immunostaining analyses, chronic mild stress decreased the HDAC2 protein levels in the CA1 and CA3 subfields of the hippocampus ($- 18 \pm 1.2 \%$ and $- 14 \pm 4.3 \%$ respectively, Figure S1A), whereas the expression of HDAC1 was not modulated. The expression of histone 3 lysine 9 di-methylated (H3K9me2) was specifically decreased in the CA3 region ($- 23 \pm 4.3 \%$, Figure S2A), whereas the expression of histone 3 lysine 9 and 14 acetylated (H3K9/14ac) was increased in the CA1 and CA3 subfields ($+ 63 \pm 12.5 \%$ and $+ 32 \pm 12.5 \%$ respectively, Figure S2B). The expression of histone 4 acetylated (H4ac) and histone 3 lysine 4 tri-methylated (H3K4me3) were not modulated by the stress (Figure S2C and S2D). Given the pronounced alterations of gene expression and epigenetic regulations, the ability of agomelatine to correct for these abnormal epigenetic modifications in the hippocampus was investigated. The figure 4 shows that chronic treatment with agomelatine could significantly prevent the stress-mediated decrease of HDAC2 and H3K9me2, as well as the increase of H3K9/14ac in the CA3 region of the hippocampus. The action of agomelatine was rather specific to the CA3 subfield, since no significant effects were observed in the CA1 and DG subfields (Figure S3). In a similar manner, some of the alteration in plasticity-related gene expression observed after chronic ultra mild stress exposure could be normalized by chronic agomelatine treatment (Figure 5). Agomelatine significantly restored the abnormal expression of the *Bdnf* gene, the *Neurod1* gene, the *Igf1* gene, the *Arc* gene, whereas the expression the *Gdnf* gene and the *Bcl2* gene could not be normalized. All in all, these data indicate that chronic stress triggered a marked reorganization at histone post-translational modifications, pointing towards an open state of chromatin. Interestingly, this permissive state of chromatin observed after chronic ultra mild stress was associated with increased transcription of genes involved in neuroplasticity, and preventive administration of agomelatine could abolish these latter alterations.

IV – Discussion

Mounting evidences suggest that stressful life events might play a part in the development and course of mood disorders, and that successful antidepressant treatments might involve a normalization of stress-related alterations in cortico-limbic structures (Calabrese et al., 2009; Touma C, 2011). In this context, the aim of the present study was to investigate the effect of prolonged socio-environmental stressors of low

intensity on mood-related behaviors and hippocampal function in mice, and explore the biological mechanisms and therapeutic potentialities of the new generation agomelatine in this stress model.

Effect of stress on neuroplasticity and behavior: In the present study, we found that a chronic, unpredictable and ultra mild stress could induce a decrease in saccharin consumption and grooming behavior, reflecting motivation and reward deficits, two core symptoms generally related to depression like behavior and observed after chronic stress exposure (Willner P, 2005). We also found a robust decrease of immobility in the TST, an increased mobility and number of burst in the FST. These behavioral measures can be seen as the expression of defense mechanisms in reaction to innate fear stimuli. Hence, in our experiments, the chronic mild stress exposure facilitated flight response as shown by tonic/energetic agitation and escape-like behaviors, which might be interpreted as an excessive emotional arousal and high vigilance state. In addition, stressed mice displayed a reduced anxiety in the open field test. Further, we observed an increased locomotor and motor activity, reflecting a hyperactive state of mice after stress exposure. Some studies have reported such locomotor activity following chronic mild stress exposure in rodents and could be interpreted as a psychomotor agitation often seen in mood disorders (Gronli et al., 2005; Pardon et al., 2000; Negroni et al., 2004). Since many studies demonstrated that physical activity and exercise could significantly enhance hippocampal neurogenesis and plasticity (Van Praag et al., 1999; Fabel et al., 2008), the increased locomotor activity observed after chronic stress might explain, at least in part, the increased in hippocampal plasticity in our mice model. In addition, stressed mice displayed a pronounced impairment in motor coordination and balance, which can be related to the fact that mice subjected to stress had difficulty to learn the task and to keep focus on movement coordination. At the neurobiological level, chronic mild stress was shown to enhance hippocampal intracellular signaling pathways such as mitogen-activated protein kinases (MAPK) known to be involved in neuronal survival, differentiation and proliferation (Thomas et al., 2004). In addition, we found robust changes in epigenetic regulation and gene transcription. The increased in chromatin permissive state and plasticity-related gene expression might correspond to the nuclear integration of the observed increased in intracellular signaling cascades. Among all the epigenetic modifications, a significant decrease of the repressive mark HDAC2 was reported. The low levels of HDAC2 in the hippocampus were shown to strongly correlate with enhanced synaptic plasticity (Guan et al., 2009). In addition, stress and environmental adversity has been shown to impact on chromatin and histone regulations to generate an epigenetic reprogramming, possibly increasing the susceptibility for mood disorders (Roth et al., 2009; Tsankova et al., 2006; Weaver et al., 2004). Further, many genes including trophic factors (*Bdnf*, *Gdnf*, *Igf1*), pro-survival factor (*Bcl2*), differentiation factor (*Neurod1*) and cell/dendritic growth factor (*Arc*), known to be critical in neuronal plasticity, were increased after chronic mild stress. Hence, the neurobiological investigations points towards drastic molecular and cellular changes underlying increased hippocampal plasticity. These results are rather provocative since it is well established that chronic stress paradigms in rodents are generally associated with impaired synaptic

integrity, reduced dendritic arborization and decreased neurogenesis in the hippocampus, as well as depressive- and anxiety-like behaviors (Alonso et al., 2004; Goshen et al., 2008; Mineur et al., 2007). However, few studies reported that chronic stress could also enhance neurogenesis as well as dendritic spines and excrescence in CA3 neurons of the hippocampus (Parihar et al., 2011; Sunanda et al., 1995). In our protocol of chronic mild stress - in contrast to other protocols using longer or stronger stressors - we used a repetition of low-intensity socio-environmental stressors for a total period of 3 weeks suggesting that the timing, duration and intensity of the stressors might be critical in the determination of the neuroendocrine and behavioral outcomes. Further, we previously reported a reduced hippocampal expression of GR mRNA in stressed mice (Froger et al., 2004). Other reports showed that downregulation of GR in the hippocampus in rodents, either by genetic knockdown or by pharmacological blockade after exposure to chronic stress, could be associated with an increased synaptic plasticity (Avital et al., 2006; Fitzsimons et al., 2013; Krugers et al., 2006).

Effect of agomelatine treatment on stress-related alterations: Agomelatine is an atypical antidepressant that acts on the melatonergic and the serotonergic systems. Like many commonly used antidepressants, agomelatine was shown to positively modulate neurogenesis, hippocampal plasticity and neurotrophic signaling in rodent models of depression (Banasr et al., 2006; Soumier et al., 2009). In our experiments we did not observe any effects of chronic agomelatine treatment on hippocampal plasticity in non-stressed mice. Interestingly, agomelatine could specifically normalize the hippocampal alterations induced by chronic mild stress, indicating that this drug has a disease-dependent action on neuronal activity. MT2 receptors, one of the key players in agomelatine action, are abundantly expressed in various cell populations of the hippocampus (Musshoff et al., 2002). Melatonin receptors are coupled to $G\alpha_i$ proteins resulting into an inhibition of cyclic AMP signaling (De Bodinat et al., 2010). Some studies showed that melatonin, most likely through the interaction via MT2 receptors, reduced neuronal firing and excitability, and inhibited long-term potentiation in mouse hippocampal slices (Hogan et al., 2001; Wang et al., 2005). This latter effect might explain the normalization of the increased intracellular signaling and associated epigenetic and neuroplastic changes observed after chronic mild stress. A previous study also showed that agomelatine could specifically normalize the increased hippocampal c-Fos levels, an indicator of increased neuronal activity, induced by prenatal stress in rat (Mairesse et al., 2013). At the behavioral level, chronic mild stress caused reward, motivation and attention deficit. These symptoms are known to be highly dependent on the mesolimbic dopaminergic system. The normalization of these behavioral alterations by agomelatine can be attributed to the inhibition of 5-HT_{2C} receptor, known to enhance dopamine release in several brain structures including the frontal cortex, striatum and nucleus accumbens (De Deurwaerdere et al., 2004; Di Matteo et al., 1999). In addition, our model of stress induced an overall increase in motor activity, which could be corrected by agomelatine. Previous studies also reported similar effects of agomelatine on hyperactivity in mice models of mood disorders (Mairesse et al., 2013; Norman et al., 2011), and systemic administration of melatonin was also shown to

reduce spontaneous locomotor activity in rats exposed to stress (Chuang et al., 1994). Finally, the emotional reactivity or vigilance is also known to be sensitive to melatonin receptor agonists, shifting the arousal state from hyperactive to sleep (Pandi-Perumal et al., 2007).

Conclusions and perspectives: In summary, the neurobiological and behavioral investigations provided clean-cut evidences that prolonged exposure to socio-environmental stressors of low intensity in C57Bl/6 mice could induce drastic molecular and cellular changes leading to an increased hippocampal plasticity, together with marked hyperactivity, hyperarousal, reward and motivation deficits, distractibility and increased risk taking. Agomelatine could prevent both neurochemical and behavioral alterations triggered by stress, providing novel insights into the molecular, cellular and behavioral mechanisms of action of this atypical antidepressant in a rodent model of mood-related disorders. In addition, the altered epigenetic regulations in the hippocampus support the notion that chromatin remodeling via histone modifications can increase susceptibility for mood disorders, and that the reversal of such changes by antidepressants are required for their therapeutic action.

Finally, the abovementioned behavioral responses described in our stress model can be assimilated to common symptoms usually observed in mixed manic state of bipolar disorders (see Table S8). Although, no compelling evidences for hippocampal hyperactivity were shown in humans, validated models of manic-like behaviors showed similar neurochemical changes in the hippocampus, including upregulation of BDNF protein and increased PI3-K and MAPK signaling (Kirshenbaum et al., 2011; Prickaerts et al., 2006). In addition, both human and animal studies established a pivotal role for circadian rhythms and seasonal changes in the pathophysiology and treatment of bipolar disorders (Arey et al., 2013; Harvey A.G, 2008; Kirshenbaum et al., 2011). Hence, this model might be of particular interest to further investigate the causal relationship between stress, circadian rhythms and hippocampal dysfunction in the development and course of manic and bipolar disorders.

V – References

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Chapter VIII

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Chapter VIII

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Figures and legends

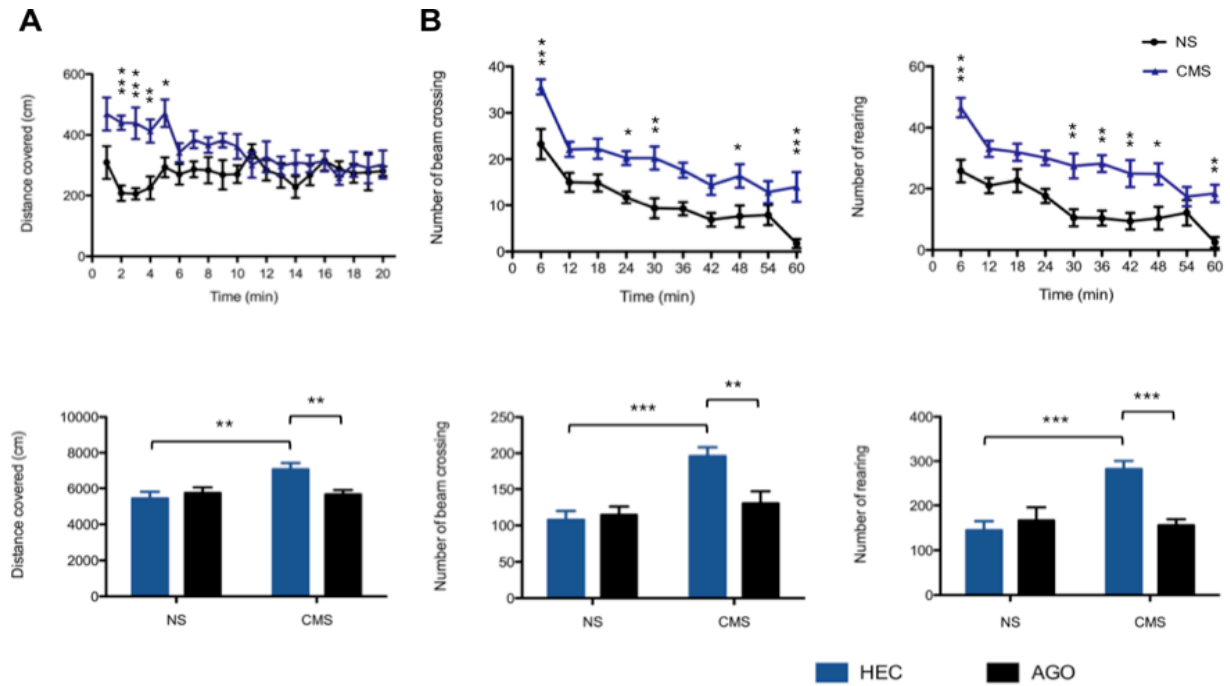


Figure 1. Effects of chronic agomelatine treatment on stress-induced locomotor dysfunction. (A) Mice were subjected to an open field test, and the distance covered in the arena was measured over the time (top panel). On the bottom panel, the total distance over a 20-minute period is represented. The two-way analysis of variance (ANOVA) indicated significant effect of Stress [$F(1,28)=5.78$; $p<0.05$], but no effect of Treatment [$F(1,28)=2.95$; $p>0.05$]. However, there was a significant interaction between the Stress and the Treatment factors [$F(1,28)=6.89$; $p<0.05$], and the Bonferroni *post hoc* test revealed a significant increase of the distance covered in stressed mice ($p<0.01$), which could be prevented by agomelatine ($p<0.01$). (B) Mice were placed in actimetry boxes and the numbers of crossings in the center of the apparatus, as well as the numbers of rearings were measured with an infrared device over one hour (top panel). On the bottom panel, the total numbers of crossings and total numbers of rearings over a one-hour period are indicated. The two-way ANOVA indicated significant effect of Stress on crossing activity [$F(1,28)=15.21$; $p<0.01$], and rearing activity [$F(1,28)=9.12$; $p<0.001$]. A significant interaction between the Stress and the Treatment factors on crossing [$F(1,28)=7.30$; $p<0.05$] and rearing behaviors [$F(1,28)=12.43$; $p<0.01$] was observed. Bonferroni *post hoc* test revealed a significant increase of the crossings and rearings in stressed mice ($p<0.001$), which could be prevented by agomelatine ($p<0.01$ and $p<0.001$ respectively). Data are expressed as mean \pm S.E.M of $n=8$ mice per group. $**p < 0.01$ CMS HEC vs CMS AGO; $***p < 0.001$ CMS HEC vs CMS AGO.

