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Marion J. F. LEVY

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Evaluation of BDNF/TrkB signaling as a common target in the treatment of major depression and Alzheimer's disease

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Monsieur le Professeur Thierry Charlier	**Rapporteur**
Monsieur le Professeur Michel Hamon	**Examinateur**
Madame le docteur Laurence Lanfumey	**Directeur de thèse**
Monsieur le Docteur Raymond. Mongeau	**Rapporteur**
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Monsieur le Professeur Harry W.M. Steinbusch	**Co-directeur de thèse**
Monsieur le Docteur Tim Vanmierlo	**Examinateur**

Unité INSERM U894 – Centre de Psychiatrie et Neurosciences
Equipe Lanfumey – « Physiopathologie des troubles de l'humeur et de l'addiction
102-108 rue de la Santé
75014 Paris – France

Department of Psychiatry and Neuropsychology - School for Mental Health and Neuroscience – Maastricht university
P.O. Box 616
6200 MD Maastricht - The Netherlands

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Chapter 1:

General introduction

1 Depression

1.1 Definition and epidemiology of depression

Around approximately 400 B.C, Hippocrates, a Greek physician also called "father of modern medicine", described melancholia as a disease characterized by physical and mental illness. This holistic view was based on the humorism system, consisting of 4 body fluids, known as humors, which would directly influence an individual's temperament and health. The word melancholia means black bile as it was assumed that an excess of black bile would cause symptoms like depressed mood. In the middle of the 20th century, Adolf Meyer proposed to use the word depression instead of melancholia and researchers suggested that depression was caused by a chemical dysregulation of neurotransmitters. Thus, it is interesting to see that a conceptually holistic view on a person's psychological state was already introduced more than 2000 years ago.

Depression is a mood disorder ranked as the leading cause of disability for both males and females, making it a significant contributor to the global burden of disease. Today, depression is estimated to affect 350 million people worldwide and in 2012 about 1 in 20 people reported suffering from an episode of depression. In addition, one million suicides have been reported to occur each year [1]. In the Diagnostic and Statistical Manual of Mental Disorders V (DSM 5) and the International Classification of Diseases (ICD-11), depression is characterized by several symptoms including sadness, anhedonia, disturbed sleep, fatigue, poor concentration, suicidal thoughts or acts. A major depressive episode is diagnosed when at least five of the aforementioned symptoms are reported for longer than two weeks.

1.2 Neurobiology of depression

Even though depression has been studied for many years, its exact pathogenesis remains not fully understood. However, with the development of new tools in research, several hypotheses have been proposed [2-5].

Monoamine hypothesis. This hypothesis is the oldest and the most studied one and was raised after the observation of a dysregulation of the monoaminergic system in depressed patients. The three major monoamine neurotransmitters involved in depression are dopamine (DA), serotonin (5-HT) and noradrenaline (NA). The most common way to treat depression is the use of antidepressants which act on these neurotransmitter systems. Monoamine oxidase inhibitors (MAOIs) inhibit monoamine oxidase (MAO) enzymes, which leads to an increase in monoamine levels by preventing the breakdown of monoamine neurotransmitters in presynaptic neurons. Tricyclic antidepressants (TCAs) block 5-HT and NA transporters, reducing 5-HT and NA reuptake from the synapse into the presynaptic nerve, resulting in an increase of these neurotransmitters in the synaptic cleft. However, as TCAs are rather unspecific, this class of antidepressants can induce considerable

side effects. To avoid them, more specific antidepressants have been developed, i.e. selective serotonin reuptake inhibitors (SSRIs), serotonin-noradrenaline reuptake inhibitors (SNRIs) and norepinephrine reuptake inhibitors (NARIs). Due to their selectivity, these classes of antidepressants have a better side-effect profile although they do not show superior clinical efficacy [6]. Indeed, about one-third of patients remain non-responsive to the treatment [7]. Furthermore, the delayed action of the antidepressants (from three to four weeks) is still a major limitation. Therefore, research has focused on other biological hypotheses of depression in order to identify new targets for drug treatment [8].

Hypothalamic-pituitary-adrenal (HPA) axis or the corticosteroid hypothesis. This theory suggests that the hyperactivity of the HPA axis along with an increase in corticosterone levels may be at the origin of depression's pathophysiology. HPA axis activation is mainly triggered by stress, which has been shown to be strongly involved in inducing depression [9]. The HPA axis is composed of the hypothalamus, the anterior pituitary gland and the adrenal gland. Corticotropin Releasing Hormone (CRH) is a hormone secreted from the hypothalamus which activates the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which in turn will release glucocorticoids from the adrenal cortex. Thus, through the HPA axis, stress induces the production and release of glucocorticoids, such as cortisol in humans and corticosterone in rodents, from the adrenal cortex. It interacts with high affinity with the mineralocorticoid receptors (MRs) and, with a lower affinity, with glucocorticoid receptors (GRs). Glucocorticoids induce a negative feedback by inhibiting CRH production through GRs at various levels within the HPA axis as well as at the level of the hippocampus and other brain regions. A deficit in this negative feedback, among others, is associated with HPA axis hyperactivity resulting in a chronic increase in cortisol levels often observed in depressed patients. In accordance with this hypothesis, several mouse models of depression were developed, among them the GR-impaired mice (GR-i mice) with a decrease in GR-specific binding and in GR mRNA expression. These mice exhibit increased levels of depressive-like behavior characterized, for example, by a decrease in the time spent in grooming behavior and an increase in the immobility time in the tail suspension test (TST), a test commonly used for screening antidepressants. [10, 11]. Antidepressants have also been shown to regulate GR mRNA expression which further validates this theory [12].

Inflammatory response hypothesis. In depressed patients, an increase of inflammation markers has often been noticed with an elevation of pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1ß and tumor necrosis factor–α (TNF-α) and their receptors. Acute-phase reactants – chemokines and soluble adhesion molecules level – are also elevated in the blood and the cerebrospinal fluid (CSF). Moreover, the blockade of either cytokines or inflammatory signaling pathway components has been proven to reduce depressive symptoms in depressed patients [13]. Furthermore, hepatitis-C patients treated with interferon-α (IFNα), a pro-inflammatory cytokine,

display severe psychiatric side effects, including clinical depression. A recent meta-analysis concluded that one in four IFNα-treated hepatitis-C patients develop a major depressive episode [14]. Interestingly, pretreatment with an SSRI could decrease the incidence and severity of IFNα-induced depression [15]. Similarly, in rodents, systemic or central administration of IL-1β or TNF-α induces sickness behavior [16], a syndrome that resembles some features of depression. In addition, several studies have shown anti-inflammatory properties of commonly prescribed antidepressants [13]. A mouse model of depression often used to study inflammation in depression consists of injecting a bacterial endotoxin, i.e. lipopolysaccharide, or LPS, in mice. Several hours after LPS injection, mice exhibit sickness behavior, characterized by an adaptive response to illness that includes, among others, social withdrawal, decreased appetite, and lethargy. This state is followed by depression-like behavior approximately 24h after LPS exposure [17]. Concomitant antidepressant treatment restores normal behavior in LPS-treated mice, supporting the idea of the involvement of inflammation in depression [18].

Neurotrophin hypothesis. Finally, another hypothesis that emerged during the last few years is the neurotrophin hypothesis. This concept is based on the observation of a decrease in neurotrophins, more specifically of the brain-derived neurotrophic factor (BDNF), in depressive patients and in diverse animal models of depression, concomitant with increased cell loss and synaptic dysfunction, associated with depressive symptoms [19, 20]. This hypothesis will be further detailed below and in Chapter 2.

2 Alzheimer's disease

2.1 Epidemiology of Alzheimer's disease

In 1907, the psychiatrist and neuropathologist Aloïs Alzheimer studied the case of a 51-year-old patient, Auguste Deter, who presented dementia characterized by memory deficits, disorientation and mutism. After her death, postmortem analysis of her brain revealed two major hallmarks that distinguished her brain from a normal one: senile plaques and neurofibrillary tangles. In 1910, this disorder was named after Alzheimer and with the help of other scientists such as Oskar Fisher and Gaetano Perusini, who observed the same characteristics, the disorder was defined as a clinical entity. However, it was not before the 1980s that the exact nature of these two AD hallmarks was identified, i.e. Aß plaques, the major component of senile plaques, and hyperphosphorylated tau protein that formed the neurofibrillary tangles.

Alzheimer's disease (AD) is a neurodegenerative disorder and is the most common form of dementia that represents a very high social, societal and economic burden [21, 22]. Indeed, in 2008, the total cost of illness for dementia disorders was estimated to be €177 billion in Europe for the approximately 11 million people diagnosed with dementia. [23]. The disorder has a characteristic

development and course. In the early stage, the symptoms are often considered a normal part of the aging process, where patients can become forgetful, lost in familiar places or exhibit some symptoms close to the ones observed in a depressed patient. In the middle stage, the patients may need personal care, as they are unable to meet their basic needs and not even to live alone safely. They become very forgetful, in particular, regarding recent events and person's names. Finally, in the late stage, patients need more assisted self-care as they cannot eat alone, experience difficulties in swallowing, are unable to recognize relatives and familiar objects, and are unable to find their way around the home [21, 24].

2.2 Neurobiology of Alzheimer's disease

So far, the main hallmarks of AD are brain atrophy, and extracellular aggregation of neurotoxic forms of amyloid-beta (Aß) and intracellular neurofibrillary tangles, consisting of hyperphosphorylated tau proteins, particularly affecting brain regions involved in learning and memory [25].

Amyloid hypothesis. Aβ molecules are derived from the amyloid precursor protein (APP), an integral membrane protein that can be cleaved at different sites by several enzymes (α-, ß-, and γ-secretases). The cleavage by α-secretase releases soluble APPα in the extracellular space and is referred to as the non-amyloidogenic pathway. In the amyloidogenic pathway APP is sequentially cleaved by ß-secretase at the N-terminus and by γ-secretase at the C-terminus. This generates Aß peptides which are inclined to form aggregates because of their hydrophobic properties. Aggregation has been shown to be toxic to neurons, and possibly to induce neuronal loss. In order to study the pathophysiology of AD, several mouse models have been developed and among them, transgenic mice, based on the amyloid hypothesis, namely the APPswe/PS1dE9 [26], have been widely studied. These double transgenic mice possess mutant forms of APP and PS1 which are genes associated with the early-onset familial AD form [27]. These mice develop amyloid ß plaques and cognitive impairment around 6 to 7 months of age [28].

Tau hypothesis. Tau proteins are microtubules-associated proteins (MAPs) that are the major phosphoprotein in the brain. It is phosphorylated at several serine and threonine residues. In the AD brain, intracellular neurofibrillary tangles (NFTs), constituted by hyperphosphorylated tau protein, are regularly observed. Hyperphosphorylation of tau, together with glycogen synthase kinase 3ß (GSK3ß) activation have been shown to mediate cell death and might be one of the primary causes of cognitive dysfunction observed in AD [29].

3 Relationship between depression and Alzheimer's disease

Emerging evidence has shown that there are increased probabilities for AD patients to develop depression and conversely, depressive patients are more susceptible to develop AD. Up to 30 – 50 % of AD patients have a previous history or express signs and symptoms of depression [30, 31]. Moreover, similar neuroanatomical changes, e.g. at the level of the parietal and temporal cortex, can be found in depression and AD. Atrophy of the hippocampus has also been observed in depressed patients as well as in AD patients [32]. In addition, studies on inflammation revealed the involvement of neuroinflammation in the development and the progression of AD with a high level of inflammatory cytokines in the CSF of patients with dementia. As mentioned above, the same dysregulation has also been observed in depressive patients [33]. Stress also seems to be a common characteristic of depression and AD, and impaired negative feedback of the HPA axis has been described in both disorders. Additionally, HPA axis dysfunction seems to occur in the early stages of AD, when the symptoms can overlap with depression [34]. Interestingly, it was suggested that Aß metabolism is affected in depression. Indeed, infusion of Aß oligomers (AßO) in mice induced depressive-like behavior along with cognitive deficits. Moreover, analysis of mouse brains injected with AßO revealed an increase in pro-inflammatory cytokines. Interestingly, SSRI treatment reversed these impairments [35]. Finally, levels of proteins involved in synaptic plasticity and neuronal survival, such as BDNF, have been shown to be lowered in both depressive and AD patients [34, 36]. This phenomenon will be described later. All in all, these results validate the strong association between depression and AD [37].

Interestingly, some pharmacological treatments revealed clinical efficacy in both depression and AD. Furthermore, experimental work showed that antidepressants can increase BDNF levels, stimulate neurogenesis, and decrease Aß-induced toxicity [38]. Moreover, work on rodent models of AD revealed that a 4-week SNRI antidepressant treatment improved learning and memory and increased non-toxic monomeric Aß [39]. On the other hand, a recent meta-analysis did not confirm the real efficacy of antidepressants on depression in AD, with two studies showing antidepressants to be more efficient than placebo over seven which did not. This lack of effect might be explained by a multitude of neurotransmission dysfunctions along with other pathophysiological processes in AD [40, 41]. Therefore, even though antidepressants did not prove consistent efficacy, the fact that studies observed some effects on Aß-peptides and on memory performance reinforces the link between depression and AD.

AD patients present cholinergic deficits, and acetylcholinesterase (AChE) inhibitors represent the first-line treatment in AD patients. These compounds inhibit the breakdown of Acetylcholine (ACh) by AChE and, as a consequence, increase ACh levels in the synaptic cleft in an attempt to improve cognitive function [42]. These drugs have also been tested in depression and have shown positive effects with partial or total resolution of depressive symptoms after several months of

treatment [40], thereby further highlighting the neurobiological link and common pharmacological targets of depression and AD. However, in AD, this symptomatic treatment only temporarily delays the progressive cognitive impairment and is not able to cure this progressive neurodegenerative disorder, underscoring the need for novel, more effective treatment strategies [43].

4 Brain-derived neurotrophic factor and the Tropomyosin-related kinase B receptor

4.1 Brain-derived neurotrophic factor

BDNF is a neurotrophic factor that plays an essential role in the maintenance of the central nervous system (CNS), regulating neuronal and synaptic plasticity. BDNF transcription is induced by neural activity. The BDNF gene is composed of eight 5' non-coding exons (I-VIII) and one 3' coding exon (IX). Each exon is controlled by a specific promoter, i.e. one of the 5' non-coding exons with the 3' coding exons, which can lead to the production of multiple splice variants. It has been suggested that *Bdnf* is synthesized preferentially in dendrites [44-46] where BDNF mRNA is translated in the endoplasmic reticulum to pre-pro-BDNF, a 30-35 kDa precursor protein. The signal peptide is then removed and pro-BDNF accumulates in the trans-Golgi network. In the Golgi, sortilin, an intracellular chaperone molecule, binds the pro-domain of BDNF in order to stimulate folding of the mature domain after which carboxypeptidase E binds to the mature domain of BDNF, thereby sorting BDNF to the regulated secretory pathway. Pro-BDNF is then cleaved into mature BDNF, although the exact cleavage location remains unclear. Indeed, the phenomenon could occur either in the secretory granules or in the extracellular medium where BDNF would be processed by metalloproteinases and the extracellular protease plasmin. BDNF is then delivered to the presynaptic axon terminal, by anterograde transport. The secretion is triggered by membrane depolarization via Ca^{2+} influx or, alternatively, by extracellular factors including neurotrophins themselves. Once released in the extracellular space, mature BDNF binds with a high affinity to its receptor, tropomyosin-related receptor kinase B (TrkB), inducing cellular survival, proliferation and differentiation. Interestingly, pro-BDNF binds preferably to the p75 receptor [44, 47, 48], which is suggested to represent a negative regulator of neuronal remodeling, synaptic transmission and synaptic plasticity within the adult CNS [49] (Figure 1). However, these results remain controversial, as p75 has been found to exert opposite effects during CNS development, i.e. increasing neurite outgrowth and axon specification [50].

4.2 Tropomyosin-related receptor kinase B

TrkB is a member of the tyrosine kinase family. Like BDNF, its gene, which contains 24 exons, can be differentially expressed as multiple splice variants that encode various protein isoforms. Four major proteins can be translated, namely the full-length TrkB ($TrkB.tk^{+}$), the C-terminal

truncated receptor (TrkB.t1), the C-terminal truncated Shc^+ receptor (TrkB.shc) and the C-terminal truncated receptor, the latter lacking exon 23 and 24, but retaining tyrosine kinase activity (TrkB-T-TK). The most abundantly expressed TrkB isoforms in the brain are $TrkB.tk^+$ and TrkB.t1. Although TrkB.t1 has the same extracellular domain as $TrkB.tk^+$, the lack of Shc, tyrosine kinase and phospholipase-γ (PLCγ) regions prevent it from showing catalytic activity. Three dimers have been found in the brain, i.e. a TrkB.tk+ homodimer, a $TrkB.tk^+$-TRkB.t1 heterodimer, and a t1 homodimer. The heterodimer $TrkB.tk^+$-TRkB.t1 has been shown to be a dominant negative receptor meaning that it inhibits $TrkB.tk^+$ activation [51, 52].

4.3 Tropomyosin-related receptor kinase B signaling

Once BDNF is released into the extracellular space, it binds to TrkB and induces receptor dimerization. This triggers the increase in the catalytic activity of the intracellular domain resulting in the autophosphorylation of tyrosines and the subsequent activation of three main signaling pathways. Phosphorylation of the Y515 tyrosine activates mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling, allowing for neuronal growth, differentiation and survival. Y816 tyrosine phosphorylation induces PLCγ signaling, promoting synaptic plasticity and neurotransmission [53]. In order to exert this neurotrophic action, the three signaling pathways require cAMP response element-binding protein (CREB) which will indirectly induce an increase in the expression of immediate early genes (IEGs) such as *cfos*, the activity-regulated cytoskeleton-associated protein (*arc*) and the *early growth response protein 1*(*EGR1*) [54, 55] (Figure 1). Interestingly, it has also been shown that TrkB can be activated in the absence of neurotrophins. This direct or indirect activation is called transactivation. It can occur through for example adenosine receptor activation (A2a), a G protein-coupled receptor (GPCR), or after the release of large amounts of zinc and glutamate. These events induce TrkB tyrosine phosphorylation through Src activation [53]. More recently, antidepressants too have been shown to transactivate the TrkB receptor [56].

