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Marina Schverer

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Université Paris Descartes – Ecole Doctorale n°563 – MTCI

Thèse de Doctorat

Spécialité : Neurosciences

Présentée par **Marina Schverer**

Pour l'obtention du grade de Docteur de l'Université Paris Descartes

Antidepressant-like effects and mechanism of action of MAP4343, a pregnenolone derivative that targets the neuronal microtubule cytoskeleton

Date de soutenance prévue : le 29 novembre 2018

Membres du jury :

Monsieur le Docteur Tanguy Maurice (Université Montpellier II)	Rapporteur
Monsieur le Docteur François Tronche (Université Paris VI)	Rapporteur
Madame le Docteur Annie Andrieux (Université de Grenoble)	Examinatrice
Monsieur le Professeur Philippe Fossati (Université Paris VI)	Examineur
Madame le Docteur Laurence Lanfumey (Université Paris V)	Directrice de Thèse
Monsieur le Docteur Nicolas Froger (Mapreg)	Invité

Abstract

Major depressive disorder is a mental illness characterized by several symptoms, including low mood and cognitive deficits, that could rely on neuroplasticity alterations. Current pharmacotherapy displays significant limitations since 30 to 40% of patients fail to achieve remission. In addition, the classical treatments do not properly alleviate the cognitive impairments observed in depressive patients. MAP4343 is a pregnenolone derivative that displayed antidepressant-like activity in animals, triggering faster efficacy than fluoxetine, a selective serotonin reuptake inhibitor currently prescribed in patients. Moreover, MAP4343 promoted cellular process outgrowth, probably through its binding to microtubule-associated protein 2 (MAP2). Given these findings, we hypothesized that MAP4343 antidepressant-like activity may encompass the reversal of cognitive deficits. Hence, the aim of this thesis was to evidence the effects of MAP4343 on stress-induced cognitive impairments, and to elucidate its mechanism of action related to the cellular pathways that trigger plasticity. MAP4343 action on depressive-like behavior was evaluated in mice subjected to chronic stress induced by social defeat, and in transgenic mice having altered stress response. MAP4343 had antidepressant-like activity in both models, and restored cognition in defeated animals. This molecule also had pro-cognitive action in unstressed mice, suggesting that MAP4343 could promote neuroplasticity. Using primary neuron cultures, we found that it promoted neurite outgrowth and growth cone bundling, and upregulated the expression of both actin- and microtubule-related proteins. Moreover, MAP4343 increased the activation and/or the expression of various intracellular kinases, including extracellular signal-regulated kinase (ERK), together with the transcription factor cyclic adenosine monophosphate-responsive element binding (CREB), considered as key actors in neuronal plasticity pathways. The emergent hypothesis was that MAP4343 activity on the cytoskeleton could be responsible for ERK and CREB activation, leading to an upregulation of brain-derived neurotrophic factor signaling pathways triggered by its binding to its high affinity receptor tropomyosin receptor kinase B.

Overall, the results obtained in this work showed that MAP4343 could be a serious candidate for depression treatment, since it directly promoted neuroplasticity and enhanced cognitive performances through an original mechanism of action involving the cytoskeleton.

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Abbreviations

5-HT: 5-hydroxytryptamine or serotonin

ACTH: adrenocorticotrophic hormone

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BDNF: brain-derived neurotrophic factor

CA: *Cornu Ammonis*

CaMKII: Ca²⁺/calmodulin-dependent protein kinase II

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CLIP: cytoplasmic linker protein

CNS: central nervous system

COMT: catechol-O-methyl transferase

CREB: cAMP-responsive element

CRH: corticotropin-releasing hormone

CSDS: chronic social defeat stress

DAG: diacylglycerol

DIV: day in vitro

DNA: desoxyribonucleic acid

ELISA: enzyme linked immunosorbent assay animation

ERK: extracellular signal-regulated kinase

GABA: γ -aminobutyric acid

GPCR: G protein-coupled receptors

GR: glucocorticoid receptor

GSK3: glycogen synthase kinase-3

HEC: hydroxyethylcellulose

HPA: hypothalamic-pituitary-adrenal

IDO: indoleamine-2,3-dioxygenase

IP3: inositol 1,4,5-trisphosphate

ITI: intertrial interval

L-DOPA: L-3,4- dihydroxyphenylalanine

LTD: long-term depression

LTP: long-term potentiation

MAO: monoamine oxidase

MAP4343: 3 β -methoxyprogesterone

MAP: microtubule-associated protein

MDD: major depressive disorder

mGluR: metabotropic glutamate receptor

MR: mineralocorticoid receptor

mTORC: mechanistic target of rapamycin complex 1

NMDA: N-methyl-D-aspartate

OFT: open-field test

PBS: phosphate-buffered saline

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

PKC: protein kinase C

PMAT: plasma membrane monoamine transporter

PSD-95: postsynaptic density protein 95

RNA: ribonucleic acid

RT-qPCR: Quantitative reverse transcriptase polymerase chain reaction

RTK: tyrosine-kinase activity receptor

S.E.M.: standard error mean

SERT or 5-HTT: serotonin transporter

SSRI: selective serotonin reuptake inhibitor

TBS: Tris-buffered saline

TrkB: tropomyosin-related kinase receptor B

VMAT2: vesicular monoamine transporter 2

WT: wild type

Preamble

The aim the present study was to characterize the antidepressant-like effects of MAP4343 and its mechanism of action. This manuscript begins with an introduction that overviews the literature principally related to this thesis project, rather than an exhaustive review of the large body of evidence reported over the past decades regarding depression and neuroplasticity.