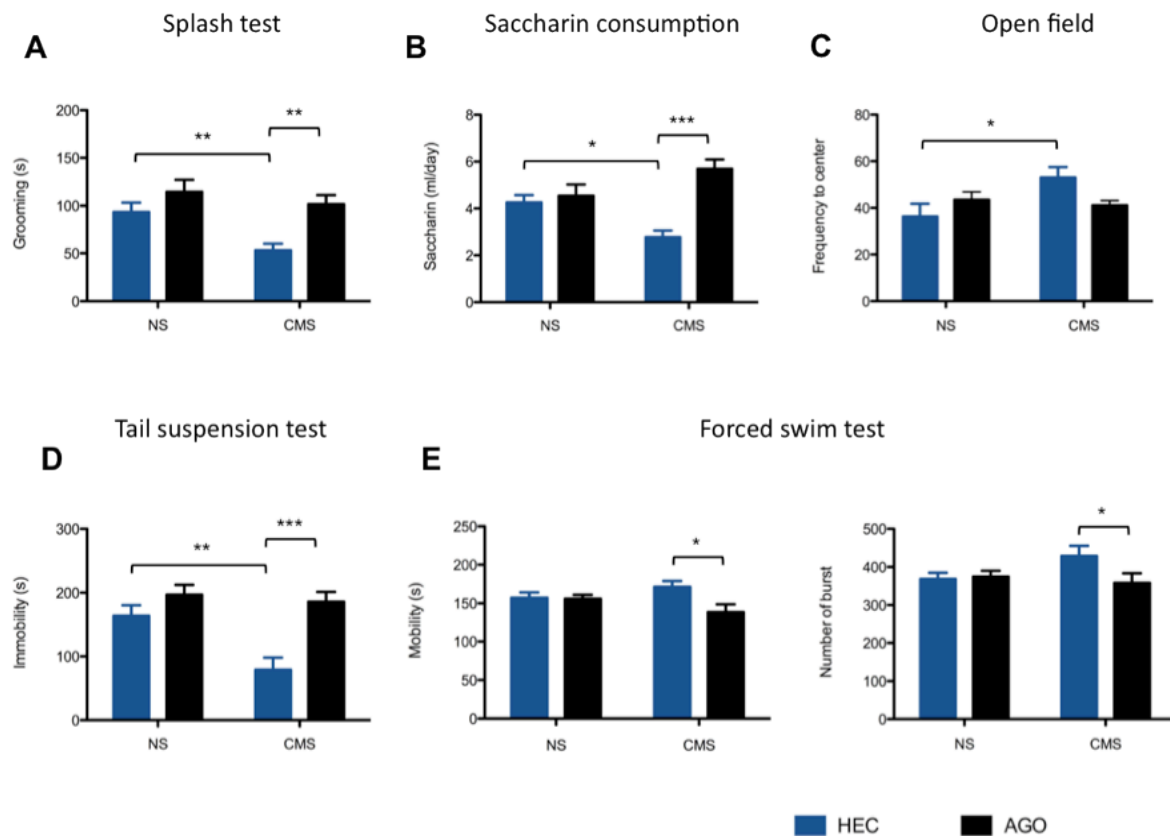


Figure 2. Effects of chronic agomelatine treatment on stress-induced affective behaviors. (A) The time spent in grooming behavior over a total period of 5 minutes was measured in mice. The two-way ANOVA indicated significant effect of Stress [$F(1,70)=13.9$; $p<0.01$] as well as Treatment [$F(1,70)=7.62$; $p<0.05$] on grooming behavior. Bonferroni *post hoc* test revealed a significant decreased in the self-grooming of stressed mice ($p<0.01$), which could be prevented by agomelatine ($p<0.01$). (B) The saccharin consumption was measured for an overnight period. There was a significant interaction between the Stress and the Treatment factors [$F(1,39)=8.29$; $p<0.01$], and Bonferroni *post hoc* test revealed a significant decreased in the saccharin intake of stressed mice ($p<0.05$), which could be prevented by agomelatine ($p<0.001$). (C) Mice were subjected to an open field test, and the number of visit to the center of the arena was measured over a 20-minute period. There was a significant interaction between the Stress and the Treatment [$F(1,26)=5.94$; $p<0.05$], and Bonferroni *post hoc* test revealed a significant decreased in the number of entries in the center of the arena in stressed mice ($p<0.05$). (D) The immobility time in the tail suspension test paradigm was measured after chronic mild stress. The two-way ANOVA indicated significant effect of Stress [$F(1,30)=7.69$; $p<0.01$] as well as Treatment [$F(1,30)=16.59$; $p<0.001$] on immobility time. There was a significant interaction between the Stress and the Treatment [$F(1,30)=4.55$; $p<0.05$], and Bonferroni *post hoc* test revealed a significant decreased in the immobility time of stressed mice ($p<0.01$), which could be prevented by agomelatine ($p<0.001$). (E) Mice were subjected to the forced swim test, and the time spent in swimming activity (mobility) and the struggling activity (number of bursts), over a 6-minute period, are represented. There was a significant interaction between the Stress and the Treatment factors [$F(1,26)=4.25$; $p<0.05$] for the mobility time, and the Bonferroni *post hoc* test revealed a significant decreased in the mobility time of stressed mice treated with vehicle as compared to stressed mice treated with agomelatine ($p<0.05$). A tendency towards an interaction for the number of burst [$F(1,26)=3.12$; $p=0.089$] was observed, and a planned pairwise comparison using Dunn's t-test indicated a significant decreased in the number of bursts of stressed mice treated with vehicle as compared to stressed mice treated with agomelatine ($p<0.05$). Data are expressed as mean \pm S.E.M of $n=7-12$ mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

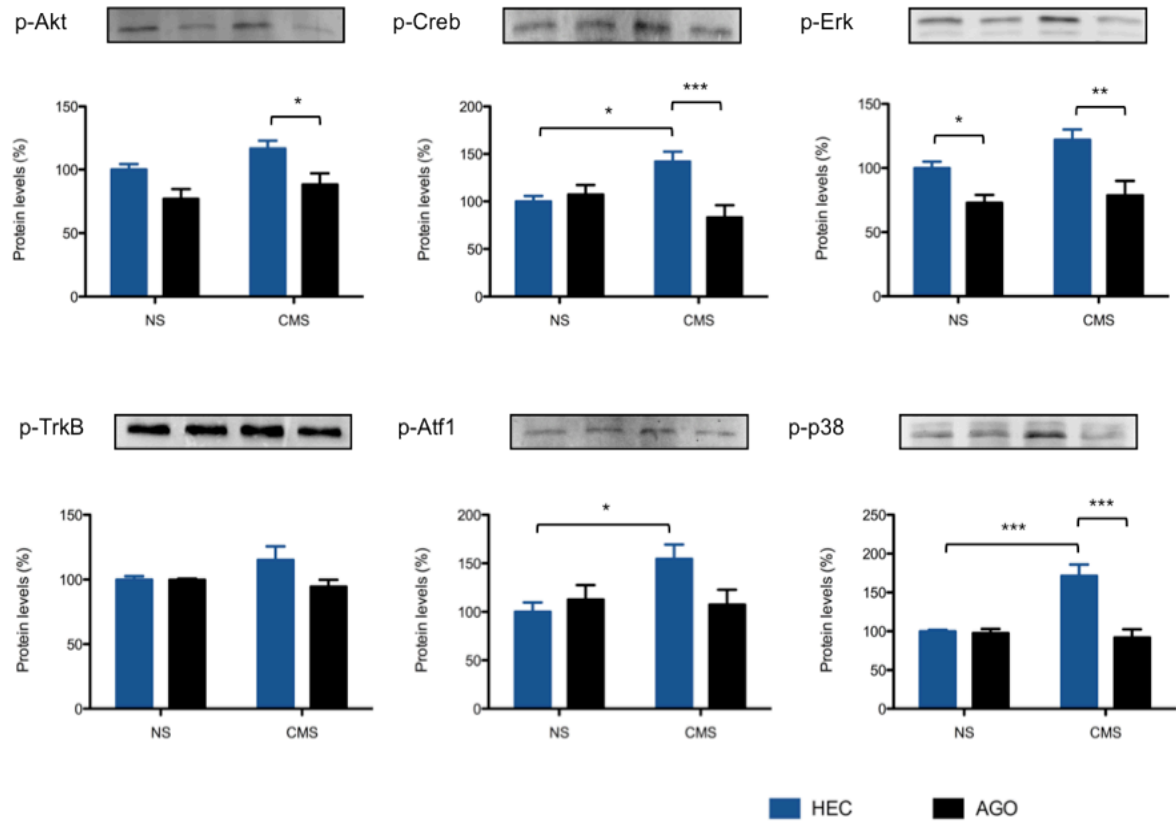


Figure 3. Effects of chronic agomelatine treatment on intracellular signaling in the hippocampus of stressed mice. Western blot analysis of phospho-Akt, phospho-Creb, phospho-Erk, phospho-TrkB, phospho-Atf1 and phospho-p38 were performed in the hippocampus of mice, and quantifications of optical density were monitored with ImageJ. A two-way ANOVA revealed a significant interaction between Stress and Treatment factors for p-Creb [F(1,28)=10.63; $p < 0.01$], p-Atf1 [F(1,11)=5.19; $p < 0.05$] and p-p38 [F(1,12)=16.22; $p < 0.001$]. There was a significant effect of Treatment on p-Akt [F(1,26)=13.72; $p < 0.001$], p-Creb [F(1,28)=6.49; $p < 0.05$], p-Erk [F(1,14)=23.82; $p < 0.001$] and p-p38 [F(1,12)=18.35; $p < 0.01$], and effect of Stress on p-p38 [F(1,12)=11.77; $p < 0.01$]. Of note, a trend for a Stress effect was observed for p-Akt [F(1,26)=4.09; $p = 0.053$], p-Erk [F(1,14)=4.55; $p = 0.051$] and p-Atf1 [F(1,11)=3.55; $p = 0.086$]. Bonferroni *post hoc* analyses revealed a significant increased between non-stressed mice treated (NS HEC) with vehicle and stressed mice treated with vehicle (CMS HEC) for p-Creb ($p < 0.05$), p-Atf1 ($p < 0.05$) and p-p38 ($p < 0.001$). Agomelatine treatment (50 mg/kg/day) could prevent the phosphorylation of Creb ($p < 0.001$) and p38 ($p < 0.001$) induced by stress (CMS HEC v.s. CMS AGO). In addition, agomelatine could decrease p-Akt ($p < 0.05$) and p-Erk ($p < 0.05$) in stressed mice (CMS HEC v.s. CMS AGO). Data are represented as percentage of non-stressed mice treated with vehicle (NS HEC), and each bar represent the mean + S.E.M of (n=4-8 mice). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