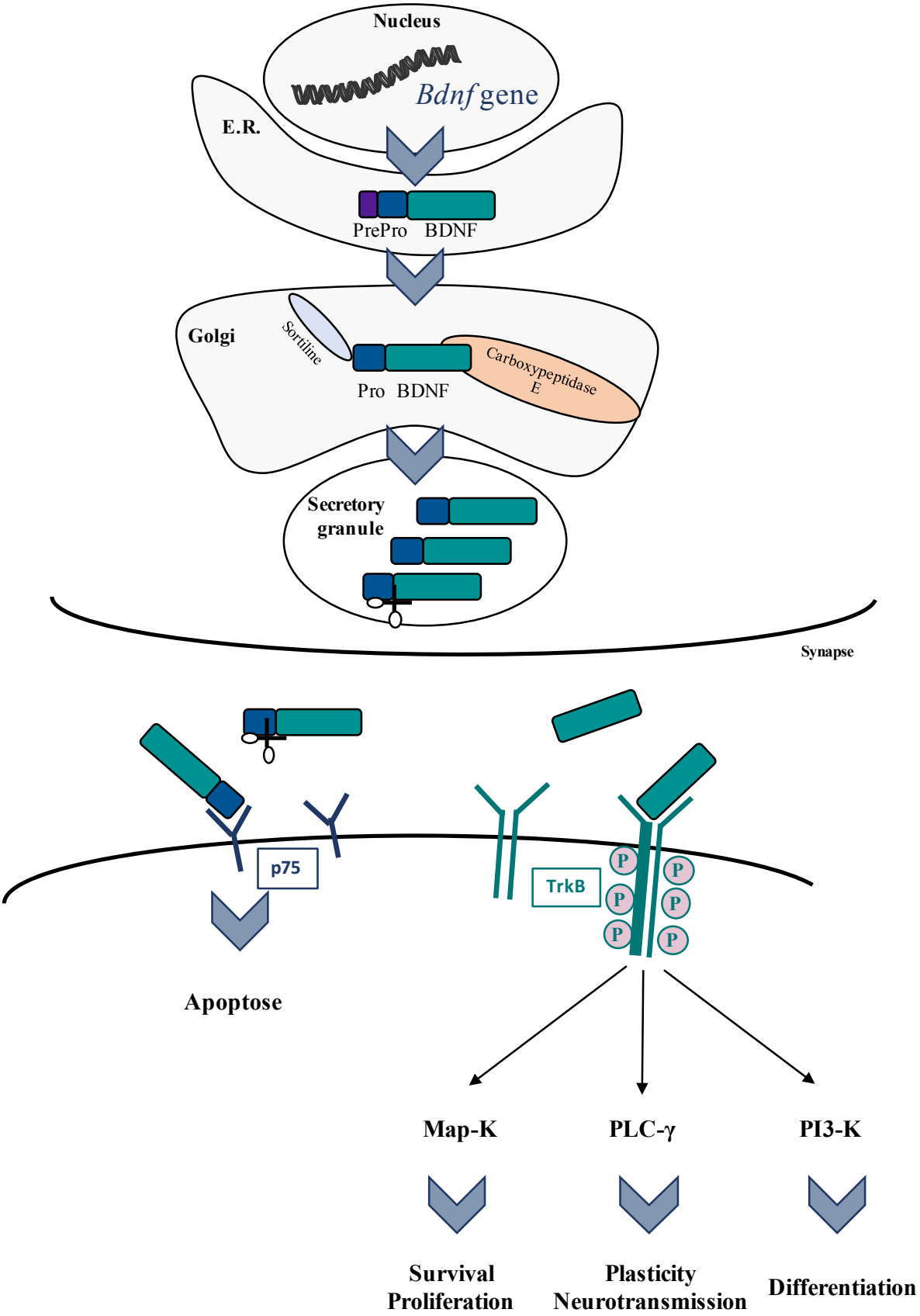

Figure 1: schematic overview of BNDF synthesis followed by TrkB signaling pathway. See text for more details. Adapted from [47, 48, 53]

5 The role of brain-derived neurotrophic factor in depression and Alzheimer's disease

5.1 Brain-derived neurotrophic factor and depression

Various clinical studies performed in post-mortem brains of suicide victims and depressed patients showed a decrease of BDNF mRNA expression and protein level in both the prefrontal cortex and in the hippocampus [57, 58]. BDNF was also shown to be decreased in serum from depressed patients [20, 59, 60]. Moreover, it has been shown that a treatment with antidepressants and ECT would normalize serum BDNF concentration in depressed patients and in rodent models of depression [60, 61]. Furthermore, a common single nucleotide polymorphism (SNP) in the BDNF gene has been shown to affect the sorting of BDNF into secretory vesicles, the synaptic localization and the activity-dependent release of BDNF. This genetic variant, called Val66Met and consisting of methionine substitution for valine at codon 66, is associated with a smaller volume of several brain regions and with impaired episodic memory function [62]. Along similar lines, rodent models of depression have revealed a decrease in BDNF protein levels, notably in the prefrontal cortex and hippocampus [63] [46]. Furthermore, infusion of BDNF into the hippocampus of rat models of depression induces antidepressant-like effects [64]. Administration of antidepressants promotes the increase of BDNF in the prefrontal cortex, and repeated application of electroconvulsive shocks increases BDNF in the hippocampus and prefrontal cortex [65]. Altogether, this data reveals the important role of BDNF in depression. However, the fact that BDNF levels were shown to be increased in the ventral tegmental area – nucleus accumbens (VTA-Nac) circuit in depression, along with other pharmacological studies targeting BDNF that have generated negative results, strongly suggests that the role of BDNF in depression remains unclear and needs further investigation.

5.2 Brain-derived neurotrophic factor and Alzheimer's Disease

In line with what is observed in depression, BDNF has also been shown to be relevant in the pathophysiology of AD. For example, it has been shown that BDNF mRNA and protein levels were decreased in regions associated with learning and memory, such as the hippocampus and cortex, in postmortem brains of AD patients when compared to age-matched controls [66]. Similar results have been found in animal models of AD [67, 68]. Furthermore, this decrease in BDNF levels was correlated with the degree of cognitive impairment in AD [69]. However, like in depression, controversial results have been published, occasionally reporting no change in brain BDNF levels and AD. More importantly, even opposite effects have been described with a three-fold increase of BDNF mRNA in the vicinity of Aß plaques [70]. Similarly, in the blood of AD patients, BDNF appears to be decreased in some studies, whereas others showed opposite effects or no difference. An explanation to this discrepancy might be that blood and plasma analyses are not fully consistent and representative

[66]. Interestingly, a study investigating the BDNF Val66Met SNP revealed a reduction of volume in several brain structures [71], which is similar to effects of both normal and AD-associated aging, showing reduced brain region volume for the hippocampus, cortex, cingulate, insula and amygdala, among others. Furthermore, 66Met carriers showed episodic memory deficits and impaired cognition. Importantly, BDNF, which has been proven to reduce Aß production *in vitro* and *in vivo* [72], can exert neuroprotective effects against Aß, including neuronal growth and survival in the hippocampus. These effects allow the rescue of spatial memory deficits in Aß-pretreated mice [73]. Therefore, even with some negative results, BDNF/TrkB signaling clearly represents a promising target to alleviate or reverse symptoms of AD.

5.3 Brain-derived neurotrophic factor as a treatment for mood disorders and neurodegenerative diseases

Given the involvement of BDNF in depression and AD, research has been focusing on the use of this neurotrophin as a treatment. Accordingly, in rodents, infusion of BDNF in the hippocampus has proven to exert antidepressant-like effects [64, 74-76]. Furthermore, direct infusion of BDNF into the hippocampus of Aß-pretreated mice showed protective effects against Aß [77]. Unfortunately, BDNF has a poor pharmacological profile, displaying a very short half-life (less than 10 min), a poor penetration of the blood-brain-barrier and a low oral bioavailability, hampering its clinical application [78]. Therefore, TrkB agonists that mimic the activity of BDNF have been developed. So far, the most studied one is 7,8 dihydroxyflavone (DHF), which was first studied as an antioxidant and subsequently validated as a TrkB agonist [79]. Several studies have been performed in *in vitro* and *in vivo* models of depression and AD, showing promising results. In various rodent models of depression, DHF has been shown to exert antidepressant-like properties [80], while it has also been shown to alleviate memory deficits in the 5XFAD and APPswe/PS1dE9 mouse models for AD [81, 82], whereas it failed to induce recovery from memory deficits in APP23/PS45 [83]. Recently, other molecules have been studied, showing TrkB agonistic properties, such as deoxygedunin, LM22A-4, TDP6 and 29D7 [84-87]. These molecules all showed neuroprotective properties, but they are primarily known for other properties, such as their antioxidant properties, besides being TrkB agonists. A novel TrkB agonist developed using a structure-based *in silico* screening approach, is TB001 [88]. Its 3D pharmacophore was based on the X-ray structure of BDNF loop L4, focusing on two lysine residues, previously reported to be involved in BDNF-induced TrkB activation [89]. This new molecule will be further studied in this thesis in the context of depression and AD.

6 Aims and outline of this thesis

The aim of this thesis is to investigate the pathophysiological role and the therapeutic potential of BDNF/TrkB signaling in depression and AD.

Chapter 2 provides an overview of the pathophysiology and the treatment of depression, focusing on the role of neurotrophic factors and related neuroplasticity. In this review, we summarize neuroanatomical and neurobiological observations seen in depression. The effect of several antidepressant treatments is also highlighted. We describe the link between depression and reduced neuroplasticity, more specifically the changes in neurotrophic factors, as well as the effects of antidepressants in this respect. Finally, a perspective towards the development of novel antidepressant drugs based on the neurotrophin hypothesis of depression is presented.

In **Chapter 3**, we study the mechanism of action of fluoxetine, the most commonly used SSRI, in relation to plasticity. First of all, the effect of fluoxetine on TrkB receptor function is analyzed. Then, *in vitro* and *in vivo* studies using serotonin transporter (5-HTT) knock out mice allow to study the involvement of 5-HTT in fluoxetine-induced neuroplasticity.

We then examine the effect of neurotrophins in mouse models of depression. Therefore, in **Chapter 4**, a newly developed TrkB agonist molecule, TB001 is investigated. First we present *in vitro* and *in vivo* studies carried out to validate the agonistic properties of TB001 on the TrkB receptor. Then, this molecule is applied on two different models of depression in order to assess its putative antidepressant properties after acute and chronic administration.

Considering the same decrease of BDNF mRNA expression and protein level observed in depression and AD, we also test TB001 on a mouse model of AD in **Chapter 5**. To this aim, its protective effects are first assessed *in vitro* in cells transfected with the TrkB receptor and incubated with Aß. Its effects on cognition as well as on proteins involved in BDNF/TrkB signaling are subsequently studied in a mouse model of AD after acute and chronic administration of TkrB agonits.

Finally, in **Chapter 6**, we summarize and discuss the main results and conclusions and we provide future directions and perspectives.

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Chapter 2:

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

Marion J.F Levy [1,2,3,*], Fabien Boulle [1,2,3,*], Joost van Haasteren [3], Harry W. Steinbusch [3], Daniel L.A. van den Hove [3,4], Gunter Kenis[3,*], Laurence Lanfumey [1,2,*]

[1] Centre de Psychiatrie et Neurosciences, Inserm U 894, Paris, France

[2] Univerité Paris Descartes, UMRS894 Paris, France

[3] Maastricht University - European Graduate School for Neuroscience EURON, Department of Psychiatry and Neuropsychology, Maastricht, The Netherlands

[4] Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Fuechsleinstrasse 15, 97080 Wuerzburg, Germany

* These authors have contributed equally

Author for correspondence

L. Lanfumey

Inserm U894, Centre de Psychiatrie et Neurosciences

102-108 rue de la Santé

75014 Paris France

E-mail: laurence.lanfumey@inserm.fr

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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 neuroanatomical level, major depression (MD) is associated with atrophy or impaired functioning of cortico-limbic regions involved in mood and emotion regulation including the hippocampus and the prefrontal cortex. In particular, it is believed that alterations in neurotrophic growth factors and associated signaling pathways underlie neuronal loss and synaptic dysfunction in cortico-limbic structures, which may be causally related to the development and course of depression. Accordingly, mounting evidence suggests that antidepressant treatment may exert its therapeutic effects by enhancing trophic signalling and their downstream effects on neuronal and synaptic plasticity. However, current antidepressants still show significant limitations including a delayed therapeutic effect, as well as lack of efficacy. Hence, a deeper understanding of the molecular and cellular mechanisms involved in the pathophysiology of depression, as well as in the action of antidepressants might provide further insight into the development of novel fast-acting and more effective therapies. Here, we summarize the current literature on the involvement of neurotrophic factors and associated signaling pathways 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, mood disorders

1 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 [1-3]. Despite recent advances in neuroscience research, the neurobiological mechanisms underlying the pathophysiology of depression remain poorly understood. The development and course of the major depressive disorders (MDD) 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 [4]. So far, many classes of antidepressants have been discovered and marketed for the treatment of depression. However, currently available antidepressants display significant limitations, including a delayed onset of action, low response rates and relapse after treatment cessation, which remain major drawbacks for a disease with relatively high suicide rates [5]. To date, clinical and preclinical studies have linked depression to structural and cellular alterations, such as neuronal loss and synaptic dysfunction, in cortico-limbic brain regions controlling mood and emotions [6]. Among many candidates, neurotrophic growth factors and related signaling pathways constitute major players in neuroplasticity, and current evidence indicates that impairment in growth factor signalling is associated with depressed mood [7]. Interestingly, currently prescribed antidepressants have been shown to increase neuroplasticity when exerting their therapeutic effects [8]. Hence, a deeper understanding of the exact molecular, cellular, and structural plasticity 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 summarize the current literature on the implication of neurotrophic factors and associated signaling pathways in depression and antidepressant treatments. First, a brief overview of those brain regions and circuits implicated in the pathophysiology of depression and in the response to antidepressant is highlighted. Further, evidence for the involvement of neurotrophic factors and associated signalling pathways in depression and its current treatment is described. Finally, a perspective towards the development of novel antidepressant drugs is given.

2 Brain regions and neurocircuits involved in depression: neuroanatomical evidence

2.1 Amygdala

The amygdala is an integrant part of the limbic system implicated in cognitive and emotional processing, in particular regarding fear and anxiety [9, 10]. Volumetric magnetic resonance imaging (MRI) studies so far revealed contrasting results, with studies showing either an increase [11-13] or a decrease in amygdala volume in depressed patients [14-16]. A reduction in glial cell number, probably due to a decrease in oligodendrocytes, was also discovered in post-mortem amygdala tissues of MDD patients [17, 18]. Functional MRI (fMRI) studies in depressed patients have shown exaggerated amygdala activity when confronted with emotional facial expressions [19, 20]. Moreover, increased activity of the right amygdala has also been observed in depressed patients during encoding of negative but not neutral or positive stimuli [21]. Depressed patients also showed an overall increased activity of the left amygdala [22]. Similarly, Drevets and colleagues using positron emission tomography (PET) imaging showed an increase in amygdala activation and metabolism in MDD patients [23]. Several studies that reported abnormal microstructure and connectivity of the amygdala and mPFC using diffusion tensor imaging in remitted MDD patients [24] or functional or reduced functional coupling between the amygdala and the supragenual PFC using magnetic resonance imaging suggested that MDD may result in part from a failed ability to co-activate a cognitive control network during emotion processing [25]. At preclinical level, an enhanced dendritic arborization, elongation and spine density, providing evidence for increased synaptic connectivity within the amygdala was also shown after a chronic stress exposure in rats [26, 27].

2.2 Prefrontal cortex (PFC)

The prefrontal cortex (PFC) in functionally connected within several brain structures, processing sensory input and mediating executive motor functions. The ventromedial PFC and the orbitofrontal cortex are involved in top-down cognitive processing of bottom-up emotional, i.e. treathful, stimuli originating from the limbic system (e.g. hippocampus, amygdala, ventral striatum, hypothalamus) and forming an integral part of memory consolidation and retrieval [28, 29]. As such, the PFC plays a major role in regulating the appropriate emotional response such as fear or anxiety. Moreover, the PFC has been associated with decision-making, personality expression and social behavior. Neuroimaging studies showed a reduction in size of multiple areas of the PFC in subjects diagnosed with MDD [30, 31]. 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 [32-34]. A study revealed that chronic restraint stress caused a significant reduction in number and length of apical dendritic branches of pyramidal neurons in some areas of the PFC in

rodent [35]. Similar results were observed in rat which received chronic administration of corticosterone. In this rodent model of depressive-like disorders, in which a sustained high corticosterone level could match that observed in MDD patients, a drastic dendritic reorganization of pyramidal neurons was also reported in the medial PFC (mPFC) [36]. PFC abnormalities observed either in MDD patients or in rodent models are partly corrected by antidepressants. A 8 weeks escitalopram treatment was shown to reduce irregular high functional connectivity in the bilateral dorsal medial PFC in MDD patients [37, 38] and, in rats, a 2 week antidepressant could restore the reduced dendritic length and spine densities, but not the cortical thickness, induced by early stress exposure [38]

2.3 Ventral Striatum

Next to the PFC and amygdala, a preponderant role for the ventral striatum has been reported in major depression. The 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 mediating the anhedonic symptoms of depression [39, 40]. Depressed patients show an attenuated activation of the VTA-NAc pathway or the NAc itself when compared to normal patients using fMRI analysis [41-44]. Rat models also indicate that deep brain stimulation (DBS) of the NAc 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 [45]. NAc DBS may also alter the morphology of the PFC, with increased apical and basilar dendrite length [46]. Both the changes within the PFC and hippocampus in response to NAc DBS show the influence of the NAc in depression over the long term. DBS targeting the NAc was shown to have antidepressant, anxiolytic and hedonic effects, notably in treatment resistant depression [47-49]. These effects have been proven to be long-lasting and stable up to four years [50, 51]. While MDD patients demonstrated reduced ventral striatal activation during anticipation of gain and loss, a treatment with antidepressant was able to normalize this hyporesponsiveness. [52]

2.4 Hippocampus

The hippocampus is a major structure within the limbic system known to be highly vulnerable to stress and other environmental factors. This region is critical in diverse cognitive processes and in the regulation of emotions. MRI analyses revealed reduced hippocampal volumes in patients suffering from both recurrent and first episode depression [53-55], and a correlation between volume reductions and total duration of major depression has been reported [56]. It has also been proposed that reductions in hippocampal volume may not antedate illness onset, but that hippocampal volume may decrease most in the early years after illness onset [57]. 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 [58]. This might indicate a decrease in cellular neuropil that might provide an explanation for the reduced hippocampal volume found in depressed subjects. Studies showed that chronic stress caused atrophy of apical dendrites of pyramidal neurons in the CA3 region of the hippocampus in rodents [27, 59, 60]. Exposure to excess glucocorticoids in rats showed decreased apical branching numbers and apical dendrite length [61], suggesting a role of the activation of the HPA axis in the remodeling 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 (DG), involving a reduced proliferation, survival and differentiation of neural stem cells [62]. Moreover, evidence that adult hippocampal neurogenesis is required for the behavioral effects of antidepressants [63, 64] provides further proof of the crucial role of the hippocampus in depression.

3 Structural and neuroplastic changes in MDD and in animal models

3.1 Neuroplasticity: definition

Neuroplasticity can be defined as the ability of the nervous system to respond to intrinsic or extrinsic stimuli by reorganizing its structure, function and connections [65]. It includes different mechanisms. One of them is neurogenesis, the formation of new born neurons happening in neurogenic areas. The regions identified so far in the adult brain are the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus [66, 67]. Another mechanism of the plasticity is the modifications of mature neurons morphology, such as axons and dendrites arborization and pruning, increase in spine density, and synaptogenesis. At a functional level, the long-term potentiation (LTP) is also a mechanism belonging of plasticity. Finally, a molecular scale, neurotrophins such as brain derived neurotrophic factor (BDNF) have been proved to be strongly involved in regulating plasticity by impacting upon neuronal survival, differentiation and proliferation. Regulation of gene transcription by epigenetic mechanisms also contributed to synaptic plasticity. Altogether, these changes lead to a regulation of synaptic strength [68].