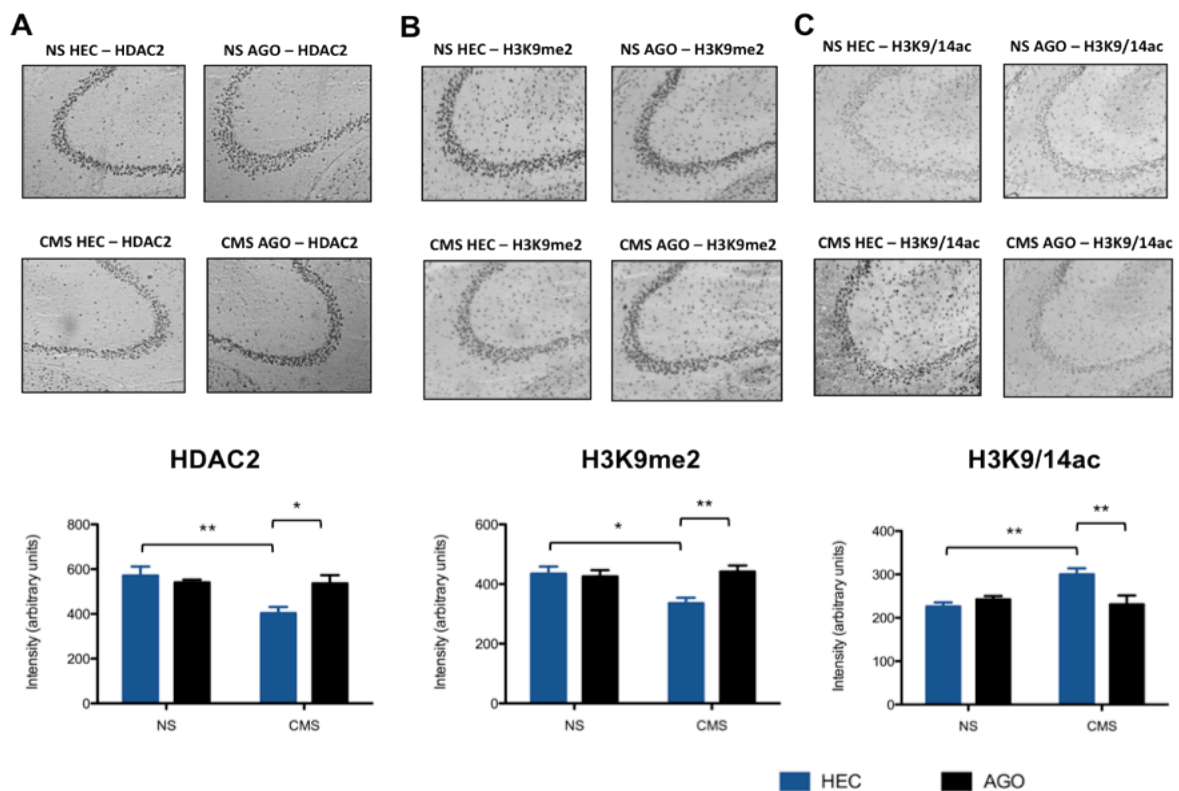


Figure 4. Effects of chronic agomelatine treatment on epigenetic remodeling in the hippocampus of stressed mice Representative photomicrographs of HDAC2 (A), H3K9me (B) and H3K9ac (C) immunolabelings in the CA3 subfield of the hippocampus (top panel) are shown, as well as the quantification of optical density (bottom panel). (A) The two-way ANOVA analysis indicated a significant effect of Stress [$F(1,16)=5.76$; $p<0.05$], but no effect of Treatment [$F(1,16)=2.02$; $p>0.05$] for the repressive mark HDAC2. There was a significant interaction between the Stress and the Treatment [$F(1,16)=5.17$; $p<0.05$], and the Bonferroni *post hoc* test revealed a significant decreased HDAC2 in the CA3 subfield ($p<0.01$), which could be prevented by agomelatine ($p<0.05$). (B) The two-way ANOVA analysis indicated a significant effect of Treatment [$F(1,17)=4.75$; $p<0.05$], but no effect of Stress [$F(1,17)=3.46$; $p>0.05$] for the repressive mark H3K9me. There was a significant interaction between the Stress and the Treatment [$F(1,17)=6.83$; $p<0.05$], and the Bonferroni *post hoc* test revealed a significant decreased H3K9me in the CA3 subfield ($p<0.05$), which could be prevented by agomelatine ($p<0.01$). (C) The two-way ANOVA analysis indicated a significant effect of Stress [$F(1,16)=5.25$; $p<0.05$] and a tendency for an effect of Treatment [$F(1,16)=3.71$; $p=0.072$] for the repressive mark H3K9ac. There was a significant interaction between the Stress and the Treatment [$F(1,16)=9.86$; $p<0.01$], and the Bonferroni *post hoc* test revealed a significant decreased H3K9ac in the CA3 subfield ($p<0.01$), which could be prevented by agomelatine ($p<0.01$). Quantifications of immunolabelings are expressed as arbitrary units and each bar represent the mean + S.E.M. of $n=5$ (NS HEC), $n=6$ (NS AGO), $n=4$ (CMS HEC) and $n=6$ (CMS AGO) mice. * $p < 0.05$; ** $p < 0.01$.

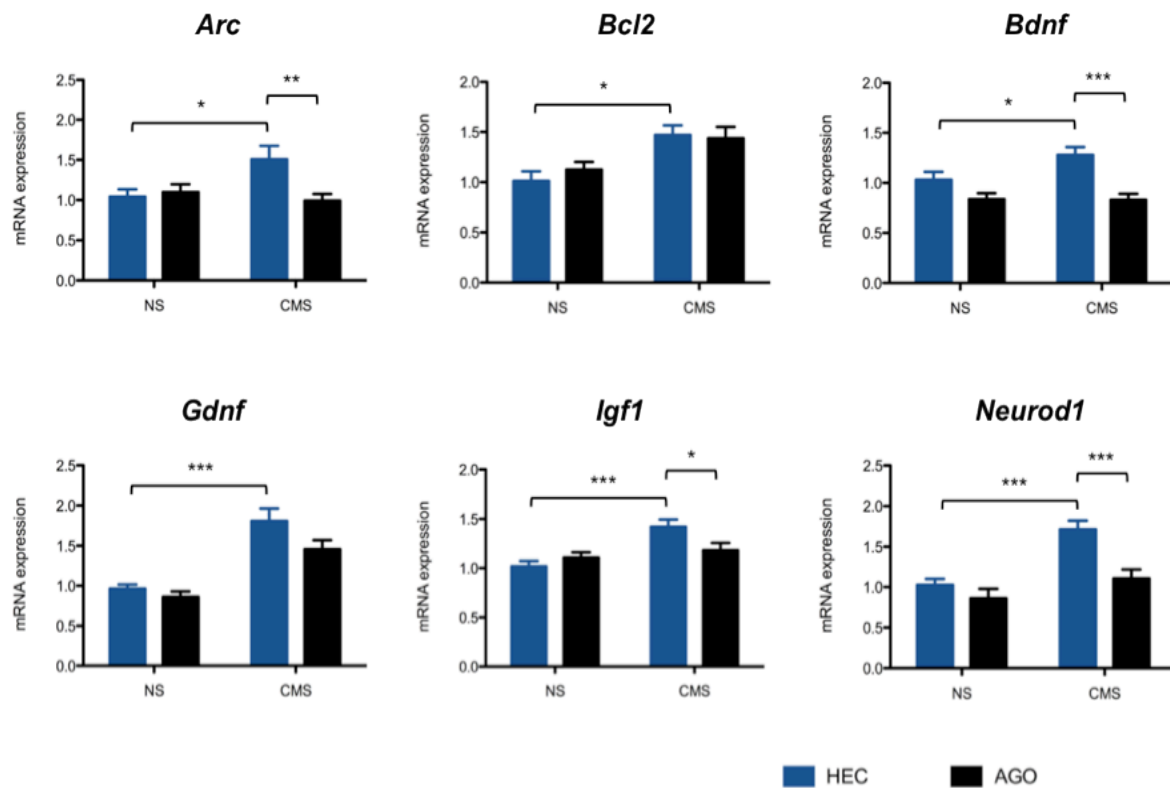


Figure 5. Effects of chronic agomelatine treatment on plasticity-related gene expression in the hippocampus of stressed mice. mRNA levels of plasticity-related genes in the hippocampus of mice were measured by quantitative real-time RT-PCR, and are expressed as fold changes as compared to the non-stressed mice treated with hydroxyethylcellulose (NS HEC). The two-way ANOVA indicated a significant interaction between Stress and Treatment factors for *Arc* [F(1,29)=7.05; p<0.05], *Igf1* [F(1,34)=6.15; p<0.05] and *Neurod1* [F(1,32)=4.80; p<0.05]. In addition, there was also an effect of Stress on *Bcl2* [F(1,26)=12.47; p<0.01], *Gdnf* [F(1,25)=56.71; p<0.001], *Igf1* [F(1,34)=13.30; p<0.001] and *Neurod1* [F(1,32)=14.41; p<0.001], and a Treatment effect on *Arc* [F(1,29)=4.50; p<0.05], *Bdnf* [F(1,31)=17.16; p<0.001], *Gdnf* [F(1,25)=5.57; p<0.05] and *Neurod1* [F(1,32)=21.05; p<0.001]. The Bonferroni *post hoc* tests indicated a significant increase of *Arc* (p<0.05), *Bcl2* (p<0.05), *Bdnf* (p<0.05), *Gdnf* (p<0.001), *Igf1* (p<0.001) and *Neurod1* (p<0.001), between non-stressed mice treated with vehicle (NS HEC) and stressed mice treated with vehicle (CMS HEC). Similarly, there was a significant difference between stressed mice treated with vehicle (CMS HEC) versus stressed mice treated with agomelatine (CMS AGO) for *Arc* (p<0.01), *Bdnf* (p<0.001), *Igf1* (p<0.05) and *Neurod1* (p<0.001). Each bar represent the mean + S.E.M of n=7-12 mice. *p < 0.05; **p < 0.01; ***p < 0.001.

Supplementary methods.

Saccharin consumption test

At the beginning of the test, plastic pipettes filled with tap water were proposed to the mice so as to measure the daily consumption of water using such kind of apparatus. Then, after habituation, two pipettes filled either with water or a 10% saccharin solution were assigned to every cage and the mice were able to freely choose between the two solutions. The pipettes were presented to the animals for an overnight period of 18 hours, after which they were removed from the cages.

Tail suspension test

The apparatus consists of three suspension units divided by walls (ID-Tech-Bioseb, Chaville, France). The mice were suspended by the tail, using an adhesive tape, and the duration of immobility was measured automatically by the software over a 6-minute period.

Splash test

The splash test, previously described by Surget et al., 2008, consists into spraying a 10% sucrose solution on the back of mice in their home cage. Because of its viscosity, the sucrose solution dirties the coat and induces grooming behavior. The total time of grooming over a 5-minute period was recorded in our experiments.

Open field test

The apparatus consisted of four open boxes of 40 x 40 cm separated by white wood walls and equipped with an infrared floor for the measurement of motor activity. The arena was subdivided into a central and a peripheral zone. Mice were placed in the open field boxes for 20 min under normal light conditions, and the motor activity of the animals were automatically scored with a camera connected to a computerized system (Viewpoint, Lyon, France). The time spent in the different zones and the distance moved were scored.

Forced swim test

In the forced swimming test (FST), originally designed by Porsolt and colleagues (1977), mice were placed into plastic buckets (19 cm diameter, 23 cm deep, filled with 23°C–25°C water) under low light conditions and videotaped for 6 min (Viewpoint, Lyon, France). The different outcome measures were “immobility” reflecting no movement at all and/or minor movements necessary to keep the nose above the water; “mobility” reflecting medium activity such as swimming; and “bursts” reflecting strong mobility and struggling behaviors.

Rotarod test

Motor coordination and balance were assessed with the rotarod test using an accelerating rotarod (Accelerating Rotarod, UGO Basile, Comerio, Italy). Mice were placed on rotating drums (3 cm diameter), and the time that each animal maintained its balance on the rod was measured. The speed of the rotarod accelerated from 4–40 rpm over a 5-min period.

Spontaneous locomotor activity

Locomotor activity was measured using a computer-based photobeam apparatus. Actimeter box (area: $300 \times 150 \text{ mm}^2$; height 180 mm; with plexiglass wall and grid floor) detected mouse movements by means of two infrared light beams. Mice were placed in the testing apparatus for 1 hour. The ambulations corresponding to the number of squares crossed in periphery and centre during the observation period, and the rearings corresponding to the number of times the mouse stands on its hind feet (and leaves the floor with the fore feet) were measured.

Elevated plus maze

The elevated plus maze (EPM), as described previously by Narboux-Neme and colleagues (2011), consisted of a plus-shaped apparatus with two open and two closed arms. The apparatus was made from dark plastic material that was transparent for infrared light, and the walls of the closed compartment were covered with opaque scotch tape. For the test, the mouse was placed into one of the closed arm facing a closed part of the apparatus, and the movements of the animal were scored automatically under dark conditions with a computerized system using an infrared video camera (Viewpoint, Lyon, France). Percentage of time spent in the open and closed parts of the maze, numbers of entries to the open and closed arms, and total distance traveled were investigated.

References:

Surget, A., Saxe, M., Leman, S., Ibarguen-Vargas, Y., Chalon, S., Griebel, G., Hen, R. and Belzung, C. (2008) Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. *Biol Psychiatry* 64, 293-301.

Porsolt, R. D., Le Pichon, M. and Jalfre, M. (1977) Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266, 730-732.

	Morning	Afternoon	Night
Monday		30° cage-tilt (1h)	Difficult access to food
Tuesday	Confinement (1h)	Paired housing (2h)	Overnight illumination
Wednesday	30° cage-tilt (1h)	Confinement (2h)	30° cage-tilt (1h)
Thursday	Paired housing (2h)	Confinement (1h)	Soiled cage
Friday	Confinement (1h)	30° cage-tilt (2h)	Reverse light/dark cycle
Week-end	Reverse light/dark cycle		

Table S1. Procedures used for the chronic mild stress paradigm. The stressors consisted into repeated periods of 30° cage tilt, confinement to small cages (11 x 8 x 8 cm), two 2 hour periods of paired housing, one overnight period of difficult access to food (without modifying the daily food ration), one period of continuous overnight illumination, and one overnight period in a soiled cage (50 ml of water/l of sawdust bedding). Animals were also placed on a reversed light/dark cycle from Friday evening to Monday morning. These stressors were scheduled over a 1-week period and repeated throughout a total period of 3 weeks.

Symbol	Sense	Anti-sense
Arc	TCATTCAGTATGTGGTGGGC	GTCCTGCACTTCCATACCC
Akt1	TATTGGCTACAAGGAACGGC	ATAAAGGTGTTGGGCCTTGG
Bdnf	AAAACCATAAGGACGCGGAC	TAGACATGTTTGCGGCATCC
Creb1	GTTGTTATGGCGTCCTCCC	TACGACATTCTCTTGCTGCC
Crf	CCTGAATTTCTTGCAGCCG	GGACTTCTGTTGAGATTCCCC
Mapk3	TACGATCCGACAGATGAGCC	GAAGATCAACTCCTTCAGCCG
Gdnf	TGGGTTTGGGCTATGAAACC	AACATGCCTGGCCTACTTTG
Glur1	TGTGACACCATGAAAGTGGG	AAAACCGCTAGGTTTACGGG
Igf1	GACTGGAGATGTAAGTGTCC	GTACTTCCTTCTGAGTCTTGGG
NeuroD1	TGAAGCCATGAATGCAGAGG	TTTCTTTTTGGGACCCCGTC
Vgf	TTGACACCCTTATCCAAGGC	ACCTGAGCTAATCCTTGCTG
cfos	AGGGAACGGAATAAGATGGC	TGCAACGCAGACTTCTCATC
Egr1	TCAATCCTCAAGGGGAGCC	ACTCGTCTCCACCATCGC
Bcl2	ATGACTGAGTACCTGAACCG	ATGCTGGGGCCATATAGTTC

Table S2. Primers used for SybrGreen protocols. One part of the amplification was made with Absolute SYBR Green ROX Mix (Thermo Scientific, Illkirch, France) using the 7300 Real Time PCR System (Applied Biosystems, Courtaboeuf, France) and the sequences of primer sets are indicated. Gene expressions were normalized using Hprt as a reference gene and the conditions for the reaction included 35 cycles in a fixed sequence of 95°C for 20 s, 62°C for 30 s, and 72°C for 30 s.

Symbol	Reference
Bmal1	Mm00500226_m1
Cbp	Mm01342452_m1
Id2	Mm01293217_g1
Nr1d2	Mm00441730_m1
Ncor1	Mm00448681_m1
Rpol1-2	Mm00485813_m1
Dnmt1	Mm01151065_g1
Hdac1	Mm02745760_g1
Hdac2	Mm00515108_m1
Hdac3	Mm00515916_m1
Hdac4	Mm01299565_m1
Hdac5	Mm00515941_g1
Hdac9	Mm00458454_m1
Mbd1	Mm01262472_g1
Mecp2	Mm01193537_g1
Myst2	Mm01304323_m1
Gapdh	Mm99999915_g1

Table S3. Primers used for Taqman protocols. The second part of gene expression was measured using TaqMan (Applied Biosystems, Courtaboeuf, France) gene expression assays, and the sequences of primer sets are indicated. Gene expressions were normalized using Gapdh as a reference gene and the conditions for the reaction were set as follow: 50 °C, 2 min; 95 °C, 10 min, and followed by 40 cycles at 95 °C, 15 s; 60 °C, 1 min.

Symbol	Name	Supplier
p-Akt	Phospho-Akt (Ser473) (587F11) 4051	Cell signaling
p-Creb	Phospho-CREB (Ser133) (87G3) 9198	Cell signaling
p-Erk	Monoclonal Anti-MAP Kinase, Activated (Diphosphorylated ERK1&2) M9692	Sigma aldrich
p-p38	Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) 4511	Cell signaling
p-TrkB	Trk B Phospho (pY817) (NTRK2) 2149-1	Epitomics
H3K9me2	Anti-Histone H3 (di methyl K9 [mAbcam 1220] ab1220	Abcam
H3K4me3	Anti-trimethyl-Histone H3 (Lys4) Antibody, clone MC315	Millipore
H4ac	Anti-acetyl-Histone H4 Antibody 06-866	Millipore
H3K9/14ac	H3K9/14ac polyclonal antibody pAb-005-044	Diagenode
HDAC1	Anti-HDAC1 antibody - ChIP Grade ab7028	Abcam
HDAC2	Anti-HDAC2 antibody - ChIP Grade ab7029	Abcam

Table S4. List of antibodies used in the experiments. The names (including the reference) and suppliers for the antibodies used for western blots and immunohistochemistry are indicated.

Symbol	Definition	NS	CMS	% over NS	P value
Arc	Activity-regulated cytoskeleton-associated protein	1.03 ± 0.08	1.59 ± 0.10	56%	*
Akt1	Thymoma viral proto-oncogene 1	1.01 ± 0.04	1.07 ± 0.04	6%	ns
Bcl2	B cell lymphoma 2	1.01 ± 0.09	1.47 ± 0.09	46%	**
Bdnf9	Brain-derived neurotrophic factor exon 9	1.00 ± 0.08	1.28 ± 0.07	28%	*
Creb1	cAMP responsive element binding protein 1	1.01 ± 0.08	1.17 ± 0.07	16%	ns
Crf	Corticotropin releasing factor	1.02 ± 0.07	1.13 ± 0.07	11%	ns
Mapk3	Mitogen-activated protein kinase 3	1.01 ± 0.08	0.87 ± 0.05	-14%	ns
Gdnf	Glia-derived neurotrophic factor	1.01 ± 0.07	1.59 ± 0.15	58%	***
Glur1	Glutamate receptor, ionotropic, AMPA1	1.01 ± 0.10	0.96 ± 0.08	-5%	ns
Gpr88	G-protein coupled receptor 88	0.99 ± 0.17	1.17 ± 0.17	18%	ns
Igf1	Insulin growth factor 1	1.01 ± 0.05	1.42 ± 0.07	41%	***
NeuroD1	Neurogenic differentiation 1	1.02 ± 0.07	1.71 ± 0.10	69%	**
Vgf	Nerve growth factor inducible	1.04 ± 0.10	0.78 ± 0.13	-26%	ns

Table S5. Effect of chronic mild stress on hippocampal plasticity-related gene expression profiles. Diverse genes involved in neuroplasticity have been measured in the hippocampus of mice subjected or not to the chronic mild stress procedure. mRNA levels were quantified by quantitative real-time RT-PCR, and data were normalized to the house keeping gene Actb. Data are expressed as mean ± S.E.M of arbitrary units representing the fold changes as compared to the non-stress (NS) condition. ns: non significant *p < 0.05 vs. NS; **p < 0.01 vs. NS; ***p < 0.001 vs. NS. Student's t test.

Symbol	Definition	NS	CMS	% over NS	P value
Bmal1	Aryl hydrocarbon receptor nuclear translocator-like	1.00 ± 0.04	0.91 ± 0.04	-9%	ns
cfos	FBJ osteosarcoma oncogene	1.05 ± 0.18	0.63 ± 0.11	-42%	*
Cbp	Creb binding protein	1.00 ± 0.02	1.01 ± 0.04	1%	ns
Egr1	Early growth response 1	1.00 ± 0.08	1.06 ± 0.14	6%	ns
Id2	Inhibitor of DNA binding 2	1.00 ± 0.05	0.89 ± 0.05	-11%	ns
Nr1d2	Nuclear receptor subfamily 1, group D, member 2	1.00 ± 0.01	1.40 ± 0.05	40%	***
Ncor1	Nuclear receptor co-repressor 1	1.00 ± 0.05	0.63 ± 0.06	-37%	**
Rpol1-2	RNA polymerase 1	1.00 ± 0.03	0.96 ± 0.12	-4%	ns

Table S6. Effect of chronic mild stress on hippocampal transcription regulators. Diverse genes involved in the control of transcription have been measured in the hippocampus of mice subjected or not to the chronic mild stress procedure. mRNA levels were quantified by quantitative real-time RT-PCR, and data were normalized to the house keeping gene Actb. Data are expressed as mean ± S.E.M of arbitrary units representing the fold changes as compared to the non stress (NS) condition. ns: non significant *p < 0.05 vs. NS; **p < 0.01 vs. NS; ***p < 0.001 vs. NS. Student's t test.

Symbol	Definition	NS	CMS	% over NS	P value
Dnmt1	DNA methyltransferase 1	1.00 ± 0.02	0.99 ± 0.05	-1%	ns
Hdac1	Histone deacetylase 1	1.00 ± 0.02	1.01 ± 0.07	1%	ns
Hdac2	Histone deacetylase 2	1.00 ± 0.03	0.78 ± 0.04	-22%	**
Hdac3	Histone deacetylase 3	1.00 ± 0.03	1.01 ± 0.03	1%	ns
Hdac4	Histone deacetylase 4	1.00 ± 0.02	1.06 ± 0.10	6%	ns
Hdac5	Histone deacetylase 5	1.01 ± 0.04	1.03 ± 0.06	2%	ns
Hdac9	Histone deacetylase 9	1.00 ± 0.02	1.04 ± 0.06	4%	ns
Mbd1	Methyl binding domain 1	1.01 ± 0.06	1.00 ± 0.04	-1%	ns
Mecp2	Methyl CpG binding protein 2	1.00 ± 0.03	0.95 ± 0.02	-5%	ns
Myst2	MYST histone acetyltransferase 2	1.00 ± 0.03	0.99 ± 0.02	-1%	ns

Table S7. Effect of chronic mild stress on hippocampal expression of epigenetic regulators. Diverse genes involved in epigenetic repression or activation have been measured in the hippocampus of mice subjected or not to the chronic mild stress procedure. mRNA levels were quantified by quantitative real-time RT-PCR, and data were normalized to the house keeping gene Actb. Data are expressed as mean ± S.E.M of arbitrary units representing the fold changes as compared to the non stress (NS) condition. ns: non significant *p < 0.05 vs. NS; **p < 0.01 vs. NS; ***p < 0.001 vs. NS. Student's t test.

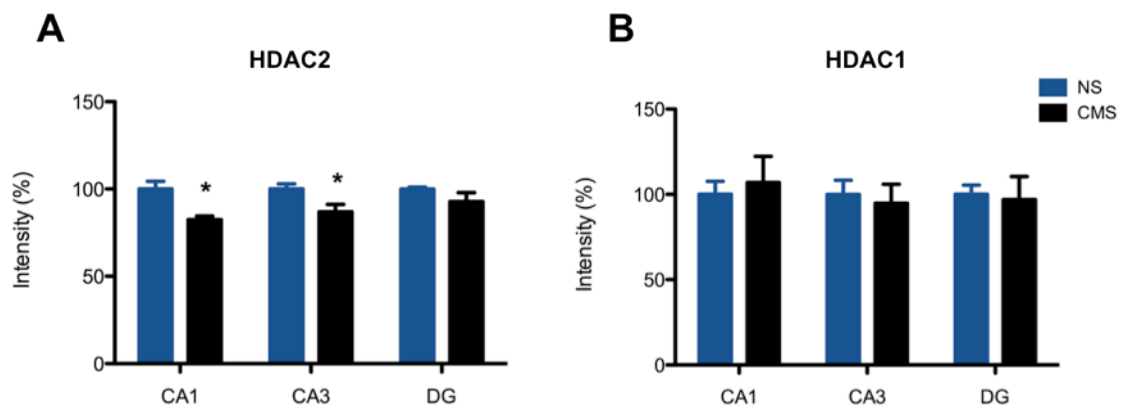


Figure S1. Effect of chronic mild stress on HDAC1 and HDAC2 expression in the hippocampus. The levels of HDAC2 (A) and HDAC 1 (B) in the hippocampus were measured by immunostaining, and the quantifications of optical density of the different hippocampal subfields are represented. Data are expressed as percentage of the non-stress (NS) condition and each bar represent the mean + S.E.M of n=5 (NS) and n=6 (CMS) mice. *p < 0.05 vs. NS. Student's t test.