3.2 Neuroplasticity changes in MDD

In MDD, changes in plasticity were pointed out and is characterized by hippocampal dendritic atrophy, cell death, LTP inhibition and BDNF expression decrease [69]. The effects of antidepressants on neuronal and synaptic plasticity has been widely described as outlined in an excellent review by Pilar Cuéllar and colleagues [70]. Both reduced hippocampal proliferation as well as increased chronic stress-induced cell death observed in rodent model of depression was reversed by chronic administration of antidepressants. Exercise has also shown beneficial effects on plasticity. Swimming, exercises have antidepressant-like effects on anhedonia in stressed rodents. Exercise could also restore the stress-induced BDNF mRNA expression decrease [71]. When exercise was combined with dietary

supplementation, stressed mice showed an improved neurogenesis, including benefits in hippocampal BDNF and in DG and CA1 volume. [72]. Recent clinical study using electroconvulsive therapy (ECT) in MDD patients showed that this treatment promoted structural plasticity in the hippocampus and the amygdala along with improved clinical responses especially in patients with a smaller hippocampal volume [73, 74].

3.3 Hippocampal plasticity and neurogenesis

Hippocampal neurogenesis is believed to be required in the therapeutic action of antidepressants in animal [63, 75-77]. Accordingly, chronic treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine increased hippocampal neurogenesis, involving the generation of newborn cells in the DG [63, 78, 79, 80, 81]. In addition, chronic fluoxetine administration increased the synaptic plasticity in naive rats [82]. A report showing that serotonin depletion reduced neurogenesis in the DG and SVZ in adult rats also supports the theory linking antidepressant treatment to neurogenesis [83]. Activation of 5-HT_{1A} receptors resulted in increased neurogenesis in the DG, whereas activation of 5-HT_{2A} receptors 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 [84]. 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 [85]. Similarly, chronic venlafaxine treatment proved to be efficient in preventing the deleterious effects of restraint stress on hippocampal neurogenesis and BDNF protein expression [86]. However, a recent study reported that duloxetine treatment did not significantly increase the number of new cells in the hippocampus of mice [87]. Finally, tricyclic antidepressants (TCA) have also shown to modulate hippocampal neurogenesis. Analysis of post-mortem brains of MDD patients treated with nortryptiline and clomipramine showed an increase of neural progenitor cells and dividing cells in the DG, as compared to healthy controls [81]. In animal models, clomipramine was able to counteract the stress-induced inhibition of proliferation in the hippocampus [88]. Chronic imipramine and desipramine treatment increased cell proliferation in rodents [63, 89, 90]. In addition, imipramine could restore the decrease in neurogenesis caused by chronic stress and chronic corticosteroid upregulation [91-95].

4 Neurotrophins and other growth factors

4.1 BDNF/TrkB complex

4.1.1 General function

BDNF is a neurotrophin which is involved in the growth, differentiation and survival of neurons and has also been shown to represent an important factor in the regulation of neurogenesis

and synaptic plasticity. It exerts its neurotrophic effects by activating the tropomyosin-related kinase receptor B (TrkB). It also binds, albeit with a lower affinity, to the P75 receptor, which is generally known to promote proteolysis and apoptosis [96]. BDNF is abundantly expressed in the mammalian brain, with the highest concentrations found in the hippocampus and cortex.

4.1.2 BDNF in plasticity

Several *in vitro* studies have been conducted in order to unravel the effects of BDNF on plasticity. Indeed, TrkB receptor transfected PC12 cells, BDNF for 48 hours increased neurite outgrowth was increased compared to the non-treated cells [97]. Furthermore, in primary hippocampal cells exposed to B27 deprivation, concomitant with reduced cell viability, a reduction in synaptic protein levels as well as the total dendritic length, BDNF stimulation was able to induce promoting effect on dendritic outgrowth and spine formation. The same effect was seen in the non-B27-deprived cells as well [98]. But also *in vivo* evidence supports the critical role of BDNF in neuronal and synaptic plasticity. The use of heterozygote BDNF +/- mice proved that BDNF was required for several forms of LTP [99]. Furthermore, BDNF infusion induced LTP and triggered synaptic strengthening. [100, 101]. A reduction in BDNF and its TrkB expression in the hippocampus and PFC has been reported in post-mortem brain analysis of suicide victims [102, 103], while lowered serum concentrations of BDNF have been associated with MDD [104-106]. 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 [107] and negatively influences hippocampal volume [55], which may increase the susceptibility to MDD. BDNF expression was also increased in post-mortem brains of depressed patients treated with antidepressant drugs as compared to non-treated patients [108, 109].

4.1.3 BDNF and antidepressant treatments

Interestingly, treatment with antidepressants or electroconvulsive therapy, largely normalizes serum BDNF concentration to the level of healthy controls [106, 110-113], indicating that serum BDNF levels could perhaps provide an indication of antidepressant response [114]. Similarly a meta-analyses revealed that ECT, could increase BDNF level in the plasma of depressed patients [115, 116]. Lee and colleagues developed a val66met mouse analogue [117] and subsequent experiments showed that the val66met genetic variant decreased fluoxetine efficacy through impaired synaptic plasticity in the hippocampus of $BDNF^{met/met}$ mice involving a decrease of LTP as well as an impairment in the survival of newly born cells [118, 119]. In addition, BDNF expression in the brain of rats was upregulated after chronic antidepressant drug exposure and ECT [120-123].

4.1.4 Region specificity in BDNF efficacy

In rodents, direct infusion of BDNF protein into the hippocampus [124-127] and midbrain [128] showed antidepressant-like effects. In contrast, an opposite, pro-depressive effect was reported

after infusion of BDNF in the VTA or the NAc [129]. The same disparity was present using region-specific knockdown of BDNF expression. Impairment of BDNF signaling in the DG of the hippocampus [130] elicited pro-depressive behavior, whereas knockdown of BDNF in the NAc had an antidepressive effect [131]. Interestingly, conditional knockout in the forebrain resulted in an increase in depressive-like behavior in female but not male mice [132] and, furthermore, decreased the efficacy of the antidepressant desipramine [133]. This conditional knockout of BDNF in the forebrain displayed the same sex-specific incongruity in stress-induced depressive-like behavior [134]. A different study using adeno-associated viral-mediated knockout of BDNF in the DG and CA1 of the hippocampus showed that a loss of BDNF function in the hippocampus attenuated antidepressant drug treatment efficacy [135]. Taken together, these data suggested that BDNF could be considered as a key therapeutic tool against depression. [136]

4.2 fibroblast growth factor or FGF

4.2.1 General function

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 SVZ and SGZ of the hippocampal DG. The FGF family is composed of 18 ligands and 4 subtypes of receptors. FGF1 is expressed mostly in neurons while FGF2 is expressed in both neurons and glial cells. FGF1-2 are the most studied ligands of this family and have been shown to be dysregulated in mood disorders. They can bind all four receptor subtypes in order to activate the phospholipase Cy (PLC), mitogen-activated protein kinase (MAPK) and AKT pathways [137, 138]. In addition, FGF1 and FGF2 were evidenced to play a critical role in the regulation of synaptic plasticity.

4.2.2 FGF in neuroplasticity

Intracerebroventricular (ICV) injection of FGF2 induced neurogenesis in both the SVZ and SGZ [139-141]. FGF2 knockout mice showed a significant decrease in newly generated neurons but no reduction in proliferating cells [142]. The additional increase in cell death in the hippocampus indicated a faulty neurogenesis following FGF2 knockout [142]. Conditional knockout experiments with FGFR1-*null* mice show defective LTP and neurogenesis [143], suggesting that the FGF2/FGFR1 interaction might represent an important mediator of neurogenesis.

4.2.3 FGF in depression

Regarding receptors, post-mortem brain analysis in human revealed a lower expression of FGF1 and FGF2 in the dorsolateral PFC and the anterior cingulate cortex of patients with MDD [144]. In addition, FGF2 was decreased in the hippocampus of depressed patients, whereas FGFR1 was increased [145]. 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 [146]. Injection of FGF2 was also shown to reduce depressive-like

behavior in rats [147]. 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 [148].

4.3 Vascular endothelial growth factor or VEGF

4.3.1 General function

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. It can bind to different receptors: VEGF receptor tyrosine kinases (VEGFR) 1 et 2 with a higher affinity for VEGFR1. In addition, mounting evidence suggests that VEGF can be considered as a potent neurotrophic factor as well inducing neurogenesis, neuronal survival and proliferation, glia survival and glia migration [149].

4.3.2 VEGF in neuroplasticity

Interestingly, experimental studies showed that VEGF displayed robust neuroprotective effects in cell models of ischemia and hypoxia [150] as well as a positive effect on neuronal growth, maturation and proliferation under normal conditions [151-155]. A role in the development of dendrites and axons has also been described for VEGF [152-154, 156]. Moreover, ICV administration of VEGF increased neuroprotection and neurogenesis in the adult rat brain after ischemia [157]. 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 [158], while VEGF-B knockout mice showed impaired neurogenesis [159]. 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 [160, 161]. Interestingly, VEGF can promote neurogenesis by stimulating endothelial cells in order to release other neurotrophic factors. In addition, ependymal cells can synthesize VEGF leading to a stimulation of the VEGFR2 and inducing proliferation of neuronal precursors and enhanced formation of new neurons in the hippocampus. [162].

4.3.3 VEGF in depression

A multitude of studies have investigated the plasma concentration of VEGF in MDD patients, but the data and interpretation remain conflicting possibly due to the differences in study design [163]. Chronic stress in rats decreased the expression of VEGF and its receptor in the hippocampus [164]. Furthermore, VEGF is required for the proliferation of neural stem-like cells in the hippocampus following ECT treatment [165, 166]. In a similar manner, VEGF also seems to be required for the behavioral action of various antidepressants drugs in rodent models of depression [167-170].

4.4 Glial cell line-derived neurotrophic factor or GDNF

4.4.1 General function

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. It is a member of the transforming growth factor β (TGF-β) superfamily and is important for neuronal survival especially for dopaminergic and serotonergic neurons. It binds to the GDNF-family receptors α1 (GFRα1) activating tyrosine kinase signalling [171].

4.4.2 GDNF in neuroplasticity

Experimental studies in animal models evidenced a neuroprotective role of GDNF, and ICV infusion of GDNF increased progenitor cell proliferation in the DG [172] and SVZ [173]. Similarly, infusion of GDNF in the striatum of rats increased progenitor cell proliferation in the hippocampus and substantia nigra [174]. Moreover, GDNF induced differentiation of DG-derived neural precursors into astrocytes *in vitro* [175]. Further, the use of an adeno-associated viral vector that induced overexpression of GDNF in the rat cortex provided neuroprotection against ischemia-induced injury [176].

4.4.3 GDNF in depression

Only a few clinical studies investigating the role of GDNF in depression have been reported, and contrasting findings between brain and blood expression remain [171]. A recent post-mortem brain analysis showed an increase of (GDNF) expression in the parietal cortex of depressed patients [177]. In contrast, decreased serum GDNF levels have been associated with depression [178-180], displaying a negative correlation with disease severity in adolescents [179]. Similarly, lowered expression of GDNF was reported in peripheral white blood cells of depressed patients [181]. Interestingly, antidepressant treatment significantly increased the serum concentration of GDNF in depressed patients [178]. Similarly, ECT in patients with drug-resistant depression [182] was shown to increase serum GDNF. In addition, antidepressant treatment increased GDNF release in a rat C6 glioblastoma cell line [183], whereas lithium treatment in rats resulted in increased GDNF concentration in the PFC and occipital cortex, but a decrease in the hippocampus [184]. In a mouse models of depression, an increase of the *Gdnf* mRNA expression was observed in the hippocampus. This modification is partly reversed by chronic administration of agomelatine, an antidepressant. [185]. Furthermore, work on the same mouse model showed that GDNF, with other neurotrophins, could be involved in the behavioral responses to antidepressants. The same article included demonstrated that the observed decrease of *Gdnf* expression and associated depression-like behaviors in the stressed mice might be regulated by epigenetic mechanisms. Those mechanisms include a hyperactivation of HDACs and an enhancement of CpG methylation at the *Gdnf* promoter in the NAc

[186]. 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.

4.5 Insulin-like growth factor or IGF-1

4.5.1 General function

Insulin-like growth factor (IGF-1) and its receptor IGF-1R are found in many tissues including the brain. Decreased serum concentrations of IGF-1 have been associated with increased neurodegeneration [187] and IGF-1 has been designated as a potential therapeutic target for neurodegenerative diseases such as depression.

4.5.2 IGF-1 in neuroplasticity

IGF-1 induced differentiation of neuronal precursors [188, 189], and proved to be neuroprotective in cerebellar granule neurons *in vitro* [190]. 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 [191]. Developmental research in mice has revealed the importance of IGF-1 in hippocampal neurogenesis and synaptogenesis [192]. Furthermore, ICV infusion of IGF-1 ameliorated the age-related decline in hippocampal neurogenesis [193], while peripheral administration of this growth factor could selectively induce hippocampal neurogenesis in rats [194].

4.5.3 IGF-1 in depression

Studies using selective knockout mice showed that a decrease in both systemic and hippocampal IGF-1 levels could increase the susceptibility to depression [195]. Interestingly, since IGF-1 can readily pass the blood brain barrier [196], its effects on the brain can be achieved by direct injection into the blood. Thus, when administrated chronically, a peripheral injection of IGF-1 in a mouse model of depression could induce antidepressant-like behaviors, comparable to commonly used antidepressants. [197]. In addition, an increase of peripheral IGF-1 by direct injection of IGF-1 or inhibition of IGF-1 binding protein displayed anxiolytic and antidepressant effect in rodents [197-200], which might be attributed, at least in part, to increased serotonin levels in the brain [201]. Clinical studies showed somewhat inconsistent findings, but mainly revealed higher IGF-1 levels in the serum of depressed patients, which declined during effective antidepressant treatment when. Of note, intranasal administration has been proposed in order to provide a shorter path for IGF-1 to enter the brain [202], avoiding unwanted effects of IGF-1 in peripheral tissues.

4.6 VGF nerve growth factor inducible

4.6.1 General function

VGF nerve growth factor inducible (non-acronymic) is a small neuropeptide that plays a role in energy homeostasis, metabolism and synaptic plasticity. It is widely detected throughout the brain with a higher expression in the hypothalamus. It is synthesized in neuronal and neuroendocrine cells

and its expression is regulated by neurotrophin signaling. Salton *et al.* proposed a model where VFG could be released as a full length protein and bind to a receptor tyrosine kinase or it could bind to a G-protein-coupled receptor if released as a smaller peptide fragment. [203].

4.6.2 VGF in neuroplasticity

Interestingly, VGF expression was shown to be increased by exogenous BDNF administration [204] and BDNF might even be required for regional VGF expression [205]. VGF is important in synaptic plasticity [204] and it is indicated that it increases synaptic plasticity in a BDNF-dependent manner [206]. The requirement of BDNF in VGF expression and the necessity of BDNF in VGF-induced synaptic plasticity show that VGF is heavily intertwined with BDNF signaling.

4.6.3 VGF in depression

A recent study showed that depressed patients displayed lower expression of VGF in leukocytes, which could be reversed by antidepressant treatment [207]. Moreover, VGF expression in the hippocampus and PFC, like the neurotrophins described above, is decreased in animal models of depression [207, 208]. Interestingly, infusion of VGF in the hippocampus [209] or lateral ventricle [210] had an antidepressant behavioral effect in rats. This effect may be related to increased neurogenesis, which occurred after only 7 days of VGF administration into the hippocampus [208]. In addition, VGF expression can be upregulated by exercise [210] and ECT [211, 212]. Antidepressant treatment also increased VGF expression in the hippocampus and PFC of rats [207].

4.7 Nerve growth factor or NGF

4.7.1 General function

Nerve growth factor [134] is a growth factor first described as a neurite outgrowth factor [213]. Later, NGF proved to be involved in neuronal repair and survival [214-217]. More recently, NGF has been implicated in neurogenesis in the striatum [218, 219], hippocampal plasticity [220]. As well as proliferation and differentiation of neuronal stem cells [221]

4.7.2 NGF in depression

To date, only a few studies have investigated the role of this trophic factor in depression. Serum levels of NGF are lowered in elderly MDD patients [222], while antidepressant treatment using duloxetine, a serotonin-norepinephrine reuptake inhibitor (SNRI), could restore altered levels of NGF in major depressed patients [223]. However, this effect was not found in a clinical study using SSRIs [224]. In the Flinders Sensitive Line (FSL) rat model of depression, ECT was found to increase NGF levels in the hippocampus [225]. This increase was not observed in the flinders resistance line, i.e. control rats. In addition, NGF injections show antidepressant effects [226].

5 Future directions: targeting growth factor signaling with synthetic small molecules.

Over the last decades, all the findings have shown that the BDNF/TrkB signaling pathway seems to be a main actor in the mechanism of action of mood disorders and more specifically depression. But because of the poor efficacy in some non responsive depressive patients and the delayed therapeutic effect, researchers keep working to find another pathway involved in depression, in order to target it with new treatments. However, the use of BDNF turned out to be rather difficult because of its unfavorable pharmacokinetic profile. Indeed, it hardly crosses the blood brain barrier and has a very short half-life. Thus, laboratories started to develop new molecules that can either bind the TrkB receptor or partially block the aforementioned receptor [227].

5.1 TrkB receptor agonists

5.1.1 7,8 dihydroxyflavone (DHF)

One of the main agonists that have been studied so far is 7,8 dihydroxyflavone (DHF). It was first described as an antioxidant and then identified as a TrkB agonist after a screening of a flavone derivatives [228]. Many experiments have been performed and have revealed promising results regarding antidepressant-like properties of this new molecule. Recent studies showed DHF had antidepressant effects in mice displaying LPS-induced depressive-like behavior in the TST and the FST [229]. In the learned helplessness model of depression in rats, a single bilateral infusion of DHF, in various subregions of the hippocampus and in the infra limbic medial PFC, induced antidepressant effects. However, these effects were not seen in the prelimbic prefrontal cortex [230]. Furthermore, DHF has been shown to normalize dendritic spines structure in an LPS mouse model of depression [229], and to increase neurogenesis in the hippocampus [231]. Besides its effect on depression, further studies have tested DHF in cognition. Indeed, restoration of memory deficits in 5XFAD and APP/PS1 mouse models of Alzheimer's disease have been shown after an acute and chronic administration with DHF [232-234], while it did not seem to exert this effect when injected chronically in APP23PS45 transgenic mice [235].Given the fact that cognitive deficits are part of MDD, it is not surprising to observe a recovery of memory deficits in animal models of Alzheimer disease after administration of DHF [236]. Liu and colleagues optimized the molecule and showed an even more pronounced antidepressant effect in both the FST and TST [231, 237]. Altogether, DHF's *in vivo* antidepressant effects in rodents makes it a promising compound to treat mood disorders more efficiently.

5.1.2 Deoxygedunin

Gedunin is a tetranirtriterponoid isolated from the Idian neem tree. Its derivative, deoxygedunin seems to be a promising selective TrkB agonist, showing protective effects against apoptosis both *in vitro* and *in vivo*, as well as antidepressant effects after subchronic treatment,

displaying reduced immobility in the FST [238].