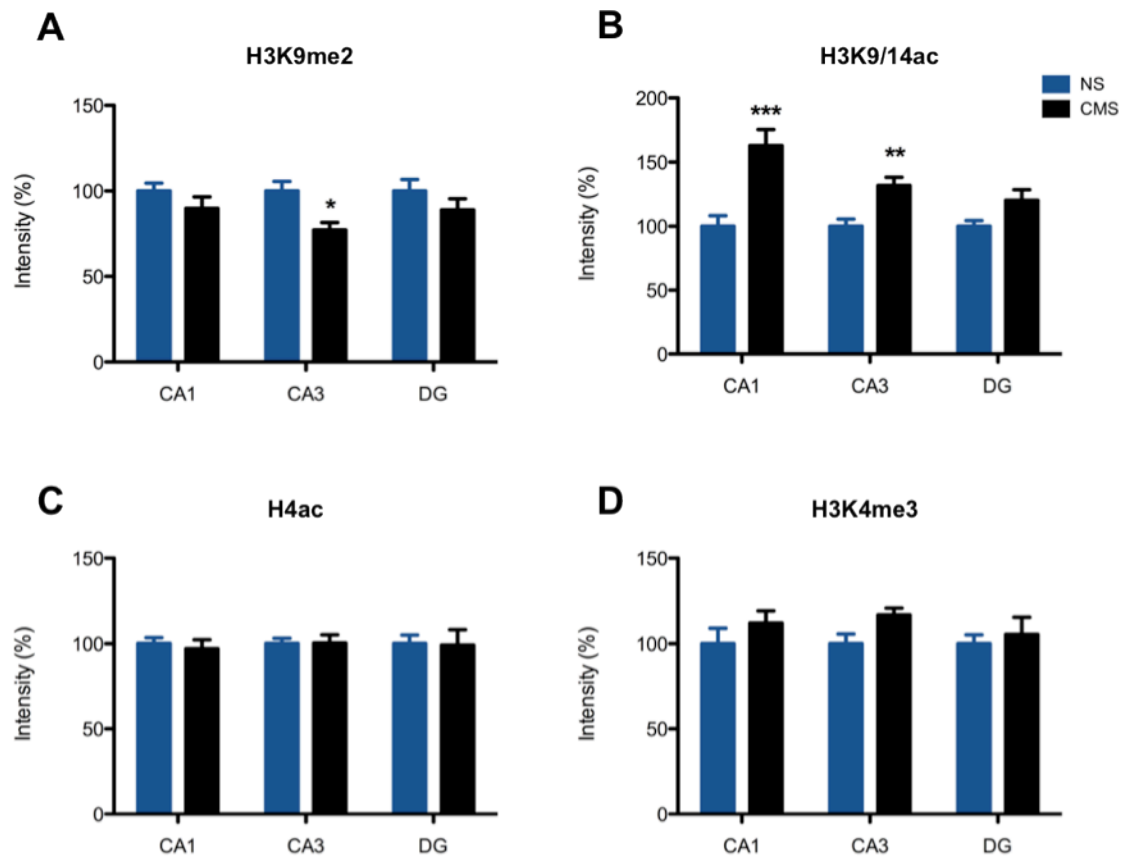


Figure S2. Effect of chronic mild stress on histone post-translational modifications in the hippocampus. The levels of H3K9me2 (A), H3K9/14ac (B), H4ac (C) and H3K4me3 (D) were measured by immunolabeling in the hippocampus, and the quantifications of optical density of the different hippocampal subfields are represented. (B) Data are expressed as percentage of the non-stress (NS) condition and each bar represent the mean + S.E.M of n=5 (NS) and n=6 (CMS) mice. *p < 0.05 vs. NS; **p < 0.01 vs. NS; ***p < 0.001 vs. NS. Student's t test.

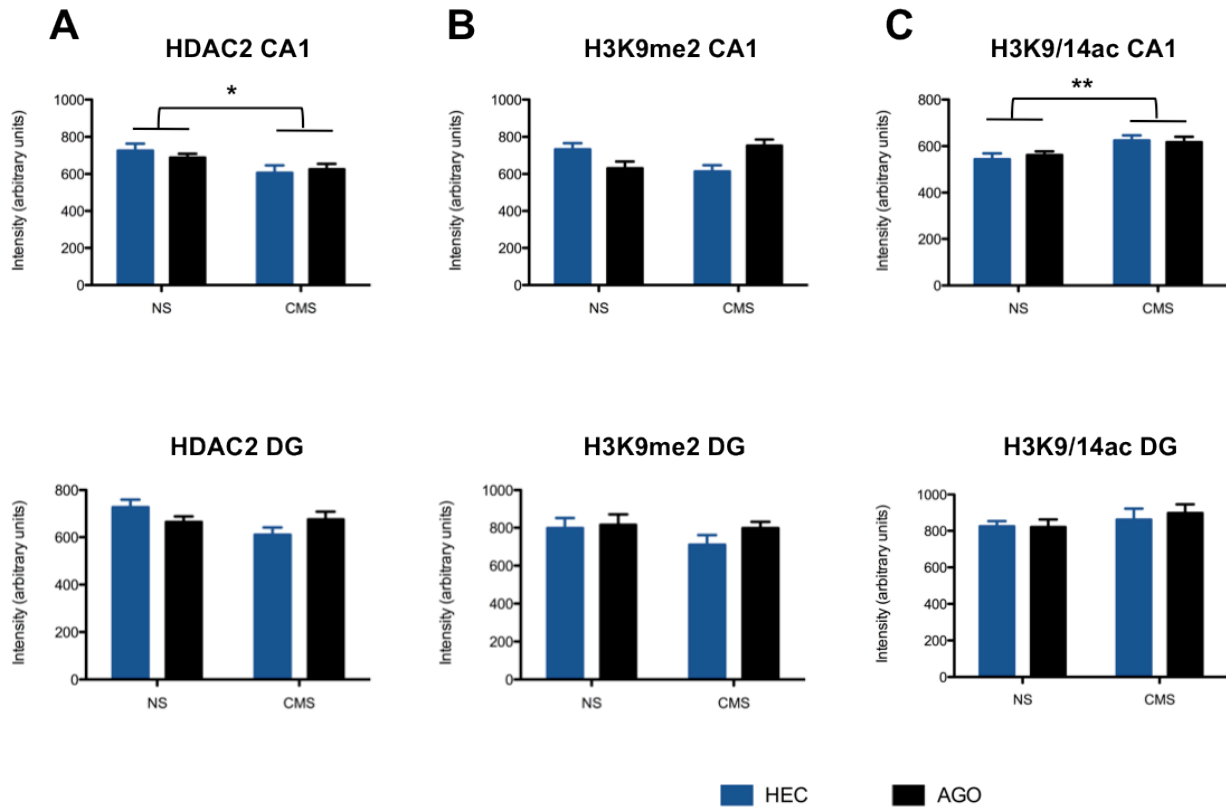


Figure S3. Effect of agomelatine on stress-induced epigenetic changes in the hippocampus. The levels of HDAC2 (A), H3K9me2 (B) and H3K9/14ac (C) were measured by immunolabeling in the hippocampus, and the quantifications of optical density of the CA1 (top panel) and the DG (bottom panel) are represented. The two-way ANOVA analysis indicated a significant effect of Stress on HDAC2 [$F(1,16)=7.03$; $p<0.05$], H3K9/14ac [$F(1,16)=8.89$; $p<0.01$], but not on H3K9me2 in the CA1 region. Further, the two-way ANOVA analysis indicated no significant effect of Stress on HDAC, H3K9me2 and H3K9/14ac in the DG. Quantifications of immunolabelings are expressed as arbitrary units and each bar represent the mean + S.E.M of $n=5$ (NS HEC), $n=6$ (NS AGO), $n=4$ (CMS HEC) and $n=6$ (CMS AGO) mice. * $p < 0.05$ CMS HEC vs CMS AGO; ** $p < 0.01$ CMS HEC vs CMS AGO.

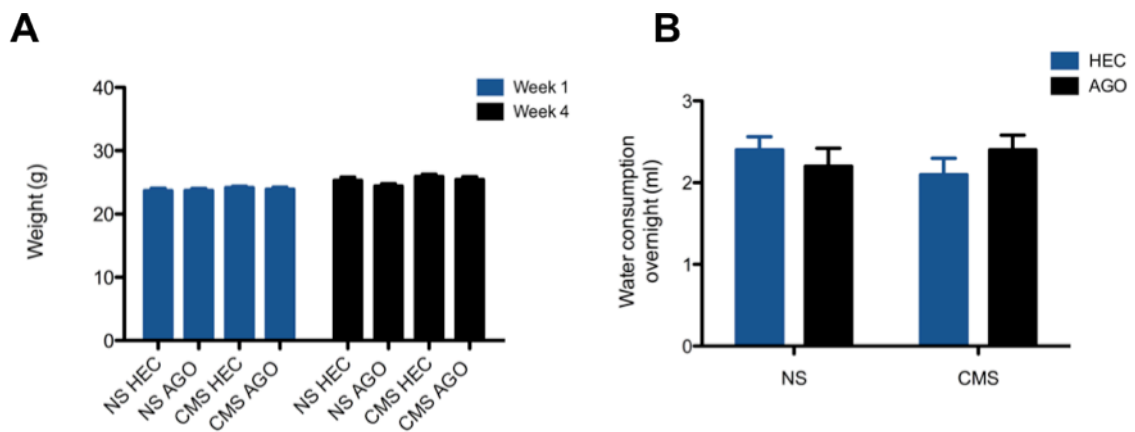


Figure S4. Water consumptions and weights. (A) The weight of mice was measure before and after the chronic mild stress procedure. (B) The consummation of water over the night is represented. Each bar represents the mean + S.E.M of n=7-8 animals per group. There were no significant modulations of the weights and water consumptions by stress or agomelatine treatment.

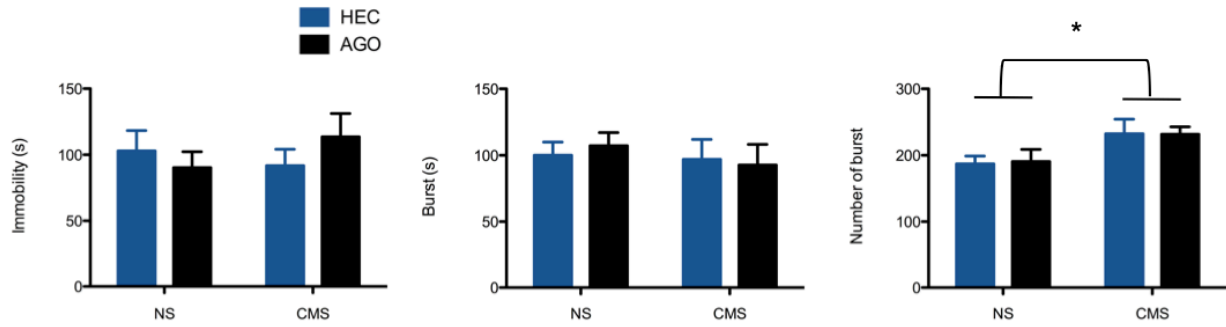


Figure S5. Effects of stress and agomelatine treatment in the FST. Mice were subjected to the forced swim test, and the time spent in immobility (right panel) and in burst activity (center panel) over a 6-minute period is represented. The numbers of bursts during the first 2 minutes is also represented (left panel). There was no significant modulation of the immobility and burst time by stress or agomelatine treatment. A two-way ANOVA indicated a significant effect of Stress factor on the numbers of bursts during the first 2 minutes [$F(1,28)=6.84$; $p<0.05$]. Data are expressed as mean + S.E.M of $n=7-8$ mice per group. * $p < 0.05$.

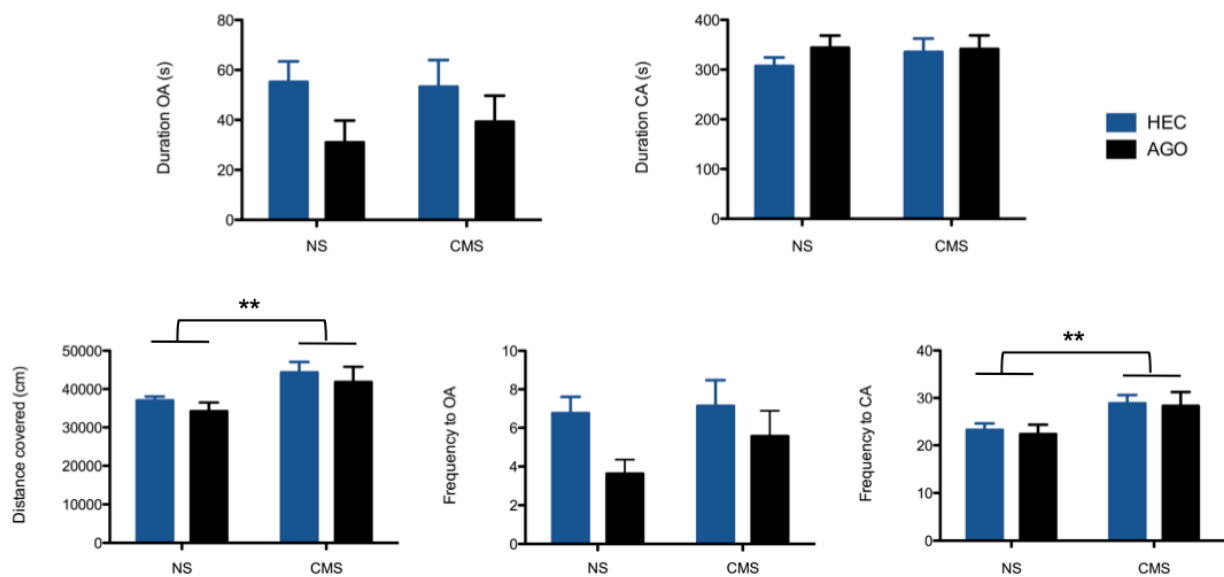


Figure S6. Effects of stress and agomelatine treatment in the EPM. Mice were subjected to the elevated plus maze test for 10 minutes. The two-way ANOVA indicated no significant difference of Stress or Treatment factors on the time spent in open arms or closed arms. However, there was a significant effect of Stress on the distance covered [$F(1,27)=7.85$; $p<0.01$] and the frequency to closed arms [$F(1,27)=8.03$; $p<0.01$]. In addition, the two-way ANOVA indicated a significant effect of Treatment on the frequency to open arms [$F(1,27)=4.68$; $p<0.05$]. Data are expressed as mean + S.E.M of $n=7-8$ mice per group. $**p < 0.01$.