5.1.3 Other promising agonist compounds

LM22A-4 TrkB agonist was designed to mimic the loop II domain of BDNF and proven to be a partial agonist. In vitro study showed that LM22A-4 has neuroprotective properties [239]. It was first shown as a good molecule to reverse respiratory abnormalities [240, 241] and improve functional recovery after a stroke [242]. Furthermore, it has also been tested in mice with compulsive alcohol drinking and shown to reverse this uncontrolled and excessive drinking. TDP6, another BDNF mimetic molecule designed by Wong and colleagues, has shown to promote oligodendrocyte myelination in vitro. [243]. 29D7 is a TrkB agonist antibody which showed an enhancement of the neuronal survival and neurotic outgrowth in vitro and in vivo and provided a long-lasting neuroprotection against neonatal model of hypoxic-ischemic brain injury [244, 245]. Another antibody targeting TrkB has been designed, TAM-163, which is a partial agonist but only tested as an agonist agent for body-weight regulatory disorders. It has been shown that, with a low dose, TAM-164 could decrease the body weight in rodents and dogs and increase it in non-human primates [246]. Another way to activate Trk receptors is to potentiate their neurotrophic-mediated activation. BMS355349 has been described as a selective potentiator of NT-3 mediated TrkA and TrkB receptor activity, and has proven to induce neuritogenesis [247, 248]. Interestingly, TrkA also seems to be a promising receptor to target. Thus the ganbogic amide which was revealed as a trkA agonist by NGF mimetic [238] could exert neurotrophic effects in an in vitro study [234].

To sum up, further investigations are required regarding the action of TrkB agonists in mood disorders, but their observed neuroprotective effects so far are promising.

5.2 TrkB receptor antagonists

Some studies use partial antagonists as a treatment for diseases related to nervous system dysregulations. From our knowledge, only ANA-12 and the cyclotraxin B, described as selective antagonists for TrkB receptor, have been tested so far.

5.2.1 ANA-12

ANA-12 is a selective partial antagonist that was first developed by Carzola and colleagues in 2011 ([47, 97] In their study, the authors identified a low-molecular weight antagonist of TrkB that could induce anxiolytic and antidepressant properties. While ANA-12 was mainly used as a tool to block the BDNF/TrkB complex in order to better understand the mechanism of action of TrkB signaling [249], two recent studies tried to use it as a therapeutic tool. Therefore, when infused bilaterally in the NAc, ANA-12 showed antidepressant properties in LPS-treated mice, as well as a blockage of the phasic stimulation which induced an inversion of social avoidance behavior [229, 250]. Moreover, when administrated intraperitoneally and alone, ANA-12 could decrease the immobility time in TST and FST in the same LPS mice.

5.2.2 Cyclotraxin B

Cyclotraxin B is an antagonist that has been mainly used to antagonize the BDNF/TrkB complex. Nevertheless, while one study has observed anxiolytic properties, this molecule did not seem to display clear antidepressant effects [251] and may be more suitable for the treatment of neuropathic pain [252]. (In spite of these interesting results, the antagonists could also induce cell death that might be an issue when used as a chronic treatment. [97, 253]

To conclude, the use of partial agonists or antagonists in order to treat mood disorders seems to be a promising avenue of research. However, whether we should agonize or antagonize the pathway is not yet well defined. Also, further investigations have to be achieved regarding the role of the BDNF/TrkB complex in mood disorders, in order to have a better knowledge of how to correct the dysregulation of this system in diseases.

6 Concluding remarks

Growth factors and associated neurotrophic signaling play an essential role in the development and maintenance of the central nervous system [254-256]. Accordingly, there is growing evidence that abnormal trophic support in cortico-limbic regions regulating mood and emotions might account in the pathophysiology of depression [257]. In addition, clean-cut evidence showed that antidepressants require neuroplasticity pathways to rescue the observed deficits in neuronal and synaptic plasticity often associated with mood disorders [7]. However, current knowledge makes it difficult to conclude whether neuroplasticity and neurogenesis represent a cause or a consequence (or both) of the pathological processes associated with depression. Hence, future research should focus on elucidating the exact involvement of neurotrophic signaling 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 [258]. In line with this hypothesis, the usage of N-methyl-D-Aspartate (NMDA) receptor antagonists showed that acute induction of neuroplasticity pathways such as BDNF and mTor signaling was sufficient to produce a robust and prolonged antidepressant effect [259, 260]. Hence, the rapid enhancement of hippocampal neuroplasticity – involving dendritic growth, spine density and synaptic transmission - represents an original strategy to circumvent the delayed efficacy of current antidepressant drugs. Finally, the use of drugs that specifically target neurotrophic signaling should provide more insights on the involvement of neuroplasticity pathways in the mediation of antidepressant responses. As growth factors and neurotrophins generally display a poor blood-brain barrier penetration and a short half-life in plasma, [261-263], identification of small non-peptidic neurotrophin mimetics albeit challenging on its own, may represent an interesting target for the development of a new class of therapeutic agents for mood-related disorders.

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Chapter 3:

Keeping 'Trk' of 5-HTT-dependent and -independent effects of fluoxetine-induced neuroplasticity

Marion J.F. Levy [1,2], F. Boulle [1,2], Michel-Boris Emerit [1], Corinne Poilbout [1], Harry W. Steinbusch [3], Daniel Van den Hove [3,4], Gunter Kenis [3], Laurence Lanfumey [1,2]

[1] Centre de Psychiatrie et Neurosciences, Inserm U 894, Paris, France

[2] Univerité Paris Descartes, UMRS894 Paris, France

[3] Maastricht University - European Graduate School for Neuroscience EURON, Department of Psychiatry and Neuropsychology, Maastricht, The Netherlands

[4] Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Fuechsleinstrasse 15, 97080 Wuerzburg, Germany

Author for correspondence

L. Lanfumey

Inserm U894, Centre de Psychiatrie et Neurosciences

102-108 rue de la Santé

75014 Paris France

E-mail: laurence.lanfumey@inserm.fr

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Abstract

Selective serotonin reuptake inhibitors (SSRIs) are among the most prescribed antidepressants. Fluoxetine is the lead molecule which has been claimed to exert its therapeutic effects, at least in part, by promoting neuroplasticity through increased brain-derived neurotrophic factor (BDNF) / tropomyosin-related receptor kinase B (TrkB) signaling. It is unclear however, to which extent the neuroplastic effects of fluoxetine are in fact mediated by inhibition of the serotonin transporter (5-HTT) and subsequent altered BDNF/TrkB signaling.

This study examines the effects of fluoxetine on neuroplasticity both in presence and absence of the 5-HTT. Using western blotting and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) approaches, we showed that in primary cortical neurons from C57BL/6J mice, fluoxetine (10µM) activated BDNF/TrkB signaling pathways and that this effect could be blocked by the parallel administration of a kinase inhibitor. In primary cortical neurons from both 5-HTT knock-out (KO) and wild-type (WT) mice of the same C57BL/6J strain, fluoxetine (10 µM) also activated BDNF and plasticity-related genes. *In vivo*, 3 weeks of fluoxetine (15 mg/kg/d; i.p.) treatment lead to an increased expression of plasticity genes, including *Bdnf*, in brains of both WT and 5-HTT KO mice as well as an increased neurogenesis especially in 5-HTT KO mice.

Our results suggest that neuroplasticity induced by fluoxetine is not solely dependent on the 5-HTT, but might in fact rely, at least in part, on 5-HTT-independent TrkB receptor transactivation.

1 Introduction

Depression is a complex and heterogeneous neuropsychiatric disorder with a high prevalence and incidence [1]. Despite decades of research, the neurobiological mechanisms underlying its pathogenesis are still not fully understood and several hypotheses have been proposed. These theories have been well described in many reviews [2, 3]. The first and most studied one is the monoamine hypothesis which postulates that depression is related to disturbances in monoaminergic neurotransmission of serotonin (5-HT), dopamine (DA) and noradrenaline (NA). More recently, other hypotheses have been introduced such as the corticosteroid hypothesis, involving stress-induced hyperactivity of the hypothalamic–pituitary–adrenal axis (HPA) axis, and the neurotrophin hypothesis. This latter theory has been described after observing decreased neurogenesis and impaired neurotrophic factor production in depressed patients. As such, depression has been linked to a decrease in brain-derived neurotrophic factor (BDNF) expression in postmortem brains and in the serum of depressed patients [4-6] which is in line with the impaired neuroplasticity observed in depression, characterized for example by hippocampal volume loss, neuron dendrite shrinkage and glial cell loss [7, 8].

Despite the limited understanding of this disorder, many treatments have, to a certain degree, proven to be efficient, including psychotherapy, electroconvulsive therapy and pharmacotherapy by means of antidepressants. However, antidepressant treatment still poses various limitations such as its delayed effect and lack of efficacy in about one third of patients [9]. One of the most prescribed antidepressants is fluoxetine, a selective serotonin reuptake inhibitor (SSRI) aimed at increasing extracellular 5-HT levels by blocking the presynaptic 5-HT transporter (5-HTT). The beneficial effects of fluoxetine have been shown to be dependent, at least in part, on BDNF and its high affinity receptor, tropomyosin related kinase B (TrkB) [10-13]. BNDF is a neurotrophin that plays a key role in the regulation and maintenance of the central nervous system. It activates three main signaling pathways, i.e. the mitogen-activated protein kinase (MAPK), phospholipase C-γ (PLCy) and phosphoinositide 3-kinase (PI3K) pathways through activation of the TrkB receptor thereby promoting cellular survival, proliferation and differentiation as well as synaptic plasticity [14].

Interestingly, the neurogenic effects of fluoxetine have recently been suggested to be partly independent of 5-HTT blockade [15]. However, to which extent such 5-HTT-independent effects involve altered BDNF/TrkB signaling is unknown to date.

The goal of the present study was to further dissect the mechanisms of action of fluoxetine in order to better understand to which extent 5-HTT and TrkB/BDNF signaling interact in mediating the neuroplastic effects of this antidepressant. To this aim, we performed both *in vitro* and *in vivo* experiments using wild-type (WT) and 5-HTT knock out (KO) mice and investigated the effects of fluoxetine on the TrkB receptor and its impact on markers of neuroplasticity

2 Materials and methods

All procedures concerning animal care and treatments were carried out in accordance with the protocols approved by the ethical committee # C2EA -05 Charles Darwin for the use of experimental animals and were licensed by the Directorate General for Research and Innovation (French Ministère de l'Enseignement Supérieur et de la Recherche), under protocol authorization # 00966.02.

2.1 Animals

C57BL/6J mice were purchased from Charles River Laboratories (L'Arbresles, France). Experiments were performed using homozygous 5-HTT KO (-/-) (KO), and WT 5-HTT+/+ (WT) littermates born from heterozygous (+/-) mutants, bred on a C57Bl/6J background. Genotyping was performed as described in detail in Mannoury La Cour et al. (2001) [42]. Experiments were started at 2-3 months of age when body weight in each genotype equally ranged between 20 and 25 g. After weaning at postnatal day 30, males were housed separately in groups of 6 animals per cage and maintained under standard laboratory conditions (22 ± 1°C, 60% relative humidity, 12-12 hour light-dark cycle, food and water ad libitum).

2.2 Drug treatment and brain collection

Ten-week-old WT and KO male mice were injected daily with fluoxetine (15 mg/kg) or saline solution (0,9% NaCl) for 3 weeks. Mice were sacrificed by cervical dislocation 24 hours after the last injection. The hippocampus and frontal cortex from the left hemisphere were collected and immediately frozen in liquid nitrogen and stored at -80°C until use. The right hemisphere was immediately frozen in isopentane -30 °C and stored at -80°C until use.

2.3 Primary cell cultures

Embryos from pregnant Swiss CD1 and C57BL/6J WT strains as well as from KO mice were dissected at embryonic day 16. The dissection of the cortices was performed in Neurobasal medium (Gibco, Thermo Fisher Scientific, Courtaboeuf, France), and, after collection, the tissues were maintained in Hank's Balanced Salt Solution (HBSS) (Gibco, Thermo Fisher Scientific, Courtaboeuf, France) until trypsinization. Dissociation of the tissues was first achieved with trypsin (Sigma Aldrich, Saint Quentin Fallavier, France), and then terminated with a mechanical dissociation with a glass pipette. Cells were counted and plated on poly-D-lysine (Sigma Aldrich, Saint Quentin Fallavier, France) -coated 6-well plates at a density of 1000 cells/mm^2 for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) purposes, or on poly-D-lysine-coated 25cc flasks at a density of 1600 cells/mm^2 for western blotting. Cells were cultured in complete Neurobasal medium supplemented with B27 (Gibco, Thermo Fisher Scientific, Courtaboeuf, France), containing 0.5 mM L-glutamine, 10 U/ml penicillin G, and 10 mg/ml streptomycin (Gibco, Thermo Fisher Scientific, Courtaboeuf, France), and used for experiments on the fifth day *in vitro* 5 (DIV5).

2.4 Quantitative reverse transcriptase PCR (qRT-PCR)

Cells were incubated with fluoxetine (10 µM) or BDNF (1 nM) (Peprotech, Neuilly-Sur-Seine, France) in presence or absence of K252a (100 nM) (Abcam, Paris, France) for 1 hour. Total mRNA extraction was performed using NucleoSpin RNA II kit (Macherey-Nagel, Hoerdt, France). To isolate the total RNA from the hippocampus and the frontal cortex of WT and KO mice, TRIzol© RNA Isolation Reagent (Invitrogen) was used. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France) according to the manufacturer's protocol. Amplification was performed with KAPA SYBR FAST qPCR kit (KAPABIOSYSTEMS, Clinisciences, Nanterre, France) using 7300 Real Time PCR System (Applied Biosystems, Courtaboeuf, France). The sequences of primers used are indicated in Supplementary table 1. qRT-PCR conditions involved 40 cycles in a fixed sequence, i.e. 95°C for 15 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 expression. Gene expression was normalized using *Actb* as a reference gene.

2.5 Western Blots

Protein expression levels for TrkB, P-TrkB, Akt, P-Akt, Erk, P-Erk and the reference protein GAPDH were assessed. Cells from WT mice were incubated for 1h with BDNF (1 nM) or fluoxetine (10 uM). After incubation, cells were washed with ice cold PBS (Gibco, Thermo Fisher Scientific, Courtaboeuf, France) and proteins were extracted using an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, phosphatase inhibitor: Na_3VO_4 (1 mM), NaF (10 nM), protease inhibitor [Roche, Meylan, France]). The lysate was then centrifuged for 15 min at 14,000 g and the supernatant was collected. The protein concentration was determined by the Lowry protein assay (DC protein assay, Bio-Rad Hercules, USA). The extract was mixed with sample buffer (5:1, v/v) containing 188 mM trisHCl, 6% SDS, 30% glycerol, 15% β-mercaptoethanol, and 0.01% bromophenol blue. The mix was heated for 5 minutes at 95°C. 20 µg of proteins were then separated in a 10% SDS-PAGE gel and transferred on a nitrocellulose membrane for 1h at 100 mV. The membranes were blocked with 1:1 v/v Odyssey blocking buffer (Li-Cor, Lincoln, USA) in PBS for 1h at room temperature (RT), incubated with the primary antibody overnight at 4°C and, subsequently, with the secondary antibody for 1h at RT. The dilution and reference of the antibodies are indicated in supplementary table 2. The membranes were finally scanned using the Odyssey system (Odyssey, Li-cor Biosciences, Leusden NL) and analyzed with ImageJ.

2.6 Immunohistochemistry

Brains were cut into 20-µm-thick coronal sections. Frozen hippocampal sections (from Bregma -1.34 to Bregma -3.20) were fixated with 4% paraformaldehyde (in 0,1 M PBS) for 10 min at 4°C, rinsed with TBS, incubated with 0.3% H_2O_2 (in TBS) for 30 min at RT, and subsequently

incubated with a rabbit anti-ki67 antibody (1/4000; Abcam, Paris, France) at 4°C overnight. The second day, the sections were rinsed with TBS-T and TBS, and incubated with the secondary antibody for 1h at RT (1/800), with Vectastain ABC kit for 45min at RT and with 3,3'-diaminobenzidine (DAB) for 10 min at RT in the dark. The sections were then rinsed successively in 70%, 90%, 100% ethanol, and Histoclear, after which they were mounted, and visualized using a microscope. Ki67 positive cells were counted using the ImageJ software.

2.7 Statistical analysis

Data are represented as mean ± SEM. For dose-response curves, a non-linear regression analysis involving a log(agonist) vs. response equation fit was used (Prism 5.0, GraphPad software, USA). A Student's t test was used for comparison between two groups. When there were more than two conditions, a one-way analysis of variance (ANOVA) was used, followed by Bonferroni *post hoc* testing in order to compare between all the groups. In addition, a two-way ANOVA (treatment x genotype) was used to investigate the effect of the chronic administration of fluoxetine in WT and KO mice. The level of significance was set as $P < 0.05$.

3 Results

3.1 Acute effects of fluoxetine in primary cortical neurons

1.1.1 Bdnf mRNA expression

In order to validate the acute effect of fluoxetine on *Bdnf* expression, we used cells from two different mouse strains, i.e. C57Bl/6J and Swiss CD1 mice. As illustrated in Figure 1A, 10 µM fluoxetine significantly increased *Bdnf* expression in primary cortical neurons from Swiss CD1 mice in a time-dependent manner (two-way ANOVA with Bonferroni post-hoc test in Swiss CD1 mice: treatment x time interaction: $F(3,85) = 12.13$, $P<0.0001$; treatment: $F(1,85) = 208.4$, $P<0.0001$; time: $F(1,85) = 12.04$ $P<0.0001$). As in the *in vivo* part of our study we used mice with a C57BL/6J background, we performed an additional batch of experiments on primary cortical neurons from this strain. As shown in Figure 1B, like in Swiss CD1 derived neurons, fluoxetine increased *Bdnf* expression (two-way ANOVA with Bonferroni post-hoc test: treatment x time interaction: $F(3,39) = 18.04$, $P<0.0001$; treatment effect : $F(1,39) = 114.1$, $P<0.0001$, time effect : $F(3,39) = 17.84$, $P<0.0001$).

1.1.2 TrkB, Akt, Erk phosphorylation

In order to know whether fluoxetine can activate TrkB signaling, primary cortical neurons from C56Bl/6J were cultured and stimulated with fluoxetine (10 µM) or BDNF (10 nM), as a control, for 1h. As expected, BDNF stimulation was able to increase TrkB activation as well as its downstream signaling, with an increase of P-Akt and P-Erk proteins (t-test: P-TrkB $t(11) = 5.40$, p =

0.0002; P-Akt T(12) = 3.26, P=0.006; P-Erk t(12) = 6.97, P<0.0001) (Figure 2, left panel). Similarly, stimulation with fluoxetine revealed an increase in P-TrkB levels (t-test: t(11) = 2.42, P = 0.034) as well as a strong tendency to P-Akt upregulation (t-test: t(13) = 2.10, P = 0.056). However, fluoxetine did not upregulate P-Erk (t-test: t(13) = 0.33, P = 0.75) (Figure 2, middle panel).