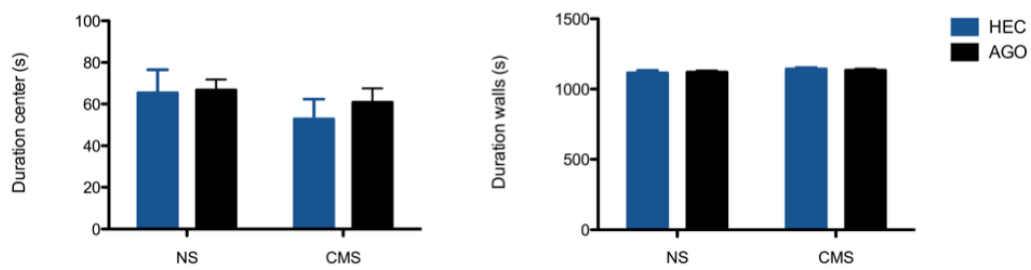


Figure S7. Effects of stress and agomelatine treatment in the OF. Mice were subjected to the open field test, and the time spent in the periphery (right panel) and the time spent in the center (left panel) of the arena over a 20-minute period are represented. Data are expressed as mean + S.E.M of n=7-8 mice per group. There was no significant modulation of the time spent in the center or periphery by stress or agomelatine treatment.

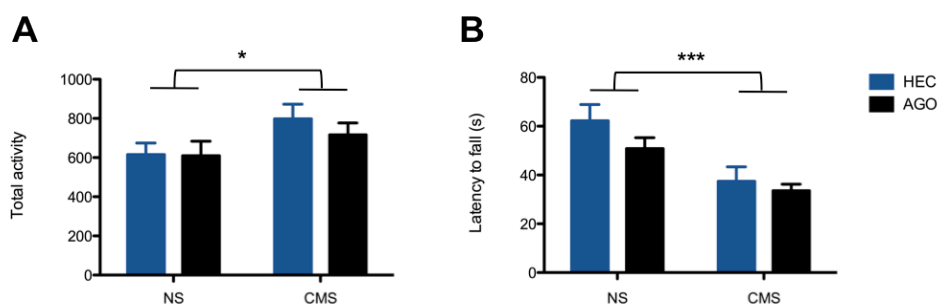


Figure S8. Effects of stress and agomelatine treatment on locomotor activity and movement coordination. (A) Mice were placed in actimetry boxes and the total locomotor activity during a 1-hour period was measured. Chronic mild stress significantly increased the locomotor activity ($p=...$). (B) Mice were placed in a accelerating rotarod apparatus, and the latency to fall was measured. Chronic mild stress significantly altered the movement balance and coordination, which might be due to an increased distractibility ($p=...$). Data are expressed as mean + S.E.M of $n=7-8$ mice per group.

Symptoms of mixed manic state	Chronic mild stress in C57Bl6 mice
Disrupted circadian rhythms	Inversion of light/dark cycles in stress procedures
Increased risk taking	Reduced anxiety in the OF
Psychomotor agitation / hyperactivity	Increased locomotion and rearing
Emotional arousal and vigilance	Increased reactivity in the FST and TST
Impulsivity	Facilitation of choice in the decision making task
Self neglect	Decreased self grooming
Distractibility / decreased concentration	Inability to keep focussed in the rotarod test
Reward deficit	Impaired motor learning
High levels of energy	Decrease saccharin intake / anhedonia
	Increased tonic agitation or bursts in the FST

Table S8. Comparison between clinical symptom of mania and behavioral response in mice exposed to chronic mild stress.

CHAPTER IX

General Discussion

Fabien BOULLE

Mood disorders are among the major health problems worldwide due to the high prevalence and recurrence in the general population, and the significant burden for individual life quality and the repercussion on healthcare systems and society (WHO, 2001). Up to date, the etiology and biological mechanisms underlying mood disorders are still poorly understood. Mounting evidences suggest that a complex interaction between genes and the environment account for the development and course of major depression, i.e. one of the most prevalent affective disorders (Keers et al., 2012). Accordingly, complex epigenetic regulations - consisting of key mechanisms by which environmental factors induce enduring changes in gene expression without altering the DNA code - have been suspected to play a pivotal role in the pathophysiology of depression (Tsankova et al., 2007). More specifically, epigenetic repression of the gene encoding for brain-derived neurotrophic factor (BDNF) - a small-secreted growth factor implicated in brain development and neuronal plasticity - may have a preponderant role in the onset of depression and other mood disorders (Boulle et al., 2012). In this context, the work presented in this thesis aimed at exploring the role of BDNF signaling and its downstream epigenetic regulation in the pathophysiology and treatment of mood disorders.

I – BDNF/TrkB signaling in the pathophysiology of mood disorders

Exon-specific regulation of the BDNF gene: BDNF has a complex gene structure, consisting of several untranslated 5' exons with independent promoters that can be connected to a 3' coding exon to form a bipartite or tripartite transcript yielding different splice variants of BDNF mRNA (Pruunsild et al., 2007). In **Chapter 6**, we investigated the expression of BDNF exon IV and exon IX in the hippocampus of rats exposed to fluoxetine during development in a model of maternal adversity. Male offspring exposed to prenatal stress showed a decrease in BDNF IV mRNA levels, while no changes were observed at BDNF IX mRNA expression. Among prenatally stressed female offspring, an increase in BDNF IV mRNA levels was observed, while expression of BDNF IX mRNA was unchanged. Maternal fluoxetine exposure on the other hand decreased BDNF IV mRNA levels in the hippocampus of female offspring, while no changes were observed at the BDNF IX exon, i.e. the coding exon. These observations are rather provocative since the BDNF IV transcript corresponds to an alternative splice variant that is not representative of the total BDNF protein level. Similarly, in **Chapter 7**, we showed that BDNF IV mRNA levels were decreased in the hippocampus of GR-i mice, further supporting the exon-specific involvement of BDNF in the stress response regulation and in mediating depressive-like behaviour. Accordingly, it has been suggested that the use of distinct BDNF mRNA splice variants differing either by the 5' or 3' extremity allows for temporal and spatial regulation of BDNF expression, which appears to be critical in the modulation of synaptic plasticity and spine development in dendrites (Baj et al., 2011). BDNF exon IV contains specific binding sites for the glucocorticoid receptor, making it a target of choice for stress-related events (Suri et al., 2013). In addition, BDNF exon IV contains specific binding sites for the cyclic

AMP response element binding protein (CREB) and methyl CpG binding protein 2 (MeCP2), making it a preferential epigenetic target (Dias et al., 2003; Martinowich et al., 2003). Accordingly, this exon has been shown to display a ubiquitous repartition in the brain and seems to play a key role in many processes related to mood, emotion, reward, learning and memory (see **Chapter 2**). Evidently, the exact role of the various BDNF transcripts and the complex regulation of BDNF mRNA and protein processing await further research.

Regulation of BDNF/TrkB signaling by stress and glucocorticoids: Stress has been proposed as a major risk factor in the development of affective disorders and the persistent elevation of glucocorticoids induced by chronic stress can significantly alter BDNF production and impact on structural and functional plasticity in the hippocampus (Berton et al., 2006; Suri et al., 2013). As such, in **Chapters 6, 7 and 8**, we investigated the hippocampal regulation of the BDNF gene in two different rodent models of stress, as well as in a transgenic mouse model displaying impaired glucocorticoid receptor (GR) signaling. Interestingly, the expression of BDNF exon IV mRNA was decreased in the hippocampus of GR-impaired (GR-i) mice. Previous experiments performed by our research group showed that GR-i mice displayed a deficit in the negative feedback regulation of the hypothalamo-pituitary-adrenal (HPA) axis under stressful conditions as well as in depressive-like behavior (Froger et al., 2004; Paizanis et al., 2010). Hence, the decrease in BDNF and downstream neuroplastic effectors in the hippocampus of this transgenic mouse model supports the notion that HPA axis impairment may play a part in the pathophysiology of major depression, and that the neuronal loss and synaptic dysfunction induced by prolonged stress exposure might involve alterations in neurotrophic signaling. In line with this hypothesis, we found that exposure to early-life (i.e. prenatal restraint) stress in Sprague-Dawley rats reduced the expression of BDNF mRNA in the hippocampus of adult male offspring, and was associated with increased depressive-like behavior (**Chapter 6**). However, the opposite effect on BDNF IV mRNA levels was observed in the hippocampus of female offspring prenatally exposed to maternal stress. This latter observation indicates a sex-specific regulation of the response and adaptation to stress, potentially leading to differential expression of the BDNF gene. In humans, a clear sex difference exists in the incidence and onset of stress-related diseases (Wang et al., 2007). In contrast, rodent models of psychiatric disorders, because of the estrus cycle and concomitant fluctuation in sex hormones in females, are based principally on male animals. However, recent studies have showed that sex hormones, especially estrogen, could induce the expression of BDNF (Scharfman et al., 2006; Solum et al., 2002). Clearly, the link between glucocorticoids, sex hormones and neurotrophic signaling should be addressed more thoroughly.

Further, exposure to repeated socio-environmental stressors of low intensity in adult C57Bl6J mice resulted in an increased BDNF IX mRNA expression and related plasticity pathways, in the hippocampus. Using this procedure, our research group previously reported a decrease hippocampal expression of GR mRNA in stressed mice (Froger et al., 2004). Endogenous decrease or pharmacological inactivation of

GR was shown to correlate with enhanced synaptic plasticity (Avital et al., 2006; Krugers et al., 2006). In addition, the mineralocorticoid receptors (MR), which bind glucocorticoids with high affinity, are most densely localized in hippocampal neurons (De Kloet ER, 2000). MRs are activated at low doses of glucocorticoids and have been associated with enhanced cognitive function and hippocampal plasticity (Avital et al., 2006; Sousa et al., 2008). Hence, the levels of glucocorticoids, as well as the balance between MRs and GRs, might play a critical role in the molecular, neuroendocrine and behavioral determination of the stress response. Of note, at the transcriptional level, GRs can affect BDNF transcription either by binding to GR binding elements (GREs) present in the promoter areas and therefore inhibit transcription (Zanchi et al., 2010), or by interfering with the transcription factors that normally activate BDNF transcription (Kassel et al., 2007).

Altogether, the data presented in **Chapters 6, 7 and 8** indicates that a clear link exists between stress, glucocorticoids and BDNF signaling, and suggest that the nature of the stressor (e.g. timing, duration, intensity), as well as genetic factors (e.g. GR polymorphism), are critically important in the determination of neuroplastic outcomes. This observation might explain, at least in part, the well-reported heterogeneity in stress responsivity and related pathologies.

Matching BDNF expression and neurocircuits to behavior: Among several growth factors, BDNF has been postulated as a key mediator of the synaptic dysfunction and neuronal atrophy often observed in the prefrontal cortex and hippocampus of mood disorder patients (Duman et al., 2012). A decrease of BDNF and its tropomyosin-related kinase receptor (TrkB) were observed in the hippocampus of suicide completers and depressed patients (Autry et al., 2012; Dwivedi et al., 2003). In **Chapters 6 and 7**, we showed that the decrease of BDNF mRNA levels was associated with increased depressive-like behavior. According, male offspring that were subjected to prenatal stress exhibited decreased expression of the BDNF exon IV, concomitant with decreased swimming behavior in the forced swim test (FST). Similarly, female offspring that were exposed to maternal fluoxetine showed decreased expression of the BDNF exon IV and increased immobility in the FST. In GR-i mice, the decreased expression of the BDNF exon IV was associated with increased immobility in the tail suspension test (TST), decreased self-grooming, and increased fear and anxiety. Altogether, these findings suggest that BDNF/TrkB signaling and its associated plasticity are important in the regulation of mood.