1.1.3 Expression of immediate early genes

To further investigate the role of TrkB in mediating the effects of fluoxetine on plasticity, primary cortical cells were incubated with BDNF or fluoxetine in absence or presence of K252a, a kinase inhibitor, and the expression of several immediate early genes, i.e. *Arc*, *Egr1* and *cFos,* as markers of plasticity, were measured. As presented in Figure 3A, one-way ANOVA showed that treatment with BDNF (1 nM for 1 hour) induced *Arc*, *Egr1* and *cFos* mRNA expression changes (*Arc*: F(3,8) = 258.4, P<0.001; *Egr1* F(3,8) = 566.6, P<0.0001; *cFos*: F(3,8) =198.4, P<0.001). A Bonferroni post-hoc test revealed a massive increase in expression of these genes after BDNF exposure (Control vs BDNF: *Arc*: t(4) = 22.80, P<0.001; *Egr1* t(4) = 32.92, P<0,001; *cFos:* t(4) = 15.20, P<0.001). Co-incubation with K252a completely blocked the expression of *Arc* (t(4) = 0.04086, P>0.05) and *Egr1* (t(4) = 1.204, P>0.05), while *cFos* expression was decreased (Control vs K252a+BDNF: t(4) = 8.847, P<0.001). When cells were incubated with fluoxetine in the presence or absence of K252a (see Figure 3B), one-way ANOVA also revealed an overall effect of fluoxetine on *Arc*, *Egr1* and *cFos* expression (*Arc*: F(3,44) = 4.459, P<0.01; *Egr1*: F(3,49) = 26.04 P<0.0001; *cFos*: F(3,8) = 31.28, P<0.0001). A Bonferroni post-hoc test showed that fluoxetine induced an increase of *Egr1* and *cFos* expression (Control vs fluoxetine: *Egr1:* t(34) = 4.798, P<0.001; *cFos:* t(33) = 3.431, P<0.0001). This effect was reversed when K252a was added to the medium with a downregulation of *Egr1* and *cFos* expression (Control vs K252a+fluoxetine: *Egr1:* t(22) = 3.313, P<0.05; *cFos:* t(21) = 5.250, P<0.0001). However the Bonferroni post-hoc test revealed that *Arc* mRNA expression was not significantly altered by fluoxetine (Control vs fluoxetine: t(34) = 1.773, P>0.05) but was decreased with the addition of K252a (Control vs K252a+fluoxetine: t(19) = 3.225, P<0.05).

1.1.4 Acute effects of fluoxetine in 5-HTT KO primary cortical neurons

In order to examine the role of the 5-HTT in mediating the plasticity-related effects of fluoxetine, we replicated the aforementioned experiments in primary cortical neurons from 5-HTT KO mice. 10 μM fluoxetine induced an increase in *Bdnf* expression in cells from both WT and 5-HTT KO mice after 4h of incubation (two-way ANOVA: effect of treatment: F(1,19) = 53.09, P<0.001). Neither an effect of genotype, nor an interaction between genotype and treatment was observed (two-way ANOVA, effect of genotype: F(1,19) = 0.05699, P = 0.8139; Genotype x treatment interaction: F(1,19) = 0.08302, P = 0.7764), suggesting that fluoxetine could exert its effects on *Bdnf* even in the absence of 5-HTT. (Figure 4).

3.2 Chronic effects of fluoxetine on plasticity in WT and 5-HTT KO mice

1.1.5 BDNF/TrkB signaling related gene expression

In order to confirm these *in vitro* results, the effects of fluoxetine were also investigated in *in vivo* experiments using WT and 5-HTT KO mice. Mice were i.p. injected with saline or fluoxetine (15 mg/kg/d) for three weeks. qRT-PCR experiments were performed on both the hippocampus and the frontal cortex of the left hemisphere of each mouse. In the hippocampus, a two-way ANOVA revealed a treatment effect with an increase in *Bdnf* mRNA expression ($F(1,35) = 11.87$, $P = 0.0015$), but no effect of genotype ($F(1,35) = 0.255$, $P = 0.616$) nor an interaction ($F(1,35) = 2.42$, $P = 0.1288$). Chronic fluoxetine did not modify the expression of *Bcl2* (two-way ANOVA, effect of treatment: $F(1,35) = 1.662$, $P = 0.2058$; effect of genotype: $F(1,35) = 0.08148$, $P = 0.7692$; treatment x genotype interaction: $F(1,35) = 3.129$, $P=0.0856$), *Creb* (two-way ANOVA: effect of treatment: $F(1,35) = 0.5533$, $P = 0.4621$; effect of genotype: $F(1,35) = 3.662$, $P = 0.0641$; treatment x genotype interaction: $F(1,35) = 0.02366$, $P = 0.8787$) *or Trkb* (two-way ANOVA: treatment: $F(1,35) = 0.3263$, $P = 0,5734$; genotype: $F(1,35) = 0.08824$, $P=0.7691$; treatment x genotype interaction: $F(1,35) = 0.06707$, $P = 0.7980$) (Figure 5A). In the frontal cortex, fluoxetine upregulated *Bdnf*, *TrkB, Creb* and *Bcl2* mRNA expression in both fluoxetine-treated WT and 5-HTT KO mice (two-way ANOVA *Bdnf*: $F(1,35) = 138.0$, $P<0.0001$; *Trkb*: $F(1,24)=20.38$ $p<0.0001$; *Creb*: $F(1,37) = 32.53$, $P<0.0001$; *Bcl2*: $F(1,35) = 13.28$, $P<0.001$). *Bdnf* and *TrkB* expression also showed a treatment x genotype interaction (*Bdnf*: $F(1,35) = 8.488$, $P = 0.0062$, *TrkB*: $F(1,24) = 12.64$ $P = 0.0016$). A Bonferroni post-hoc revealed a higher expression of *Bdnf* and *TrkB* in the fluoxetine-treated 5-HTT KO mice (*Bdnf*: Control vs fluoxetine: WT mice: $t(20) = 6.709$, $P<0.001$; 5-HTT KO mice: $t(15) = 9.795$, $P<0.001$; *TrkB*: Control vs fluoxetine: WT mice $t(13) = 0.7041$ $P>0,05$; 5-HTT KO mice $t(11) = 5.510$, $P<0.001$). There was no effect of genotype (*bdnf*: $F(1,35) = 0.05875$, $P = 0.8099$; *TrkB*: $F(1,24) = 0.7872$, $p<0.3838$). Regarding *Creb* and *Bcl2*, together with the treatment effect, an effect of the genotype was observed with a decreased expression in the 5-HTT KO mice (two-Way ANOVA: *Creb*: $F(1,37) = 174,3$ $P<0,0001$; *bcl2*: $F(1,35) = 124.6$ $P<0.0001$), but no genotype x treatment interaction (two-way ANOVA: *Creb*: $F(1,37) = 2.503$ $P<0.0001$; *bcl2*: $F(1,35) = 0.0009581$, $P<0.0755$) (Figure 5B).

1.1.6 Hippocampal cell proliferation

We used the right hemisphere of the same mice to study fluoxetine-induced plasticity at a cellular level. To this aim, we investigated the effect of fluoxetine on the number of proliferative cells within the hippocampus of WT and 5-HTT KO mice. As shown in Figure 6, the data revealed an increase of the number of proliferative cells as a results of fluoxetine treatment (two-way ANOVA, treatment effect $F(1;21)=4,380$ $P=0,0487$). No genotype effect, nor a genotype x treatment interaction was observed, although the observed statistical trends suggest an increase in cell proliferation

specifically in fluoxetine-treated 5-HTT KO mice (two-way ANOVA, genotype effect: $F(1,21) = 3.830$, $P=0.0638$; genotype x treatment interaction: $F(1,21) = 3.830$, $P = 0.0638$).

4 Discussion

Although fluoxetine is one of the most prescribed antidepressants, its effects on neuroplasticity are still not fully understood. In the past decades, studies revealed that together with its ability to increase the extracellular concentration of 5-HT by inhibiting the 5-HTT, fluoxetine exerts anti-inflammatory, anti-apoptotic and antioxidant actions via different mechanisms of action [16]. More generally, fluoxetine has been shown to be strongly involved in increasing neuroplasticity [17], but it is yet unclear whether these effects are mediated solely by 5-HTT inhibition.

In our study, we showed that the effects of fluoxetine on several markers of neuroplasticity were partially independent of inhibition of the 5-HTT. Indeed, in cultured primary cortical neurons derived from 5-HTT KO mice, fluoxetine was still able to upregulate *Bdnf*, a neurotrophin strongly involved in neuronal proliferation, survival, and differentiation as well as synaptic plasticity and related neurotransmission [14]. Moreover, *in vivo*, an increased mRNA expression of *Bdnf* and other plasticity-related genes was observed in fluoxetine-treated 5-HTT-deficient mice. Further investigation showed that fluoxetine could activate the TrkB receptor and increase BDNF-induced immediate early gene expression *in vitro*. The activation of these genes was blocked by the simultaneous administration of a kinase inhibitor, strongly suggesting a vital role for TrkB in mediating this neuroplasticity-promoting effect. Thus, it is proposed that fluoxetine partially exerts its effects on neuroplasticity via a 5-HTT-independent enhancement of BDNF/TrkB signaling.

4.1 The effects of fluoxetine on plasticity are partially 5-HTT-independent

Our data clearly suggests that fluoxetine can promote plasticity in the absence of 5-HTT. *In vitro*, fluoxetine induced an increase in *Bdnf* mRNA expression between 2 and 6 hours of incubation in neurons derived from both 5-HTT WT and KO mice. This result was already observed in a study by Seo and colleagues, [18] who treated primary hippocampal cultures with different doses of fluoxetine for 4 days. They showed that under toxic conditions, induced by B27 deprivation, 0,1 to 10 µM of fluoxetine could already increase *Bdnf* expression levels, while only 10 µM of fluoxetine was able to increase *Bdnf* expression under control conditions. Furthermore, another study using corticosterone-exposed PC12 cells showed that 5 µM fluoxetine could restore *Bdnf* mRNA expression after 24h of stimulation [19]. However, to our knowledge, this data has never been reported in primary cell cultures where the main target of fluoxetine, the 5-HTT, has been inactivated. As such, our data strongly suggests that the ability of fluoxetine to upregulate *Bndf* mRNA expression *in vitro* is, at least in part, 5-HTT-independent. In order to further investigate this hypothesis, we performed an *in vivo* study using adult WT and 5-HTT KO mice. After a 3-week treatment with fluoxetine at 15

mg/kg/d, we observed strong neurotrophic effects of this SSRI in both genotypes. Subsequently, we performed qRT-PCR experiments to study the expression of neuroplasticity-related genes in both the frontal cortex and the hippocampus as well as immunohistochemistry experiments to stain for proliferative cells in the subgranular zone of the hippocampus. Interestingly, our data indicated that fluoxetine upregulated *Bdnf, TrkB, Creb* and *Bcl2* mRNA in the frontal cortex of both WT and KO mice, while this gene expression assessment did not show significant change, except for an increased *Bdnf* expression, within the hippocampus of fluoxetine-treated mice. This result suggests a region-specific action of this antidepressant. In particular, the higher increase of *TrkB* and *Bdnf* expression observed in the frontal cortex of fluoxetine-treated 5-HTT KO mice pointed out that fluoxetine seemed to have a better effect on the plasticity-related gene expression in the frontal cortex and that it might exert its action on this structure via BDNF/TrkB. This is in line with a study using chronic mild stress (CMS) mice, another model of depression, and showing that chronic administration of fluoxetine was able to prevent the CMS-induced decrease in burst-firing rate in the pyramidal neurons of frontal cortex. This effect was coupled with an increase of BDNF protein level in the frontal cortex of CMS mice. Thus, with our results, this study highlights the involvement of the frontal cortex in the pathogenesis of depression [20].

Furthermore, we didn't observe an increase of hippocampal cell proliferation in the fluoxetine-treated WT mice. In that respect, these results are not fully in line with previous studies describing plasticity-like effect of this SSRI in the hippocampus of WT mice [21, 22]. However, in support of our findings, some studies reported that fluoxetine did not modify plasticity markers in naive conditions, i.e. in the absence of stress [23]. As such, the observed discrepancy might be related to the state of the control mice (level of stress in the animal facility) or the duration of the treatment. Indeed, while a 3-week treatment failed to induce an increase of plasticity-related genes and proliferation cells in control mice [23-25], plasticity was shown to be improved only after a 4-week treatment in mice [26-28].

Interestingly, for the first time to our knowledge in these transgenic mice, we observed an increase in *Bdnf* mRNA expression after the 3-week treatment with fluoxetine together with an increase of cell proliferation. These results suggest that in absence of 5-HTT, fluoxetine is still able to act on neuroplasticity. In fact, others have suggested that the absence of 5-HTT would speed up the action of fluoxetine, based on a study in which a more rapid antidepressant-like response was found after partial 5-HTT silencing by the use of 5-HTT siRNA [29] **.** Along similar lines, it has been shown that brain 5-HT concentrations were much higher in 5-HTT KO mice compared to WT mice treated with fluoxetine [30]. This increased 5-HT concentration in 5-HTT-deficient mice could potentiate the effect of fluoxetine on plasticity, perhaps through a faster desensitization of 5-HT1A autoreceptors. This theory has been supported by a study showing that the 5-HT1A receptor in the dentate gyrus is critical for exerting the effects of fluoxetine on neurogenesis and *Bdnf* mRNA expression [31]. Furthermore, whereas 5-HT1A KO mice responded to tricyclic antidepressants, these same mice did

not respond to fluoxetine. This last result pointed out that 5-HT and norepinephrine might exert their actions on neurogenesis via different pathways [21]. Moreover, when using mice with impaired 5-HT synthesis, the efficacy of fluoxetine on plasticity is strongly reduced [28]. Thus, these results are in line with the hypothesis that fluoxetine could induce plasticity with a better efficiency when 5-HT levels are high. However, this effect didn't occur in glial cells. Indeed, in an astroglia cell culture, 24h fluoxetine increased BDNF mRNA expression but in a 5-HT-independent way [32]. In this regard, the involvement of 5-HT in the action of antidepressants on the plasticity did not seem to be essential or, at least, always required.

However, in 5-HTT Met 172 transgenic mice, which represents a knock-in mouse model, whereby high-affinity interactions of many antidepressants at the 5-HTT have been ablated via knock-in substitution without disrupting 5-HT recognition or uptake [33], fluoxetine was shown to lose its plasticity effects. Although these results did not support our first hypothesis of fluoxetine-induced plasticity with a predominant 5-HTT-independent mechanism of action, since it highlights the crucial role of the 5-HTT, this study still supports our notion on 5-HT facilitating the effect of fluoxetine on plasticity, since increased plasticity was not observed in the absence of an increase in serotonin concentration.

4.2 Possible pathways by which fluoxetine acts to induce neuroplasticity

To further explore this hypothesis, we performed a series of *in vitro* investigations which strongly suggest that fluoxetine could exert its effect via a 5-HTT-indepdent increase of BDNF/TrkB signaling. Over the last few years, the idea of transactivation of the TrkB receptor by antidepressants and especially fluoxetine has garnered much attention. Indeed, it seems that TrkB can be activated indirectly and independently from neurotrophins, i.e. transactivation [34]. Both the *in vitro* increase of TrkB receptor activation and the upregulation of immediate early genes after 1h of fluoxetine incubation, an effect that was blocked in the presence of a TrkB inhibitor, confirmed this hypothesis. This notion was further supported by several other studies reporting transactivation of the TrkB receptor by antidepressants [13, 35, 36]. For example, the study by Rantamaki and colleagues (2011) showed a rapid action of imipramine (30 min, 30 mg/kg), a tricyclic antidepressant, on TrkB phosphorylation both in the presence and absence of BDNF, suggesting that antidepressants do not require BDNF to activate TrkB. Furthermore, the injection of fluoxetine for 1h at a dose of 30 mg/kg showed TrkB activation even in 5-HTT-deficient mice [37] Interestingly, in our hands, fluoxetine did not seem to activate the MAPK pathway and only moderately increased Akt phosphorylation, which might be due to the delayed action of fluoxetine on the downstream signaling pathways, only occurring after the initial TrkB phosphorylation. Although a large panel of studies agreed about a transactivation of the TrkB receptor by fluoxetine, we cannot rule out the involvement of other pathways that fluoxetine might induce to, more indirectly, activate TrkB signaling.

Evidently, in addition to the BNDF/TrkB pathway, other pathways could be involved in mediating the effects of fluoxetine. In particular, many lines of evidence point out the role of glial cells in depression. Indeed, post mortem brain analysis revealed a decrease in the number of astrocytic-like elements and decreased astrocyte density as well as an alteration of astrocyte morphology [38]. It has also been shown that fluoxetine was dependent on astrocytic function. In mouse models of depression, with deletion of Aquaportin 4 (AQP4), which has a vital role in the regulation of astroglia function, a 4-week treatment with fluoxetine could not rescue depressive-like symptoms [39]. Additionally, it is now known that glial cells can synthesize neurotrophic factors such as BDNF. This has been first proven in cultured microglial cells that could express *Bdnf* mRNA under basal conditions. This expression was increased when the cells were activated with 1µM lipopolysaccharide (LPS) for 24h [40]. These results were validated with cells activated with 100 ng/mL of LPS for 6h [41]. Moreover, fluoxetine has also been described to be able to increase *Bdnf* mRNA expression in astrocytes [32]. Therefore, fluoxetine could also exert its effect on plasticity via glial cells.

4.3 Conclusions and perspective

The present study shows that fluoxetine might induce neuroplastic effects through the 5-HTT-independent activation of BDNF/TrkB signaling. Whether this effect is mediated through a direct transactivation of the TrkB receptor and how exactly BDNF/TrkB signaling, 5-HT, glial cell function and/or other processes and pathways are interconnected in regulating fluoxetine-induced neuroplasticity awaits further research. Altogether, our data suggests that targeting BDNF/TrkB signaling represents a promising strategy for the development of new therapeutic approaches for depression.