Generally, impaired BDNF levels and plasticity (i.e. neurogenesis) in the hippocampus of rodents have been associated with increased depressive-like behavior (Castren et al., 2010; Jha et al., 2011; Mirescu et al., 2006). However, depression is a very complex disease and is difficult to model in rodents. Since animals might not be able to express the complete symptomatology of the disease, a rational approach should consist into matching symptom-related endophenotypes to hypothetically malfunctioning brain circuits. The hippocampus is a major component of the limbic system known to be highly vulnerable to

stress and environmental variations, and is critically implicated in learning, memory and mood (see **Chapter 3**). However, in our experiments, stress or genetic variation may affect BDNF levels in various regions, and the observed changes in the hippocampus may not be solely attributed to the broad range of behavioral responses. Interestingly, approaches using region-specific knockdown or local infusion showed that BDNF-mediated plasticity in the hippocampus could be mostly associated with cognitive performance, fear memories and anxiety (Heldt et al., 2007; Quesseveur et al., 2013; Taliáz et al., 2010). Hence, it is possible that the observed decreased BDNF levels in the hippocampus are implicated predominantly in anxiety and in the cognitive symptoms of depression. In the hippocampus, BDNF/TrkB are abundantly expressed on neurons receiving serotonergic and glutamatergic afferences (Lessmann V, 1998; Martinowich et al., 2008). Accordingly, the neuroplastic effects of BDNF on i.e. dendritic arborization, axonal growth, neuronal survival and neurotransmitter release might critically affect the development and functioning of specific neurotransmitter systems, which would explain at least in part some of the behavioral performances observed in our models.

In contrast, the findings presented in **Chapters 7** bring some novel insights into the region-specific regulation of BDNF/TrkB signaling in the pathophysiology of depression. We confirmed that the chronic blockade of BDNF/TrkB signaling by peripheral administration of Ana12, a recently developed TrkB inhibitor, could result in antidepressant-like effects in GR-i mice. These findings are rather provocative because a growing body of literature indicated similar effects after administration of BDNF directly in the hippocampus, or peripheral administration of TrkB agonists (Liu et al., 2010; Shirayama et al., 2002). However, as recently described by Cazorla and colleagues, Ana12 diffused preferentially in the striatum to inhibit TrkB (Cazorla et al., 2011). Interestingly, clean-cut evidence from both rodent and human studies demonstrated that stress and/or major depression is associated with increased BDNF levels in the mesolimbic dopaminergic system i.e. the nucleus accumbens (Krishnan et al., 2007). The ventral striatum, including the nucleus accumbens, plays a major part in reward, pleasure, interest, impulsivity and fatigue/energy. Accordingly, BDNF-mediated plasticity in the nucleus accumbens might be implicated in the hedonic symptoms of depression (Martinowich et al., 2008; Krishnan et al., 2007).

Finally, in **Chapter 8**, we found that chronic exposure to low-intensity stress could be associated with hyperactivity. In this study, BDNF IX mRNA levels as well as neuroplasticity were increased in the hippocampus of mildly stressed mice. The relationship between hyperactivity and the hippocampus is not fully understood yet, but some evidence indicates that physical activity and exercise could significantly enhance hippocampal neurogenesis and plasticity (Van Praag et al., 1999; Fabel et al., 2008). In addition, a recent study provided evidence on the involvement of BDNF/TrkB signaling in enkephalinergic striatopallidal neurons in the control of locomotor behavior (Besusso et al., 2013).

Altogether, our data point to a complex region-specific regulation of BDNF/TrkB signaling in the pathophysiology of depression. BDNF/TrkB signaling is critically important in regulating plasticity in cortico-limbic structures. Hence, dysregulation of this neuroplastic signaling may impact on neuronal connectivity and neurotransmission in diverse brain regions, leading to a broad range of behavioral disturbances.

II – BDNF/TrkB signaling in the mechanism of action of antidepressants

Putative mechanism of the adverse effects of fluoxetine during early development: Fluoxetine, an antidepressant belonging to the class of SSRIs, is widely used in the clinic for the treatment of mood disorders during pregnancy and the post-partum period (Cooper et al., 2007). There is growing evidence that the use of this antidepressant during development has negative consequence on brain development and adult psychopathology (Pawluski et al., 2009; Laine et al., 2003; Oberlander et al., 2009). In adulthood, it is well established that fluoxetine increases BDNF levels (including BDNF IV mRNA levels) in cortico-limbic structures to induce antidepressive and anxiolytic effects in rodents (Martinowich et al., 2007). However, in **Chapter 6**, we found that early-life exposure to fluoxetine led to decreased levels of BDNF IV mRNA in male and female offspring in adulthood. These observations are rather interesting, and suggest that the molecular mechanisms involved in early-life versus e.g. adulthood intervention with fluoxetine are distinct. Serotonin plays an important role in the development of the brain modulating neuronal differentiation, axon guidance or dendritic pruning (Gaspar et al., 2003; Homberg et al., 2010). The serotonergic system is complex, and involves at least fifteen identified receptors associated with different intracellular transduction cascades (Gaspar et al., 2003). Hence, the pattern of expression of serotonin receptor subtypes might be critical during development, and in the determination of fluoxetine effects. In addition, during postnatal development, certain populations of neurons express a transient serotonergic phenotype that no longer exists in adulthood, and the modulation of this partial serotonergic system might interfere with the fine-tuning process of neuronal networks (Cases et al., 1998; Gaspar et al., 2003). Moreover, fluoxetine was shown to interact with several targets including serotonin receptors (2A, 2C and 2B) and ion channels (Mostert et al., 2008), in addition to the serotonin transporter (5-HTT). The differential patterns of expression of these systems in early-life might also be determinant in the long-term biological action of developmental fluoxetine exposure.

Hence, future research should investigate the precise mechanisms of action of fluoxetine treatment during development in order to conclude whether the observed changes at BDNF signaling in adulthood are mediated via a direct effect of serotonin and its downstream signaling in developing neurons, via additional target systems of fluoxetine or via other compensatory mechanisms occurring later in life.

Mechanism of action of agomelatine in the treatment of mood disorders: As mentioned above, conventional antidepressants, via the modulation of monoaminergic signaling, are known to enhance

BDNF expression and neuroplasticity in cortico-limbic structures to exert their therapeutic effects (Castren et al., 2010). However, little is known about the neuroplastic mechanisms of action of the new generation antidepressant agomelatine. This atypical antidepressant acts as a mixed melatonin receptor 1 and 2 agonist and serotonin receptor 2C antagonist (De Bodinat et al., 2010). In **Chapters 7 and 8**, we particularly focused on the molecular and neurotrophic actions of agomelatine in two mouse models of affective disorders. In GR-i mice, the decreased BDNF IV mRNA levels and deficit in cell proliferation in the hippocampus could be reversed by chronic agomelatine treatment. In addition, the marked decrease of Bcl2 and cFos mRNA levels in GR-i mice could be restored by chronic agomelatine treatment. All this neurochemical changes correlated with the reversal of depressive- and anxiety- like behavior in GR-i mice. These findings support the notion that antidepressant medications, including agomelatine, require BDNF/TrkB signaling to rescue the impairment of hippocampal plasticity and exert their therapeutic effects. However, in our model of chronic mild stress, where BDNF IX mRNA levels and neuroplasticity were increased, we found that chronic administration of agomelatine could restore a normal hippocampal function by decreasing the levels of BDNF and associated signaling pathways. Of note, agomelatine did not produce such effects in naïve animals. This finding is of particular interest and indicates that agomelatine, rather than directly targeting the BDNF/TrkB system, acts as a normalizing drug, thereby restoring/rebalancing gene expression specifically in pathological conditions. Accordingly, agomelatine is thought to exert its effects via a re-synchronization of circadian rhythms (De Bodinat et al., 2010). Disruption of circadian periodicity, as well as seasonal changes, have been proposed as a major player in the onset of various mood disturbances (i.e. bipolar disorders, mania), and successful antidepressant treatments have been associated with normalization of disturbed biological rhythms (Harvey AG, 2008; Lanfumey et al., 2013). Moreover, in our model of chronic mild stress, which appears to mimic common symptoms usually observed in the mixed manic state of bipolar disorder, and shows neurobiological changes observed in mouse models of mania (including increased hippocampal BDNF levels), agomelatine was efficient in rescuing both the neurochemical and behavioral alterations. Hence, we carefully suggest that the modulation of melatonin receptors, possibly with additional blockade of serotonin 2c receptors, may provide a specific and innovative way to treat bipolar disorders.

III – Epigenetic regulation of BDNF/TrkB signaling in mood disorders

BDNF/TrkB signaling and chromatin remodeling in neuronal plasticity: The interaction of BDNF with its receptor TrkB plays a preponderant role in the induction and maintenance of neuronal plasticity (Chao MV, 2003). The action of BDNF on neuronal architecture and networks is tightly regulated by epigenetic re-arrangements at the chromatin level (Karpova N, 2013). Accordingly, BDNF has a complex gene structure that has been reported to be highly sensitive to environmental variations and concomitant epigenetic modifications (Tsankova et al., 2007) underlying changes in expression of downstream target genes. In **Chapter 5**, we explored the role of epigenetic regulation downstream of BDNF/TrkB signaling

using a pharmacological approach. We showed that chronic blockade of the BDNF receptor TrkB downregulates plasticity-related gene expression concomitant with epigenetic changes (e.g. post-translational modifications at histone tails and MeCP2 binding) in cultured neuronal cells. These findings strongly suggest that TrkB signaling can underlie epigenetic remodeling at plasticity-related genes, and that its selective blockade is able to induce histone modifications in order to significantly alter neuronal plasticity. One might also suspect that the downregulation of BDNF/TrkB signaling observed in cortico-limbic structures after severe or chronic stress might critically impact on the epigenetic program of neuronal cells, leading to reduced plasticity and thereby increasing the vulnerability towards mood disorders.

Early-life events, brain development and epigenetic imprints: The modulation of gene expression by epigenetic mechanisms is essential during development, where a “cellular memory” is created enabling an appropriate pattern of gene expression and a specific cellular phenotype (Probst et al., 2009). Hence, early-life events, behavioral experience or genetic variations can influence epigenetic remodeling, and modulate neuronal architecture and networks during development (Roth et al., 2011). In **Chapter 6**, we showed that early-life exposure to maternal fluoxetine is associated with persistent epigenetic changes, e.g. H3K27 tri-methylation at the BDNF promoter IV, concomitant with decreased expression at the corresponding BDNF mRNA, in the hippocampus of adult female offspring prenatally exposed to stress. Of note, this effect was observed only in female offspring prenatally exposed to stress, indicating that maternal adversity increased the vulnerability for fluoxetine-induced epigenetic reprogramming in the hippocampus in a sex-dependent manner. In **Chapter 7**, we explored epigenetic processes in the hippocampus of the GR-i transgenic mice, in which a transgene affecting the GR expression is present throughout the lifespan. We found a marked alteration in the machinery controlling histone post-translational modifications and DNA methylation, including e.g. decreased expression of the DNA methyltransferase 1 (DNMT1) gene, the DNA methyltransferase 3a (DNMT3a) gene, the histone-lysine N-methyltransferase HRX 3 (MLL3) gene and the histone-lysine N-methyltransferase HRX 4 (MLL4) gene. In addition, GR-i mice showed deficits in neurogenesis and decreased BDNF-related signaling in the hippocampus in adulthood. Glucocorticoid-related signaling is critical during brain development, by remodeling axon and dendrite networks as well as modulating neuronal maturation and survival (Lupien et al., 2009). Hence, it is tempting to speculate that changes in the machinery controlling epigenetic regulation during development are responsible for an epigenetic reprogramming associated with the observed permanent deficit in functioning of the adult hippocampus. Altogether, the data presented in **Chapter 6** and **7** provide further evidence that early-life events, including exposure to drugs or stressful situation, are critical for brain development, and can have negative consequences involving persistent epigenetic imprints up to adulthood, that most likely increase the susceptibility for mood disorders.

Stress and epigenetic regulation in adulthood: Chromatin remodeling via histone modifications occurs in response to diverse environmental exposures, among which, stress insults have been most extensively described (Mifsud et al., 2011). At the molecular level, the sustained elevation of glucocorticoids observed in stressful situations might interfere with various intracellular signaling and transcriptional processes, and prolonged exposure to stress in adulthood have been shown to interact with the epigenome (Maze et al., 2013). In **Chapter 8**, we report that chronic exposure to a low-intensity socio-environmental stress could impact on chromatin regulation. We found that stress was associated with robust changes in epigenetic regulation and plasticity-related gene transcription. Chronic stress was associated with decreased HDAC2 and H3K27me3 levels in the hippocampus, while H3K9/14ac was increased in this same region. HDAC2 is a critical enzyme implicated in histone regulation, and low levels of this protein in the hippocampus have previously been shown to strongly correlate with enhanced synaptic plasticity (Guan et al., 2009). Further, in our model, there was also a robust increase in plasticity-related intracellular signaling pathways such as the mitogen-activated protein kinase (MAPK) cascade, after chronic exposure to stress. Hence, it remains speculative to conclude whether the changes in histone modifications correspond to the nuclear integration of the activated intracellular signaling cascades in response to stress, or whether stress directly affects histone-modifying enzymes, thereby inducing aberrant neuroplastic signaling. However, modifications at histone tails are considered as dynamic regulatory processes involved in gene transcription. Importantly, the alteration in the machinery controlling chromatin regulation and histone modifications, involving e.g. histone acetylating and methylating enzymes, might maintain stable patterns of gene expression and a persistent aberrant phenotype.

In conclusion, it remains difficult to determine whether stress-induced histone modifications in adulthood represent a cause or a consequence of the disease progression, but it appears to be well established that such kind of chromatin regulations forms a core are part of the neurobiological alterations observed in adult animal models of stress and related mood disorders. In this context, genetic association studies investigating the role of functional single nucleotide polymorphisms (SNPs) of genes involved in epigenetic regulations in mood disorder patients should be addressed.

IV – Perspectives for the development of novel antidepressant drugs

Targeting BDNF/TrkB signaling to modulate epigenetic-mediated neuroplasticity: As described above, BDNF/TrkB signaling plays a critical role in neuronal plasticity, and appears to be required for the therapeutic action of antidepressants (Castren et al., 2010). However, the use of BDNF as a therapeutic treatment encounters many limitations, BDNF has a poor blood brain barrier penetration, low oral bioavailability and short half-life in plasma (Poduslo et al., 1996). In addition, the interaction of BDNF with pro-apoptotic p75 receptor might result in undesirable effects (Zhang et al., 2008). As such, the

development of specific small-molecule TrkB ligands has recently emerged, and so far, only a limited amount of candidates have been identified and characterized (see **Chapter 3**). Peripheral administration of small synthetic TrkB agonists could induce neurogenesis and antidepressant-like properties in rodents (Liu et al., 2010). Nevertheless, we found that Ana12, a small non-peptidic, TrkB inhibitor could induce antidepressant effect in GR-i mice. These data are in accordance with a hypothetic region-specific regulation of BDNF in the pathophysiology of depression, and provide novel insights into the therapeutic role of TrkB inhibitors in mood disorders. However, the use of TrkB antagonists as a therapeutic treatment for mood disorders should be regarded with caution, since we also observed signs of increased anxiety after chronic treatment with Ana12 (**Chapter 7**).

Nevertheless, modulating the expression of specific BDNF exons in a targeted fashion may represent a promising strategy to restore enduring changes in gene expression induced by e.g. repeated environmental insults. In view of the region-specific distribution of BDNF transcripts in the brain and their functionally distinct roles, developing compounds that modulate the transcription of specific BDNF exons will provide considerable advantages and possibly avoid undesirable side effects. In this context, it was recently shown that a neutralizing inhibitor of miR-206, a small non-coding RNA regulating the expression of BDNF, could target specific BDNF transcripts to prevent detrimental effects of amyloid beta in a mouse model of Alzheimer disease (Lee et al., 2012). This finding should spur researchers to further investigate the possible role of such experimental strategies e.g. RNA interference therapy in the treatment of mood-related disorders.

Since aberrant BDNF signaling might play a critical role in the pathophysiology and/or treatment of mood disorders, directly targeting epigenetic modifications at the BDNF gene promoters represents another promising strategy to enduringly reverse abnormal BDNF expression and engender long-lasting clinical benefits. Interestingly, in **Chapters 5** we show that cyclotraxin-B, a specific TrkB inhibitor, induces a lasting downregulation of the expression of a subset of genes involved in neuroplasticity pathways, concomitant with post-translational modifications at histone tails as well as altered MeCP2 binding at those genes. Hence, our findings provide further insight into the neuroplastic and epigenetic effects of TrkB modulators, and point out that the ability of TrkB antagonists to reverse abnormal BDNF-mediated epigenetic remodeling in the dopaminergic system could bear therapeutic value.

Bcl2 as a promising therapeutic target for mood disorders: In the research work presented in this thesis, we studied BDNF/TrkB signaling and its downstream signaling pathways in various models, and using different approaches. It is interesting to note that, among all the TrkB target genes examined, the b-cell lymphoma 2 (Bcl2) gene was most consistently affected in our models. Accordingly, in **Chapter 7**, we found that GR impairment was associated with a robust decrease of Bcl2 mRNA levels in the hippocampus, and that this deficit was restored by chronic antidepressant treatment. Further, we found

that the Bcl2 gene expression was decreased by pharmacological intervention aiming at blocking the TrkB receptor (**Chapter 5** and **7**). In addition, the chronic downregulation of BDNF/TrkB signaling was associated with lasting epigenetic remodeling at the Bcl2 gene promoter (**Chapter 5**). Bcl2 has been described as an important regulator of cell survival (Davies, 1995), and antidepressants and mood stabilizers have been shown to enhance the expression of Bcl2 in rodents (Hunsberger et al., 2009; Kosten et al., 2007). Hence, compounds that specifically increase Bcl2 levels in cortico-limbic areas might be a therapeutic strategy for the treatment of mood disorders.

Chromatin-modifying drugs in the treatment of mood disorders: As mentioned in the previous sections, our findings on epigenetic dysregulations in the hippocampus support the notion that chromatin remodeling via histone modifications can increase the susceptibility for mood disorders. In **Chapter 7** and **8**, we provide further insight into the epigenetic potential of antidepressants. We showed that the altered HDAC2 levels, as well as post-translational modifications at histone tails (e.g. H3K27me3 and H3K9/14ac) in adult mice subjected to prolonged exposure to stress can be restored by chronic treatment with agomelatine. The reversal of chromatin-related alterations correlated with behavioral remission indicating that epigenetic processes are required for the therapeutic action of antidepressants. In contrast, chronic agomelatine treatment could not restore the alterations of epigenetic regulation (e.g. DNMT1, DNMT3a, MLL3 and MLL4) in GR-i mice. Although these results are conflicting, other studies have reported that antidepressants require epigenetic regulation to mediate their neurochemical and behavioral effects. The positive effect of chromatin modifying drugs in animal models of mood disorders further support this hypothesis. In particular, HDAC inhibitors and DNMT inhibitors represent potentially powerful agents for the treatment of psychiatric disorders through regulation of plasticity-related gene expression (Sales et al., 2011; Covington et al., 2009). On the other hand, these molecular agents are expected to exert their effects on a substantial proportion of the genome, thereby most likely inducing numerous negative side effects. More specifically, inhibitors of DNA methylation have been suggested to promote cancer metastasis, systemic lupus erythematosus or other autoimmune diseases. Nevertheless, several epigenetic drugs targeting DNA methylation and histone deacetylation enzymes have recently been approved for clinical application in oncology, while some are still being tested in clinical trials (Arrowsmith et al., 2012). Hence, in a first step of research, future clinical studies should investigate whether such drugs exhibit better remission rates on depressed mood in cancer patients suffering from comorbid depression. This would provide a first line of clinical evidence for potential therapeutic effects of epigenetic drugs in the treatment of mood disorders.

IV – Concluding remarks

In the present thesis, we have investigated the involvement of BDNF/TrkB signaling and its downstream epigenetic regulation in the pathophysiology and treatment of mood disorders using different rodent models and approaches. Our findings indicate that epigenetic regulation at BDNF/TrkB signaling is critically important in the establishment and maintenance of neuronal plasticity. Moreover, adverse environmental exposures, especially when occurring during development, are able to induce stable and enduring epigenetic reprogramming involving aberrant BDNF/TrkB signaling and impaired neuroplasticity, thereby increasing the vulnerability to stress and related mood disorders. Interestingly, antidepressants require TrkB to exert some of their neurochemical and behavioral effects. Hence, targeting the BDNF receptor TrkB in order to restore plasticity-related gene expression and associated neuronal functioning appears to be a promising strategy for the treatment of mood disorders.

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SUMMARY

Mood disorders are among the major health problems worldwide due to the high prevalence and recurrence in the general population, and the significant burden for individual life quality and the repercussion on healthcare systems and society. Up to date, the etiology and biological mechanisms underlying mood disorders are still poorly understood. Mounting evidences suggest that a complex interaction between genes and environment might account in the development and course of major depression i.e. one of the most prevalent affective disorders. Accordingly, complex epigenetic regulations - consisting of key mechanisms by which environmental factors induce enduring changes in gene expression without altering the DNA code - have been suspected to plays a pivotal role in the pathophysiology of depression. More specifically, epigenetic repression of the gene encoding for brain-derived neurotrophic factor (BDNF) - a small-secreted growth factor implicated in brain development and neuronal plasticity - may have a preponderant role in the onset of depression and other mood disorders.

In this context, the research presented in this thesis aimed at exploring the role of BDNF signaling and its downstream epigenetic regulations in the pathophysiology and treatment of mood disorders.

Our findings indicate that epigenetic regulation at BDNF/TrkB signaling is critically important in the establishment and maintenance of neuronal plasticity. Moreover, environmental variations, especially when occurring in development, are able to induce stable and enduring epigenetic reprogramming involving aberrant BDNF/TrkB signaling and impaired neuroplasticity, thereby increasing vulnerability to stress and mood disorders. Interestingly, antidepressants require TrkB to exert some of their neurochemical and behavioral effects. Hence, targeting the BDNF receptor TrkB to restore a normal epigenetic regulation and neuronal functioning appears to be a promising strategy for the treatment of mood disorders.

Key words: epigenetic, mood disorders, BDNF, plasticity, antidepressants

RESUME

Les troubles de l'humeur font partie des problèmes de santé majeurs dans le monde, du fait de leur forte incidence et récurrence dans la population générale, de la nuisance pour la qualité de vie des patients ainsi que la répercussion majeure sur les systèmes de santé. A ce jour, l'étiologie ainsi que les mécanismes biologiques sous-jacents les troubles de l'humeur sont encore très mal connus. Un nombre grandissant de preuves suggère qu'une interaction complexe entre les gènes et l'environnement serait à l'origine de la mise en place et évolution des épisodes dépressifs majeurs – un des troubles de l'humeur les plus répandus. Par conséquent, des régulations épigénétiques complexes, qui consistent en des mécanismes clefs par lesquels l'environnement induit des changements persistant sur l'expression des gènes (sans modifier le code génétique), joueraient un rôle prépondérant dans la pathophysiologie de la dépression. De manière plus spécifique, la répression épigénétique du gène codant pour le brain-derived neurotrophic factor (BDNF) – un facteur de croissance impliqué dans la plasticité neuronale et développement du système nerveux central – serait un mécanisme clef dans la mise en place de la dépression et autres troubles de l'humeur.

Dans ce contexte, les travaux de recherche présentés dans cette thèse visent à explorer le rôle des régulations épigénétiques au niveau de la signalisation BDNF/TrkB dans la physiopathologie et traitement des troubles de l'humeur.

Les résultats montrent que les régulations épigénétiques au niveau de la signalisation BDNF/TrkB sont fortement impliquées dans la mise en place et maintenance de la plasticité neuronale. De plus, les variations environnementales, particulièrement au cours du développement, sont capables d'induire une reprogrammation épigénétique stable et persistante au niveau du complexe BDNF/TrkB ainsi qu'une altération de la neuroplasticité, conduisant à une augmentation de la vulnérabilité au stress et troubles de l'humeur. De manière intéressante, la signalisation du récepteur TrkB est nécessaire pour les effets neurobiologiques et comportementaux des antidépresseurs. De ce fait, une approche pharmacologique ciblée sur le complexe BDNF/TrkB et ses régulations épigénétiques sous-jacentes apparaît comme stratégie thérapeutique prometteuse pour le traitement des troubles de l'humeur tel que la dépression.

Mots clefs: épigénétique, troubles de l'humeur, BDNF, plasticité, antidépresseurs

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ABOUT THE AUTHOR

Fabien Boullé was born on November 24th, 1985 in Curepipe (Republic of Mauritius). He attended the Lycee La Bourdonnais in Mauritius, where he completed a scientific Baccalaureate program.

In 2003, he started a Bachelor program in Cell Biology and Physiology at the University Paul Sabatier (Toulouse, France). Then, he specialized in the field of pharmacology at the Paris-Sud University (Paris, France) where he obtained his master degree in “Pharmacology, pharmacokinetics and pharmacogenetics” in 2009. During his master internship, he has investigated the pharmacological properties of newly developed TrkB ligands at the “Center for Psychiatry and Neuroscience – INSERM U894” (Paris, France) under the supervision of Dr. Joel Premont. In particular, he has been focusing on the (trans)activation of the TrkB receptor and the possible therapeutic value of compounds directly targeting the TrkB receptor.

In 2010, he officially started a collaborative PhD project initiated by the Paris-Descartes University (Center for Psychiatry and Neuroscience; Paris, France) and the University of Maastricht (Department of Psychiatry and Neuropsychology; Maastricht, The Netherlands) coordinated by Prof. Dr. Harry Steinbusch and Prof. Dr. Laurence Lanfumey. During his doctoral internship, he has been focusing on the epigenetic regulation of BDNF/TrkB signaling in the pathophysiology and treatment of mood disorders. In particular, he has been investigating the role of epigenetic regulation at BDNF/TrkB signaling in the development and maintenance of neuronal plasticity, under the supervision of Dr. Daniel van den Hove and Dr. Gunter Kenis at the department of Psychiatry and Neuropsychology (University of Maastricht). At the Center for Psychiatry and Neuroscience (Paris, France), he has been studying the role of BDNF/TrkB signaling in the mechanism of action of antidepressants under the supervision of Prof. Dr. Laurence Lanfumey.

Since October 2013, he is actively involved in a MBA program at the IONIS School of Technology and Management (Paris, France). The MBA courses focus particularly on the management of biotechnology and pharmaceutical industry.

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