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Figures

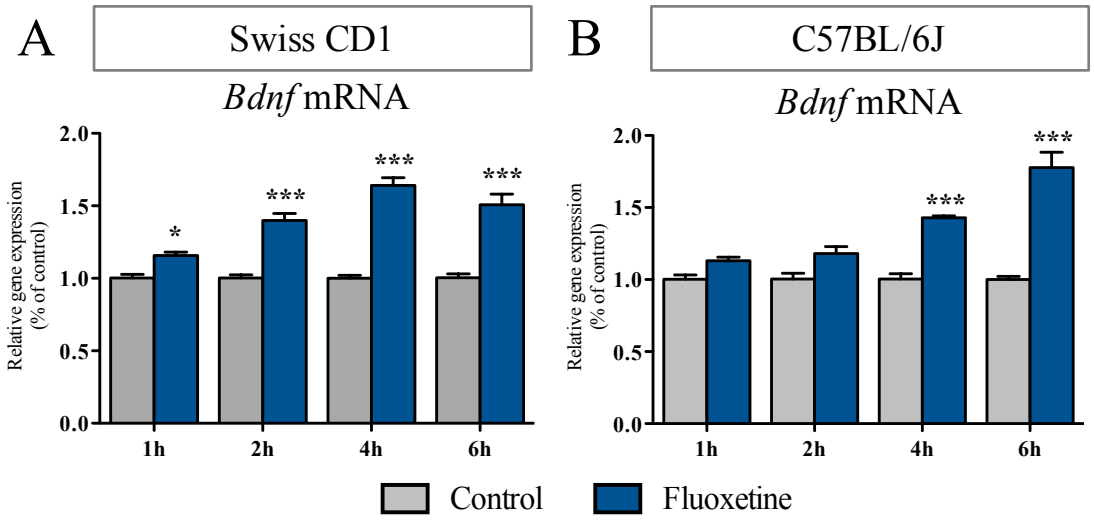


Figure 1. Effects of fluoxetine on *Bdnf* gene expression in cultured cortical neurons from Swiss CD1 and C57BL/6J mice

Cortical neurons were cultured for 5 days before the start of the experiments. Cells were incubated for 1h, 2h, 4h or 6h with 10 uM of fluoxetine in cultured medium. The expression of *Bdnf* was measured by qRT-PCR. (A) In cells issued from Swiss CD1 mouse strain, we observed an increase of *Bdnf* mRNA expression at 2h, 4h and 6h after the incubation with fluoxetine. (B) In cells issued from C57BL/6J mouse stain, an increase of *Bdnf* mRNA expression at 4h and 6h after the incubation with fluoxetine was seen. Two-way ANOVA with a Bonferroni post-hoc test. Data are expressed as mean + SEM of n=5-6. *p < 0.05, **p < 0.01, ***p<0.001 vs. CTL. WT: Wild type mice; KO: 5-HTT knock out mice

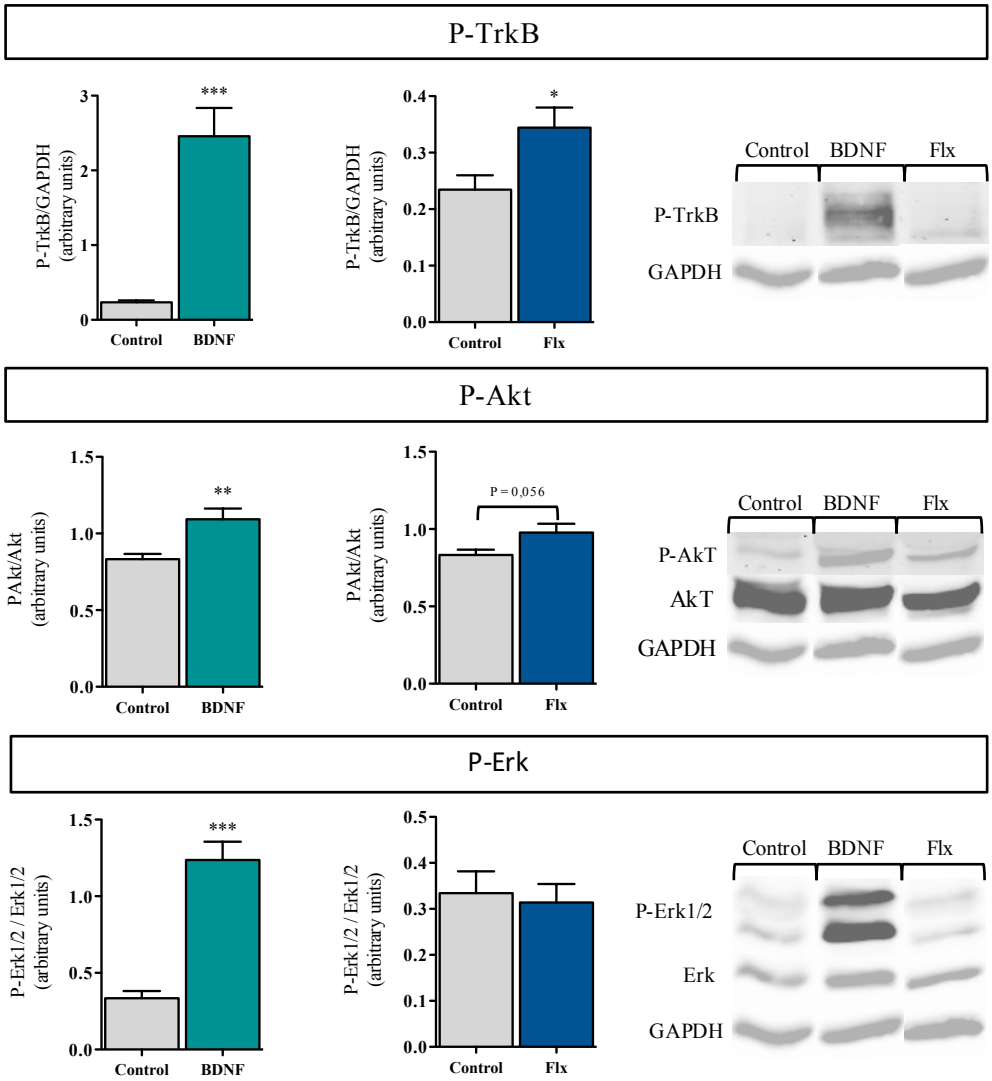


Figure 2. Effects of BDNF and fluoxetine on TrkB receptor activation and downstream signaling.

Cortical neurons were cultured for 5 days before the experiments. Cells were stimulated for 1h with 1 nM of BDNF or 10 uM of fluoxetine in cultured medium. The phosphorylation of TrkB, Erk and Akt were measured by western blot. Data indicated an increase of TrkB, Akt and Erk phosphorylation after BDNF stimulation and an increase of TrkB phosphorylation after fluoxetine stimulation. Fluoxetine failed to increase the phosphorylation of Akt and Erk. Student t-test. Data are expressed as mean + SEM of n=6-8. *p < 0.05, **p < 0.01, ***p<0.001 vs. CTL. P-TrkB: Phosphorylation of TrkB; P-Erk: Phosphorylation of Erk; P-Akt: Phosphorylation of Akt.

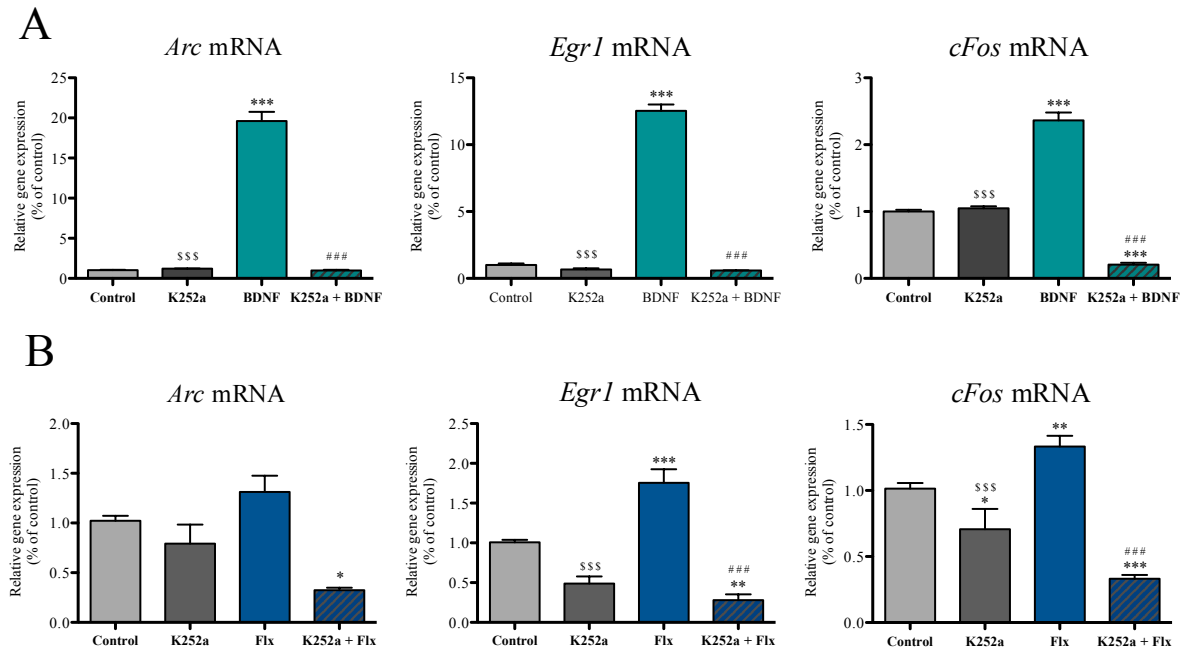


Figure 3. Effects of BDNF and fluoxetine with or without K252a on the expression of immediate early genes in cultured cortical neurons

Cortical neurons were cultured for 5 days before the experiments. Cells were stimulated for 1h with 1 nM of BDNF, 10 uM of fluoxetine, 500 nM of K252a or both K252a with fluoxetine or BDNF in cultured medium. The expression of immediate early genes was measured by qRT PCR. (A) Data indicated an increase of *Arc*, *EGR1* and *cFos* mRNA expression after BDNF stimulation which is blocked by K252a. (B) The results showed an increase of *EGR1* and *cFos* mRNA expression after fluoxetine stimulation which is blocked by K252a. Fluoxetine failed to increase *Arc* mRNA expression. One-way ANOVA with a Bonferroni post-hoc test. Data are expressed as mean + SEM of n=3-18. *p < 0.05, **p < 0.01, ***p<0.001 vs. CTL; ### p<0.001 BDNF/flx vs BDNF/flx+K252a; $$$ p<0.001 BDNF/flx vs K252a. flx: fluoxetine

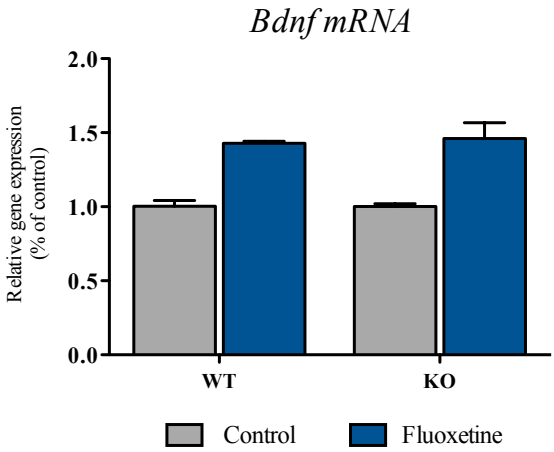


Figure 4 Effects of fluoxetine on *Bdnf* gene expression in cultured cortical neurons from WT and 5-HTT KO mice

Cortical neurons were cultured for 5 days before the experiments. Cells from WT and 5-HTT KO mice were stimulated for 4h with 10 uM of fluoxetine in cultured medium. The expression of *Bdnf* was measured by qRT PCR. Results indicated an increase of *Bdnf* mRNA expression 4h after the stimulation with fluoxetine for both cell from WT and 5-HTT KO mice. Two-way ANOVA with a Bonferroni post hoc test. Data are expressed as mean + SEM of n=5-6. *p < 0.05, **p < 0.01, ***p<0.001 vs. CTL. KO: 5-HTT knock out mice

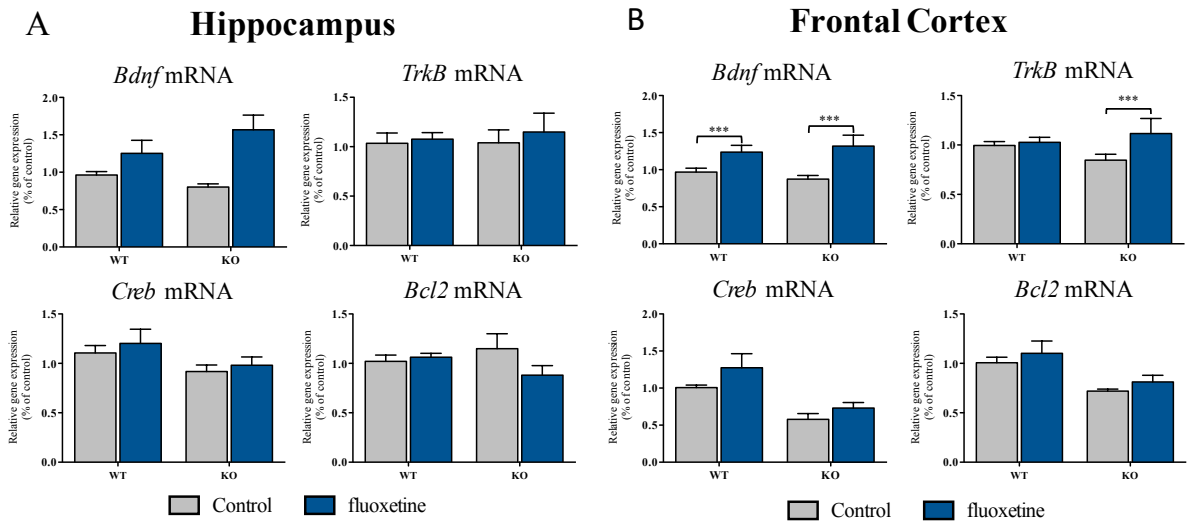
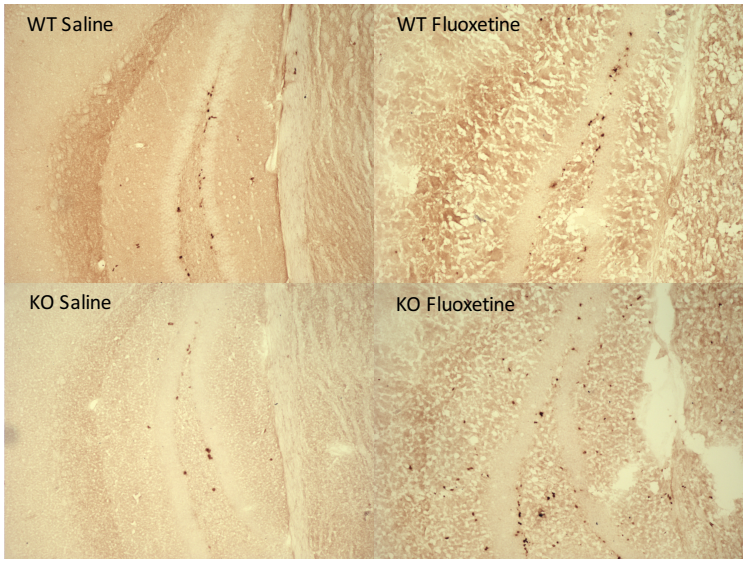


Figure 5 Effects of chronic treatment of fluoxetine on plasticity-related genes in WT and 5-HTT KO mice.

Male C57Bl/6J WT and 5-HTT KO mice were injected with fluoxetine (15mg/kg) daily for 21 days. Hippocampus and frontal cortex from the right hemisphere were collected and plasticity-related genes expression was measured by q RT PCR. (A) in the hippocampus, results indicated an increase of *Bdnf* mRNA expression in the fluoxetine treated KO mice. No significant change was observed regarding *TrkB*, *Creb* and *Bcl2*. (B) in the frontal cortex results showed that *Bdnf*, *Creb*, *Bcl2, TrkB* mRNA expression are increased in the fluoxetine-treated mice both in the WT and the KO mice. Two-way ANOVA with a Bonferroni post-hoc test. Data are expressed as mean + SEM of n=6-12. *p < 0.05, **p < 0.01, ***p<0.001 vs. CTL. WT: Wild type mice; KO: 5-HTT knock out mice.



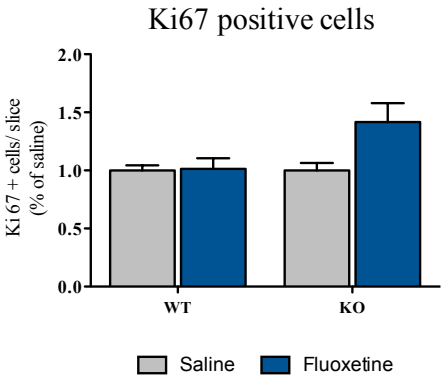


Figure 6. Effects of chronic treatment of fluoxetine on cell proliferation in WT and 5-HTT KO mice.
Male C57Bl/6J WT and 5-HTT KO mice were injected with fluoxetine (15mg/kg) daily for 21 days. Half brains were collected and Ki67-positive cells were stained by immunohistochemistry. Results indicated a significant increase in the number of Ki67 positive cells / section in the 5-HTT KO mice. Two-way ANOVA Data are expressed as mean +/- SEM of n=4-8. WT: Wild type mice; KO: 5-HTT knock out mice

Supplementary data

Table S1. Overview of primers.

gene	Forward	Reverse
Actb	CCACCATGTACCCAGGCATT	CGGACTCATCGTACTCCTGC
Bdnf	AAAACCATAAGGACGCGGAC	TAGACATGTTTGCGGCATCC
Trkb	TAGAGTGCATCACCCAGGGA	GAAGGAGGGTGTGGATGCTC
Arc	TCATTCAGTATGTGGTGGGC	GTCCTGCACTTCCATACCC
Egr1	TCAATCCTCAAGGGGAGCC	ACTCGTCTCCACCATCGC
cFos	AGGGAACGGAATAAGATGGC	TGCAACGCAGACTTCTCATC
Creb	GTTGTTATGGCGTCCTCCC	TACGACATTICTCTTGCTGCC
Bcl2	ATGACTGAGTACCTGAACCG	ATGCTGGGGCCATATAGTTC

Table S2. Overview of antibodies.

Target	Catalogue name	Supplier	Dilution
Primary antibodies Western Blot			
Erk	mouse anti-Erk1/2	Merck Millipore BV, Amsterdam-Z.O. NL	1:1,000
pErk	rabbit anti-phospho-Erk1/2	Cell Signalling Technology, Beverly USA	1:1,000
Gapdh	mouse anti-Gapdh	Fitzgerald, Huissen NL	1:2,000,000
TrkB	rabbit anti-TrkB	Abcam, Cambridge UK	1:1,000
pTrkB	rabbit anti-phospho-TrkB, Y816	Merck Millipore BV, Amsterdam-Z.O. NL	1:500
Akt	rabbit anti-Akt	Cell Signalling Technology, Beverly USA	1:500
P-Akt	mouse anti-phospho-Akt	Cell Signalling Technology, Beverly USA	1:1,000
Secondary antibodies Western Blot			
Mouse	IRDyc700 anti-mouse	Li-cor Biosciences, Leusden NL	1:10,000
Rabbit	IRDye800 anti-rabbit	Tebu-bio, Heerhugowaard NL	1:10,000

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Chapter 6:

General discussion

Both depression and Alzheimer's disease (AD) have been associated with a dysregulation of brain-derived neurotrophic factor (BDNF) / tropomyosin receptor kinase B (TrkB) signaling. For example, both disorders have been linked to a decrease in BDNF mRNA and protein expression as well as to decreased TrkB levels within the brain [1-3], concomitant with a dysregulation of signaling pathways downstream of TrkB [4, 5], thereby contributing to problems in cellular proliferation, differentiation, and survival, as well as synaptic plasticity [6, 7]. Although several studies have already been conducted, the exact role and action of BDNF in these disorders as well as its beneficial effects in view of its therapeutic potential still need to be further explored. Thus, this thesis aimed at investigating the role of BDNF/TrkB signaling in depressive-like behavior and cognition in these two different, yet strongly related diseases.

Interactions between fluoxetine, brain-derived neurotrophic factor / tropomyosin-related receptor kinase B, and serotonin

Chapter 2 presents an overview of the role of diminished neuroplasticity in depression, focusing on neurotrophic changes ranging from an anatomical to a molecular level. This review first highlights the key role of neurotrophins in mood disorders, more specifically major depression, as well as the complexity of neurotrophins' mechanisms of action. It also provides supporting evidence on the use of neurotrophins either as biomarkers, in order to allow for a better and faster diagnosis, or in therapeutic approaches. In **Chapter 3,** we examined the link between antidepressants - the main pharmacological approach used to treat depression - and neurotrophins, in particular BDNF, in more detail. For this purpose, we evaluated the effects of fluoxetine, a selective serotonin re-uptake inhibitor (SSRI), on neuroplasticity, and examined to which extent its effects were mediated by the TrkB receptor. Furthermore, this study examined to which extent fluoxetine-induced effects on BDNF/TrkB signaling were dependent or independent of the serotonin transporter (5-HTT), as a 5-HTT-independent mechanism of action had been recently suggested [8, 9]. As a main result, we concluded that, *in vivo*, fluoxetine was still able to increase plasticity-related gene expression, including that of *Bdnf*, in the cortex and the hippocampus, as well as to increase cell proliferation in the hippocampal dentate gyrus (DG), in 5-HTT-deficient mice. Furthermore, in 5-HTT-deficient primary cortical neurons, 1 hour incubation with fluoxetine still increased TrkB phosphorylation and, at least in part, signaling pathways downstream of TrkB *in vitro*. These results suggest that some of the neuroplasticity-promoting effects of fluoxetine are independent of 5-HTT and are partially mediated by fluoxetine's action on the TrkB receptor. An important point to consider in this respect is the interaction between BNDF/TrkB signaling and serotonin (5-HT). As such, we hypothesized that the increased levels of extracellular 5-HT seen in 5-HTT KO mice [10] could increase BDNF and associated hippocampal cell proliferation after chronic treatment with fluoxetine in these mice. Several studies support this hypothesis and showed evidence of a 5-HT-dependent upregulation of *Bdnf* mRNA expression in the adult rat [11-14]. This suggests a mechanistic link between 5-HT and BDNF

signaling, involving an autoparacrine loop promoting the maintenance and differentiation of serotonergic neurons. Such a mechanism of action has been particularly described for the hippocampus [12]. Furthermore, a study using BDNF $^{+/-}$ mice displaying a 50% reduction in BDNF showed an increase in 5-HT concentration in the ventral hippocampus postulated to be caused by a dysfunction of the 5-HTT [13]. In double mutant 5-HTT $^{-/-}$ x BDNF $^{+/-}$ mice, a decrease in both *Bdnf* and *TrkB* expression was measured, concomitant with a reduction in brain 5-HT levels. Interestingly, these mice exhibited an increase in anxiety-like behavior as well [14]. Furthermore, it has been shown that a pretreatment with Tryptophan hydroxylase inhibitor abolished the ability of citalopram (another SSRI) to induce rapid TrkB activation in the cortex [15]. Another study using 5-HTT KO mice but with no increase of the 5-HT in the extracellular medium showed that fluoxetine was not able to induce plasticity [16]. As such, it is tempting to speculate that both TrkB activation induced by fluoxetine and an increase in 5-HT levels promote neuroplasticity in 5-HTT KO mice. Thus, in order to better understand fluoxetine's mechanism of action and to confirm this hypothesis, it would be interesting and necessary to use conditional double transgenic 5-HTT/TrkB KO mice, or e.g. TPH2/TrkB $^{+/-}$ mice deficient in the gene encoding the rate-limiting enzyme for 5-HT synthesis, i.e. tryptophan hydroxylase 2 (Tph2), resulting in a reduction of 5-HT.

BDNF as a treatment for depression and Alzheimer's disease

In Chapter 3, we have shown that fluoxetine could exert its neuroplasticity-promoting effects, at least in part, through increased BDNF/TrkB signaling. In order to confirm the direct or indirect role of BDNF in depression, and because BDNF is unable to cross the blood-brain-barrier and has a short half-life, we studied the role of a new TrkB agonist in different mouse models of depression. In **Chapter 4** we used a new molecule that has been specifically designed to activate TrkB, i.e. TB001. After assessing the agonistic properties of TB001 *in vitro*, in primary cortical cultures, as well as *in vivo,* in naive WT mice*,* we tested this molecule in the tail suspension test (TST), a test generally used to screen antidepressants [17]. One hour after a single injection of TB001, naïve mice showed a decrease in depressive-like behavior. The tests subsequently performed in the two mouse models of depression were not as conclusive as in naïve mice. In the lipopolysaccharide [18] mouse model of depression, a model based on the inflammatory hypothesis of depression, TB001 displayed an antidepressant-like effect in the TST and the coat state test. However, TB001 did not always rescue the LPS-induced increase in pro-inflammatory cytokine expression, and did not seem to persistently activate TrkB or its downstream signaling pathways. Furthermore, chronic administration of TB001 in glucocorticoid receptor (GR) impaired (GR-i) mice, a model based on the hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis observed in depressed patients [19], did not show clear antidepressant- or anxiolytic-like behavioral effects. This made us questioning about the pathway that TB001 uses to induce its antidepressant effects. The fact that TB001 seemed to have a higher efficacy in LPS-exposed mice when compared to GR-i mice, might reflect a specific TB001 mechanism of

action primarily targeting inflammation-related processes and/or GR-dependency of TB001. As such, other studies using a different animal model of depression involving distinct disease mechanisms would be necessary. The mechanism of action of TB001 might be further investigated in different transgenic mice known to exhibit increased depressive-like behavior. For instance, in order to examine the effects of altered 5-HT function upon BDNF/TrkB signaling, the effects of TB001 in 5-HTT KO mice could represent an appropriate approach. Furthermore, heterozygous BDNF $^{+/-}$ mice appear to be an ideal model that directly involves impaired BDNF/TrkB signaling [20]. These mice have been shown to develop enhanced aggressiveness and hyperphagia with significant weight gain, which correlates with 5-HT hypofunction. In this regard, the use of double transgenic BDNF $^{+/-}$/5-HTT KO mice known to present increased anxiety-like behavior might also be a good option as it would also help us to better understand the interaction between BDNF, 5-HT and depression [21]. Thus, these models might indicate whether TB001 could restore the behavioral effects induced by a decrease in BDNF levels.

Given the strong links between stress, depression and AD and, in particular, regarding the observed dysregulation of BDNF/TrkB signaling in both depression and AD, we decided to test this new molecule in a mouse model of AD as well. To this end, as described in **Chapter 5,** we injected APPswePS1dE9 (APP/PS1) mice, a mouse model of AD, known to exhibit Aß plaques and memory deficits around 6 months of age, with TB001. We performed a behavioral test to study spatial memory, i.e. the object location task (OLT), as well as another test to assess working memory, i.e. the Y-maze. Both after acute and chronic treatment, TB001-treated APP/PS1 displayed intact spatial memory function, while vehicle-treated AD mice did not. Moreover, the Y-maze data suggested an improvement of working memory function in response to TB001. Surprisingly, using a treatment with another TrkB agonist, 7,8 dihyroxyflavone (DHF), gave mixed results, as these mice only showed a tendency for spatial recognition and no effect on working memory. After the behavioral tests, BDNF/TrkB signaling-related protein levels and the Aß42/Aß40 ratio were assessed in the hippocampi of these same mice. The *ex vivo* results were in fact rather surprising. Indeed, while in other studies, mouse models of AD present a decrease of P-TrkB (phosphorylated TrkB protein) levels, which is usually rescued by administration of DHF [22], our experiment showed the opposite effect with an increase of P-TrkB levels in APP/PS1 mice, which was normalized by both TB001 and DHF. Thus, the differences in memory performance observed for TB001-treated and DHF-treated mice, but similar results at the protein level, raise the question of TB001's and DHF's mechanisms of action. A first explanation might lie in the effect of TB001 on Aß-related toxicity. Although neither TB001 nor DHF were able to decrease the Aß42/Aß40 ratio in our AD mice, our *in vitro* study performed on SH-SY5Y-TrkB cells showed that only TB001 could prevent Aß-induced toxicity. Another explanation that may explain the difference between TB001 and DHF, might be that DHF, in addition to its agonistic properties, has also been shown to possess antioxidant properties [23] and would therefore be less specific for the TrkB receptor than TB001. As mentioned in the discussion of Chapter 5, the

behavioral differences observed between these two molecules might also be explained by the stress induced by the drug injections. As such, TB001 in addition to its cognitive action might also exert e.g. stress-reducing properties that would favor the recovery of memory deficits. Interestingly, DHF has shown anxiolytic properties in a binge alcohol exposure model exhibiting anxiety-like behavior during the withdrawal period [24]. However, it does not support our first findings reported in Chapter 4, where no strong anxiolytic effects were observed in the open field test after either an acute injection in naïve mice or a chronic injection in Gr-i mice. Yet again, these results were observed using different models of depression involving different mechanisms of action than those used in our model of AD, and should be considered carefully. Therefore, further experiments should be addressed in order to describe the exact role of TB001 and DHF in both models for depression and AD. Furthermore, when taking into account that inflammation is known to be increased in AD, the assessment of inflammation markers in treated and non-treated AD mice would be relevant to better precise which mechanisms of action TB001 could explain the alleviation of memory deficits in those mice. Finally, in order to analyze the effects of TB001 on tau hyperphosphorylation, it would have been interesting to test this agonist in a model of AD exhibiting neurofibrillary tangles.

An interesting point that has been raised in Chapter 4 is the potential antagonistic properties of TB001 in the presence of BDNF. It is often observed that in the presence of the endogenous ligand, a synthetic molecule presents antagonistic properties. This was e.g. shown in a study where they screened several potential TrkB agonists in cells stably transfected with TrkB, in order to validate the agonistic effects of these molecules on TrkB. When the cells were solely incubated with the synthetic molecules, an increase of P-TrkB was observed. However, in presence of BDNF, the molecules lost their agonistic effect and showed an antagonistic effect with a decrease of P-TrkB [25]. In our study, we did observe a competitive inhibition of TrkB activation which suggests TrkB might be a promising candidate as a therapeutic target, as, in the case of a depressed patient, an increase of BDNF levels in the VTA would promote an antagonistic activity of TB001 possibly inducing a normalization of downstream BDNF/TrkB signaling pathways and thereby participating to the recovery of the patient. Although this result might explain the normalization of the increased P-TrkB in the APP/PS1 mice after a chronic injection of TB001 and DHF, these are just speculations since as we did not directly measure BDNF levels. With respect to this latter point, measuring BDNF levels in both mouse models of depression as well as in AD mice represent another important step to take.

Region-specific action of BDNF

Taken together, these results highlight the complexity of the role of BDNF/TrkB signaling pathway in depression and AD. Indeed, while there is strong evidence of the involvement of BDNF in depression and AD, pharmacological studies have shown negative results or did not prove a causal relationship between BDNF levels and mood [26]. An explanation for this inconsistency might be the region-specific effect of BDNF.

This specificity of BDNF has been evidenced in this thesis, in particular, in Chapters 3 and 4. In Chapter 3, the examination of plasticity-related gene expression, including *Bdnf*, *TrkB*, *Creb* and *Bcl2,* after chronic treatment with fluoxetine revealed an increase of these genes in the frontal cortex of both WT and 5-HTT KO mice, while only *Bdnf* was increased in the hippocampus if these mice suggesting a more profound effect of fluoxetine in the cortex. This result may be surprising since the variations in BDNF expression following acute of chronic stress and/or antidepressant treatment were mostly similar in the hippocampus and the cortex. This has been shown in female naïve mice [27, 28] or in different models of depression such as, the chronic unpredictable mild stress (CUMS), where chronic treatment with fluoxetine could reverse the decrease of BDNF observed in stressed mice in both the hippocampus and the cortex [27, 29]. Similar effects were observed in mice exposed to repeated injections with corticosterone, where fluoxetine was able to reverse the cortiscosterone-induced decrease of BDNF in both these structures [30]. Furthermore, another study using this model displayed a fluoxetine-induced BNDF increase only in the hippocampus [31]. On the other hand, several studies reported an effect of antidepressants on BDNF levels in the cortex only [32-35], e.g. in naïve rats after chronic treatment with fluoxetine [32] as well as in CMUS mice where a chronic treatment with different antidepressants was able to normalize the decreased BDNF mRNA and protein levels in the frontal cortex, but not the hippocampus [35]. Furthermore, in another chronic stress study, an extracellularly electrophysiological recording showed that fluoxetine could prevent the stress-induced decrease in burst-firing rate and decrease of BDNF levels in the frontal cortex [33]. Another study reported a more rapid and pronounced increase in P-TrkB in the cortex after acute and chronic administration of fluoxetine and imipramine while a smaller increase was observed in the hippocampus, only after chronic administration [36]. A possible explanation to this higher effect in the cortex would be that antidepressant would increase BDNF level in the cortex which could impacting up the release of glutamate in the cortex' glutamatergic system [37]. Furthermore, TB001 was shown to activate TrkB preferentially in the frontal cortex. In studies using different mouse models of depression (e.g learned helplessness rat model and LPS-induced model of depression), chronic treatment of DHF increased phosphorylation of TrkB (P-TrkB) in the CA3, the dentate gyrus (DG) but not in the CA1 of the hippocampus. An elevation of P-TrkB was also observed in the frontal cortex [38, 39]. Thus, the fact that we measured the whole hippocampus while it seemed that BDNF is regulated differently in different subfield within the hippocampus (CA1, DG, CA3) might simply explain the non-effect of TB001 observed in the hippocampus [38-40]. Prefrontal cortex activity on PET and fMRI has revealed that this region was involved in working memory including the temporary storage and manipulation of visual and verbal material [41]. Thus, considering the evidence on the importance BDNF/TrkB signaling in the frontal cortex, particularly in view of the action of TrkB agonists, it would be necessary to assess P-TrkB in the cortex in our APP/PS1 mice (Chapter5).

Furthermore, while other studies have shown that BDNF is mainly downregulated in the hippocampus and the cortex, it is up-regulated in the nucleus accumbens (NAc) and the ventral

tegmental area (VTA), regions implicated in the reward system, in different animal models of depression as well as in post-mortem brains of depressive patients [42, 43]. Furthermore, infusion of BDNF in the VTA-NAc proved to increase depressive-like behavior [44], possibly through an activation of dopaminergic neurons. Mice in which the *Bdnf* gene was locally deleted in the VTA showed decreased levels of depressive-like behavior [45]. This region-specific effect of BDNF could explain the anxiolytic-like properties of cyclotraxin-B and the antidepressant-like effect of ANA-12, all known to be specific TrkB antagonists [46, 47]. Notably, when a bilateral NAc infusion of ANA-12 was used in LPS-exposed mice or in a learned helplessness rat model of depression, these rodents presented reduced depressive-like behaviors [39, 42]. So far, this phenomenon has not been explained, but some hypotheses have been raised. A first hypothesis concerns the distribution of the two receptors for BDNF, i.e. TrkB and p75. Knowing the fact that TrkB promotes neuronal and synaptic plasticity, whereas p75 is primarily promoting apoptosis, researchers proposed that p75 would be expressed primarily in the VTA-NAc, while TrkB would be more abundant in the hippocampus and cortex. However, it has been shown that overexpression of TrkB in the NAc induced antidepressant-like effects while overexpression in the hippocampus did not. These antidepressant-like effects, shown in the forced swim test [48], could find an explanation in the fact that TrkB overexpression would have an effect mostly on motivation (to escape) in the FST, as the NAc plays a critical role in motivated behavior. [49]. Regarding p75, it was shown to be mainly restricted to the cholinergic neurons of the basal forebrain at a basal level: neurons innervating the hippocampus, prefrontal cortex, amygdala and NAc. However, increased levels of p75 mRNA have been found in the brains of suicide victims, suggesting a synaptic depression promoted by p75, underlying the pathophysiology of depression. [50]. Another point to consider is the role of the TrkB signaling pathway in this area. Indeed, one of the pathways activated by TrkB, the phospholipase Cγ (PLCγ) pathway, has been shown to cause either increased or decreased depressive-like- behavior depending on the targeted subregion of the VTA [51]. In addition, the pro-depressive effect of BDNF in the VTA might be caused by only parts of the pathways activated by BDNF and not by all of them [52]. Knowing the opposite characteristics of the NAc-VTA in depression, further studies on TB001 should focus on this area in addition to the hippocampus and the cortex.

Conclusion

In this thesis, we have highlighted the important role of the BDNF/TrkB signaling in both depression and AD. In the first chapters, we have shown that the BDNF/TrkB complex plays a central role in the mechanism of action of antidepressants and we have confirmed the possibility of transactivation of TrkB by antidepressants, and, more specifically, by fluoxetine. Furthermore, we have shown, by the use of a new TrkB agonist specifically designed to target TrkB, i.e. TB001, that, in addition to activating TrkB, this molecule was able to reduce depressive-like behavior in a mouse model for depression and, furthermore, was able to alleviate memory deficits in AD mice.

Depression and AD are particularly hard to diagnose during their early phases, since they ensue particularly from psychological symptoms. Thus, research is currently trying to identify new biomarkers in order to timely initiate and improve the treatment of these diseases. For example, epigenetic BDNF signatures as well as the BDNF Val66Met polymorphism have been proposed as biomarkers in this respect [53, 54]. Furthermore, in view of all its beneficial properties, BDNF/TrkB signaling represents a promising target to increase the efficacy of antidepressants, e.g. regarding the delayed onset of action of currently prescribed antidepressants. Targeting TrkB may also aid in treating or at least slowing down the course of AD. However, more research is needed on the precise role of BDNF, and, particularly, its region-specific mechanisms of action, in order to optimize a BDNF/TrkB-based treatment. TB001 seems a promising candidate, but more work is needed to shed light on its exact mechanism of action, in particular in view of its partial agonistic as well as potential condition-dependent partial antagonist properties. Finally, as the non-familial forms of both these disorders are quite heterogeneous in terms of their development, course and symptomatology, and likely depend on several, distinct processes affected within the brain, either acting in isolation or in an additive or synergistic manner, more personalized treatment strategies targeting these different processes might be considered.

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Valorization

Summary

Acknowledgements

List of publications

About the author

1. Individual and socio-economic relevance

1.1. Prevalence

The World Health Organization (WHO) has categorized depression among the most disabling clinical diagnoses in the world [1]. Depression is ranked fourth in 2010 [2] when measured in disability-adjusted life years (DALYs), which is the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability. The WHO estimates that 350 million people are affected by depression worldwide [1] and the population attributable risk (PAR) due to mental disorders is estimated at 14,3% which indicates that about 8 million of deaths worldwide can be attributed to mental disorders [3]. Regarding dementia, the WHO has estimated the number of patients with dementia to be 35,6 million in 2010 worldwide predicting that this number would be doubled every 20 years. Furthermore, the total number of new cases each year worldwide is nearly 7.7 million, which corresponds to one new case every four seconds. Among the 30 million patients with dementia, 60 to 70 % have been reported to be Alzheimer's disease (AD) patients which ranks AD as the most prevalent form of dementia. [4-6]

1.2. Caregiver burden

Social support and particularly emotional support from a close relative or friend is an important protective factor for mental health problems. However, it has been demonstrated that caregivers use tireless effort, energy and empathy when supporting patients with a chronic condition like depression, obviously impacting the caregivers' daily life. Furthermore, these people are more susceptible to lose their work due to absenteeism or simply may not search for work. All these conditions may lead to a high prevalence of depressive symptoms (53,75%) in caregivers [7]. The same observations apply for AD where it has been noticed that the primary caregiver, often a family member, reported distress such as anxiety, worry, guilt. As such, this is associated with the same consequences of providing care mentioned above including decreased recreational time, outside work conflicts, financial difficulties, family conflict, altogether, reflecting the high caregiver's burden [6, 8, 9].

1.3. Cost of these diseases

Given the depression- and AD-induced burden for both patients and caregivers, it is not surprising that these diseases contribute to a significant economic cost worldwide. Indeed, on the one hand, depression significantly predicts overall reduced work performance which has been evaluated in the range of $30.1 billion to $51.5 billion [10]. In Europe, the total annual cost of depression was estimated at €118 billion in 2004, with €42 billion of direct costs including outpatient care, medication and hospitalization. This makes depression the costliest brain disorder in Europe, accounting for 33% of the total healthcare costs. [11]. On the other hand, the total estimated economic burden of AD has

been evaluated at $604 billion in 2010 worldwide with 40% associated with informal care, 40% with formal social care and only 15% for direct medical costs. AD reaches the third place in terms of total disease-related costs, after cancer and cardiovascular disorders [6, 12 , 13].

Thus, the high prevalence of depression and AD in the general population and the high socio-economic impact urge to reduce this burden, first by acting on the prevention of depression and AD, and, second, by improving diagnosis and treatment [1, 6].

2. Improving diagnosis: research of biomarkers

A biomarker is defined as a biologic feature that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention. Thus, a biomarker might help to identify whether a patient developed or is likely to develop a specific disorder or not. This is particularly true in diseases like depression and AD since these diseases are considered as mental and cognitive disorders which are diagnosed with qualitative diagnostic criteria. For psychiatric disorders, including depression, these criteria are described in the fifth version of the Diagnostic and Statistical Manual of Mental Disorders (DSM 5) or in the tenth revision of the International Statistical Classification of Diseases and Related Health Problems (ICD 10) [14, 15]. For AD, diagnosis is based on the patient's anamnesis, clinical presentation and neurocognitive tests [16]. While clinical diagnosis to distinguish patients with AD from people without dementia has good sensitivity and specificity (>80%), this diagnosis has shown inconsistent ability to differentiate AD from other forms of dementia (23-88%). [17] Hence, the final diagnosis of AD can only be made post-mortem by examining the protein aggregate signature in the brain. An early diagnosis of AD might help to better treat and take care of the patient in order to slow down disease progression [18]. Therefore, the search of strong biomarkers in both depression and AD remains a necessity as well as a real challenge.

In depression, neuroimaging outcomes such as those obtained via structural magnetic resonance imaging [19] have been considered as suitable diagnostic biomarkers [20]. Thus, meta-analyses on structural imaging studies revealed structural abnormalities in different brain regions (reviewed in [21]) such as patients with depression having smaller hippocampal volumes compared to healthy subjects [22], or patients with psychotic depression showing a smaller volume of the left posterior subgenual prefrontal cortex [23]. In order to refine the diagnosis of depression with biomarkers, future research should combine these analyses with protein level assessment in serum, cerebrospinal fluid (CSF) or urine of patients. Although metabolites of monoamine neurotransmitters have failed to demonstrate consistent alterations [21], when comparing healthy patients with depressive patients, changes in cortisol level, pro-inflammatory cytokines [21] and in neurotrophic factors such as GDNF and IGF-1 level expression have been detected. As for BDNF, increasing

evidence suggests that serum BDNF levels are decreased in depressed patients [24-26]. This may reflect decreases of BNDF in the hippocampus and prefrontal cortex of postmortem brains of suicide victims and depressed patients. [27, 28]. Such changes have also been described in rodent models of depression [29]. More interestingly, both treatment with antidepressants and electroconvulsive therapy have proven to restore BDNF levels in serum of depressed patients [26, 30-33] and in brains of rodent models of depression [34-37]. Therefore, these data support the potential of serum BDNF as a biomarker in the diagnosis of depression.

Regarding AD, structural and functional imaging approaches highlight changes occurring in AD such as atrophy of specific brain areas, tau protein accumulation and amyloid plaques, aiding in diagnosing AD before dementia onset and to track its progression. One emerging technique is positron emission tomography (PET) amyloid scanning that can identify amyloid plaques in a living brain. Furthermore, in CSF, levels of tau protein and its phosphorylated form are elevated and can be predictive measure of AD development [17]. Serum based biomarkers have also been considered for AD. Hence, O'Bryant and colleagues analyzed 121 proteins from serum of a large cohort of AD patients and healthy individuals. They were able to generate an algorithm with a biomarker risk score that was highly accurate for detecting AD [38]. Interestingly, BDNF is one of the proteins that have been studied as a biomarker and a decreased serum concentration of BDNF has been consistently described in AD patients compared to age-matched healthy individuals [39, 40]. Furthermore, a reduced BDNF level was associated with lower cognitive performance [41, 39].

The current thesis did not provide direct evidence about BDNF as a biomarker in both depression and AD. Hippocampal *Bdnf* mRNA expression was not changed in the LPS model of depression. However, we did assess the BDNF/TrkB signaling by measuring TrkB and CREB protein levels in both our LPS and AD models. We observed a decrease in TrkB protein in the AD model, although levels of the BDNF/TrkB related proteins were not changed in the depression models. This further highlights the complexity of the BDNF/TrkB system and underscores the important of measuring, besides the ligand, multiple signaling molecules when examining growth factor signaling disturbances. Nevertheless, the development of new molecules that are either agonists or antagonists of TrkB, such as TB001, the molecule we studied, might help to study BNDF and its role in depression or AD. Such a molecule could even help to evaluate BDNF as a biomarker in those diseases or other disease where BDNF has been shown to be involved, such as addiction, schizophrenia [42] or Huntington disease [43]. Therefore, via the investigation on new molecules that mimic BDNF and its mechanism of action, the present study might highlight the complex role of BDNF in various diseases.

As mentioned in Chapter 1, depression and AD share, to a certain extend and depending on disease state, similar (neuro)biological dysfunctions. For example, structural atrophy of the hippocampus or changes in the cortex have been found in both depression and AD [44]. Furthermore, the involvement of neuroinflammation or dysfunctions of the HPA axis have been proven in both

depression and early stage of AD [45, 46]. Regarding BDNF/TrkB signaling a decrease in BDNF level was also observed in postmortem brain of depressive patients and of AD patients [27, 28, 47]. Thus, all these observations would explain the high prevalence of depression in AD patients [48, 49]. It is however unlikely that these characteristics are useful in the search for biomarkers distinguishing these disorders. BDNF alone will not be able discriminate and should therefore be combined with other biomarkers (e.g. imaging, CSF/serum protein measurements). Furthermore, it is not yet known to which extent the level of BDNF in the serum reflects its level in the brain [39]. Nevertheless, it is tempting to speculate that decreased serum BDNF levels may indicate an ongoing dysregulation of this growth factor system, and that these patients may especially benefit from an appropriate treatment, i.e. with BDNF mimetics.

3. Improving treatments

Since disturbances in growth factor signaling, in particular BDNF, have been extensively described in depression and AD, the use of BDNF or small molecule TrkB agonists as a treatment strategy for these disorders has been put forward.

As mentioned earlier, the efficacy of treatments for depression, and especially antidepressants, is still very low with only one third of patients being responsive to the treatment. In addition, there is a considerable delay in the clinical effect of antidepressants [50]. Thus, there is a clear need for more effective antidepressants with a faster onset of action. Currently, it is hypothesized that the clinical effects of antidepressants rely on the stimulation of growth factor pathways, such as BDNF/TrkB, with concurrent increases in hippocampal (neuronal and synaptic) plasticity [51, 52], rather than on the direct modulation of monoaminergic neurotransmission. The studies in this thesis further examined this hypothesis and evaluated whether small molecule BDNF mimetics are able to activate BDNF/TrkB signaling, thereby inducing antidepressive effects. We indeed showed that the commonly used antidepressant fluoxetine increased *bdnf* mRNA expression within few hours *in vitro*, and after chronic treatment *in vivo*. Regarding AD, similar results were reported in Chapter 5 using a mouse model of AD with a great recovery of spatial memory upon treatment with TrkB compounds, but inconclusive results in the assessments of protein of interest. It is worth noting, though, that TB001 was able to reduce the Aβ-induced toxicity. Thus, these promising results showed the critical involvement of BDNF in these disorders, and highlight the therapeutic potential of targeting BDNF/TrkB signaling in these disorders.

It is now increasingly recognized that psychiatric and neurodegenerative disorders are heterogeneous in nature, meaning that the expression of behavioral and neurocognitive symptoms, but also the underlying pathobiology, is very diverse in different patient groups. In the case of depression, varying degrees of alterations in many (neuro)biological systems, either in isolation or in conjunction, have been implicated in subpopulations of patients. For example, while it is clear that a state of

inflammation contributes to depression, not all patients show a state of chronic mild inflammation. Likewise, dysregulation of the hormonal stress system is not necessarily seen in all patients. This issue is seen in Chapter 4 where TB001 seemed to work in an inflammation-induced mouse model of depression while the same molecule was less effective in another model of depression related to HPA axis dysfunction. These observations support the hypothesis of a treatment that would be given specifically according to biomarker assessments. Regarding AD, research in new treatments has mainly focused on removing the main hallmarks of AD, namely the amyloid plaques and the neurofibrillary tangles. However, the multifactorial and heterogeneous nature of the disorder makes us think that a general treatment including an inhibition of neurodegeneration and an activation and restoration of the neuroplasticity would be more efficient [53]. In this regard, BDNF seems to be a promising target considering its neuroplasticity-inducing capacity, and its indirect effect on amyloid plaque reduction [54]. Furthermore, in view of the strong link between depression and AD, such an approach could help to develop a treatment that reverses depressive symptoms as well as cognitive dysfunction in AD, and our first results bring promising evidence that BDNF/TrkB signaling would be a first target to consider.

4. Conclusion

Altogether, this project might be an important step forward in the search for new biomarkers for depression and AD, and, above all, in finding new potential treatments for these diseases based on modulating growth factor signaling cascades.

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English

Depression and Alzheimer's disease (AD) are both important contributors to the global burden of disease and affect people in all communities across the world with a significant social and economic impact on patients, their relatives and society in general. On the one hand, depression is a mood disorder for which the etiology and biological mechanisms, such as dysregulation of the monoaminergic system and hyperactivation of the hypothalamic-pituitary-adrenal axis, are not fully understood. On the other hand, AD is a neurodegenerative disease for which the main hallmarks are the onset of amyloid beta plaques and intracellular neurofibrillary tangles. Their origin and role in the disease still remain unclear. Although these diseases seem different, several lines of evidence show that there is an increased probability for AD patients to develop depression and conversely, depressive patients are more susceptible to develop AD. Furthermore, they both share similar dysfunctions, one of which being the dysregulation of brain-derived neurotrophic factor (BDNF) / Tropomyosine-related receptor kinase B (TrkB) signaling. BDNF is a neurotrophin that plays a key role in the regulation and the maintenance of the central nervous system. It binds to its receptor, TrkB, in order to promote neuronal proliferation, differentiation, and survival, and to enhance neurotransmission.

Therefore, this thesis aimed at investigating the pathophysiological and potential therapeutic role of BDNF/TrkB signaling in depression and AD.

After presenting an overview of the pathophysiology and treatment of depression focusing on the role of the neurotrophic factors and related neuroplasticity, the link between fluoxetine (an antidepressant), BDNF and the serotonin transporter (5-HTT, the primary target of fluoxetine) was investigated, together with the effect of fluoxetine on plasticity. Fluoxetine was able to increase *Bdnf* gene expression, as well as cell proliferation in 5-HTT-deficient mice. Furthermore, *in vitro*, fluoxetine could activate TrkB receptors in cultured neurons derived from both wild-type and 5-HTT-deficient mice. These data suggest that some of the neuroplasticity-promoting effects of fluoxetine are, at least partially, related to 5-HTT-independent activation of TrkB. A new TrkB agonist, TB001, was tested in two different models of depression and one model of AD. After demonstrating the agonistic properties of TB001 both *in vitro* and *in vivo*, we showed that TB001 could induce antidepressant-like effects in an LPS model of depression, whereas this effect was not reproduced in another model of depression, i.e. GR-i mice. However, when tested in APPswe/PS1d9 mice, a genetic model for AD, TB001 was able to alleviate memory deficits, and showed superior efficacy as compared to 7,8-DHF.

In conclusion, this thesis provided further evidence about the important role of BDNF/trkB signaling in animal models of depression and AD.

Français

La dépression et la maladie d'Alzheimer (MA) sont toutes deux d'importants contributeurs à la charge mondiale de morbidité dans toutes les communautés à travers le monde, avec un impact économique considérable sur les patients, leur famille et la société en général. La dépression, d'une part, est un trouble de l'humeur où l'étiologie et les mécanismes biologiques, tels que la dérégulation des systèmes monoaminergiques et la suractivation de l'axe hypothalamo-hypophyso-surrénalien restent toujours mal compris. La MA, d'autre part, est une maladie neurodégénérative, principalement caractérisée par l'apparition de plaques amyloïdes et de dégénérescences neurofibrillaires dont les origines et les rôles dans la maladie sont encore très mal connus. En dépit des différences apparentes entre ces deux maladies, plusieurs études ont suggéré que les patients Alzheimer auraient de fortes probabilités de développer un état dépressif majeur et qu'à l'inverse les patients dépressifs seraient plus susceptibles de développer la MA. De plus, on retrouve dans ces deux maladies les mêmes dysfonctions, l'une d'elle étant la dérégulation de la signalisation du complexe Brain-Derived Neurotrophic Factor (BDNF)/ Tropomyosine-related receptor kinase B (TrkB). Le BDNF est une neurotrophine qui joue un rôle clé dans la régulation et le maintien du système nerveux central. Il se lie à son récepteur, le TrkB, induisant prolifération, différenciation, survie et neurotransmission cellulaires.

Aussi cette thèse a eu pour but de comprendre le potentiel rôle thérapeutique et physiopathologique de la voie de signalisation BDNF/TRkB dans la dépression et la MA.

Après avoir présenté la physiopathologie et les traitements de la dépression ciblant le rôle des facteurs neurotrophiques dans la neuroplasticité, nous avons examiné le lien entre un antidépresseur - la fluoxétine - le BDNF et le transporteur de la sérotonine (5-HTT), ainsi que l'effet de la fluoxétine sur la plasticité. Nous avons montré que la fluoxétine a gardé sa capacité d'augmenter l'expression du gène du *Bdnf* ainsi que la prolifération cellulaire chez des souris n'exprimant pas sa cible primaire, le 5-HTT. De plus, *in vitro,* la fluoxétine a activé le récepteur Trkb en absence de 5-HTT suggérant que certains des effets qui induisent la plasticité seraient indépendants du 5-HTT et qu'ils seraient, en partie, médiés par l'action directe de la fluoxétine sur le récepteur TrkB. Dans un second temps, l'essai d'un nouveau ligand du TrkB, le TB001, *in vitro* et *in vivo*, sur des souris naïves a permis de valider les propriétés agonistes de la molécule sur le récepteur TrkB. De plus, TB001 injecté chez un modèle murin de dépression induit par le lipopolysaccharide (LPS) a démontré des effets de type antidépresseur. Cependant, ces effets n'étaient pas significatifs chez un autre modèle de dépression, les souris GR-i. Enfin, chez un modèle de souris de la MA, les souris APPswe/PS1d9, TB001 a amélioré plus efficacement les déficits de mémoire que la molécule de référence, le 7,8-DHF.

Ainsi, cette thèse a apporté des réponses quant à l'importance du rôle de la voie de signalisation BDNF/TrkB sur des animaux modèles de la dépression et de la MA.

To me, this part is one of the most important ones in my thesis. All this work would have not been possible without the help, advise, guidance and support of many people and I am truly happy that finally, I can formally thank them.

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Effect of fluoxetine and MIML4-11, a novel tropomyosin-related kinase receptor agonist, in a lipopolysaccharide-induced model of depression

Marion J.F. Levy; Fabien Boulle; Vincent S. Martin; Corinne Poilbout; Didier Rognan; Laurence Lanfumey

Published, *European Neuropsychopharmacology*, Volume 25, Issue null, Pages S292-S293

Acute and chronic intervention with a TrkB receptor agonist rescues spatial memory deficits in an Alzheimer's disease mouse model

Marion J.F. Levy; Artemis Iatrou; Nick Van Goethem ; Maxime Cazorla; Daniel L. Van den Hove; Didier Rognan; Laurence Lanfumey; Gunter Kenis

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

Marion J.F Levy; Fabien Boulle; Joost van Haasteren; Harry W. Steinbusch; Daniel L. van den Hove; Gunter Kenis; Laurence Lanfumey

To be submitted

Keeping "Trk" of 5-HTT-dependent and -independent effects of fluoxetine-induced neuroplasticity

Marion J.F. Levy; Fabien Boulle; Michel-Boris Emerit; Corinne Poilbout; Harry W. Steinbusch; Daniel L. Van den Hove; Gunter Kenis; Laurence Lanfumey

To be submitted

Antidepressant properties of TB001, a small BDNF mimetic

Marion J. F. Levy*; Fabien Boulle*; Corinne Poilbout; Maxime Cazorla; Didier Rognan; Harry W. Steinbusch; Gunter Kenis; Daniel van den Hove; Laurence Lanfumey

* Equal contribution

In advanced preparation

Small molecule TrkB agonists enhance memory performance in an animal model for Alzheimer's Disease

Marion J.F. Levy*; Artemis Iatrou*; Anja Muurmans; Nick van Goethem; Maxime Cazorla; Didier Rognan; Laurence Lanfumey; Harry W. Steinbusch; Daniel L. van den Hove; Gunter Kenis

* Equal contribution

In advanced preparation

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Summary

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About the author

Marion Levy was born on June, 6th 1989 in Melun (France). She attended the Lycée Jacques Amyot in France where she completed a scientific baccalaureate program.

She started her education by performing a bachelor degree in Biology and Health at the university of Paris-Sud 11 in Orsay (France). She had the opportunity to achieve an internship in the center of neurosciences of Paris-Sud by establishing a behavioral protocol to study the episodic memory in the rat. She was very interested in understanding how memory works and more generally everything related to neurosciences. Thus, she took more options in physiology and neurosciences and logically, she continued her Master degree in the same university, with a focus on neurosciences and cellular signaling. She had the opportunity to make an optional internship in the same center in order to learn different techniques such as western blotting, immunohistochemistry and image analysis through a very interesting project about hypothyroidia associated to Alzheimer disease. For her last internship, Dr Laurence Lanfumey gave her the opportunity to work at The Psychiatry & Neurosciences Center, which is linked to the national institute of the health and the medical research (INSERM) and Paris Descartes University. Hence, using a large panel of technics such as primary cell culture, qRT PCR and immunocytochemistry, she could evaluate the *in vitro* regulation of BDNF/TrkB signaling pathway by antidepressants.

Following this work, she started her PhD in the same laboratory with a cotutorship with Maastricht University under the supervising of Dr Laurence Lanfumey and Pr. Harry Steinbusch, co-supervised by Dr. Gunter Kenis and Dr. Daniel Van Den Hove. Her research aimed at evaluating the BDN/TRkB pathway as a common target in the treatment of major depression and Alzheimer disease. Thus, she explored the role of fluoxetine in the modulation of BDNF/TrkB signaling and the role of serotonin transporter in the fluoxetine-induced plasticity. Furthermore, she investigated on the effects of a recently developed agonist of TrkB receptor in model of depression (Paris) and Alzheimer Disease (Maastricht).