First, the current knowledge in depression will be reviewed, in particular through the description of historical hypotheses that could sustain major depression etiology. Indeed, the emergence of the monoamine, glucocorticoid and inflammation hypotheses was the starting point of major depression pathophysiology understanding. This will be followed by a brief assessment of the pharmacological treatments prescribed in patients, illustrating that new compounds are needed to attempt to cure this disease. Current research focuses on neuroplasticity mechanisms, majorly because of their role in cognition, which is altered in depressed patients, as it will be demonstrated in the present introduction. Since neuroplasticity processes are wide and largely described in the literature, a particular attention will be focused on the microtubule cytoskeleton, which is a target of MAP4343, and on the neurotrophic hypothesis of major depression, which encompasses interesting targets for the treatment of this pathology. This report will end on the effects of MAP4343 and related neurosteroids on neuroplasticity, and their involvement in depression, making this new molecule an innovative potential antidepressant compound.

At the end of the introduction, a review article about how the non-genomic action of neurosteroids can modulate neuroplasticity and play a role in pathological conditions will be presented. This chapter will be followed by data divided in two research articles encompassing the experimental results collected during this thesis project. All these data will be finally discussed, opening new perspectives to further investigations.

Introduction

1. Major depressive disorder: general considerations

1.1. Diagnostic

1.1.1. Clinical symptoms

Major depressive disorder (MDD) is a mental illness characterized by a negative alteration of the mood. It is defined by symptoms listed in the fifth edition of the Diagnostic and Statistical Manual published by the American Psychiatric Association in 2013. The diagnosis requires the presence during a two-week period of at least five symptoms (among a nine-item list), including depressed mood and/or anhedonia, which refers to the inability to feel pleasure in normally pleasurable activities (Figure 1). Although prior depressive episode constitutes the most significant risk for MDD, other factors such as genetic, medical, social, and environmental are also of importance to trigger this disease (Bentley, Pagalilauan, & Simpson, 2014). To date, MDD diagnostic is only based on oral evaluation, since no reliable biomarker has been evidenced so far.

- A. Five (or more) of the following symptoms present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) anhedonia
- B. Symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning
- C. Episode is not attributable to the physiologic effects of a substance or another medical condition
 1. Depressed mood most of the day (eg, feels sad, empty, hopeless)
 2. Markedly diminished interest or pleasure in almost all activities nearly every day
 3. Significant appetite changes or significant weight loss or gain
 4. Insomnia or hypersomnia nearly every day
 5. Psychomotor agitation or retardation
 6. Fatigue or loss of energy
 7. Feelings of worthlessness or excessive guilt
 8. Diminished ability to think or concentrate or indecisiveness

Figure 1. Diagnostic and Statistical Manual (fifth edition) criteria for diagnostic of major depression. From Bentley et al., 2014.

1.1.2. The role of stress in MDD

Homeostasis, which is mandatory for maintaining life, is the auto-regulation of the internal balance by the organism. It is continually challenged by internal or external threats, called “stressors”. Stress, perceived as danger or physical and psychological barrier, is the reaction of the body toward an event or situation that disturbs the internal balance (McEwen, 2000). An acute stress can be positively perceived and the downstream effects can lead to enhanced function of the body, hence constituting an adaptive strategy (Sterling & Eyer, 1988).

However, if recovery is not complete by homeostatic return, this defense mechanism can result in harmful consequences. Indeed, when the stressor is perceived as irritating or imposing significant danger, it may induce short- or long-term physiological and psychological alterations. Chronic exposure to stress is considered as the major environmental risk factor for MDD (Lupien, McEwen, Gunnar, & Heim, 2009), therefore classified as a stress-related disorder. The cumulative response to stressors, mainly mediated by the brain, can also have deleterious repercussions, as discussed in the section 2.2.

1.2. Animal models of MDD pathophysiology

Most of MDD pathophysiology features cannot be investigated without invasive methods, explaining why a wide amount of neurochemical data collected over the years regarding this disease is coming from animals rather than patients. Animal models can be based on pathophysiological processes that lead to the disease in human (construct validity), on their ability to reproduce symptoms observed in patients (face validity), or on their response to pharmacological treatments used in clinic (predictive validity) (Abelaira, Reus, & Quevedo, 2013; Paul Willner, 1984). Regarding MDD, a fourth criterion has been proposed as the pathological validity. It encompasses the observation in animal models of the same physiological, cellular and molecular changes than those found in *post-mortem* brain of depressed patients (Krishnan & Nestler, 2011). However, because these alterations are not clearly established, it is difficult to satisfy this criterion.

1.2.1. Modeling MDD in rodents

The most predominant symptoms of depressive patients are subjective feelings that cannot be measured in animals. Hence, modeling depression remains a challenging issue. The main strategy is to induce a phenotype that will disturb the “normal” behavior of rodents, which includes social interaction, food quest, grooming, exploration, reproduction, and hiding. There is a plethora of animal models used to study depression. In this section, the focus will be only on animal models relevant for the project of the present thesis.

Several spontaneous MDD rodent models have been generated over the years. For example, they can be based on genetic modifications leading to neurobiological and behavioral outcomes that parallel those observed in MDD patients. The most common strategy to induce a spontaneous depressive-like phenotype in mice is to alter the hypothalamic-pituitary-adrenal (HPA) axis activity (described in the section 2.2.3), by targeting the glucocorticoid receptor (GR). Given the fact that knockout the gene encoding for GR causes perinatal lethality (Cole et al., 1995), several approaches were developed to specifically impair central GR function. One of them consists in tissue-specific mutations of the gene encoding for GR protein, using the Cre/loxP-

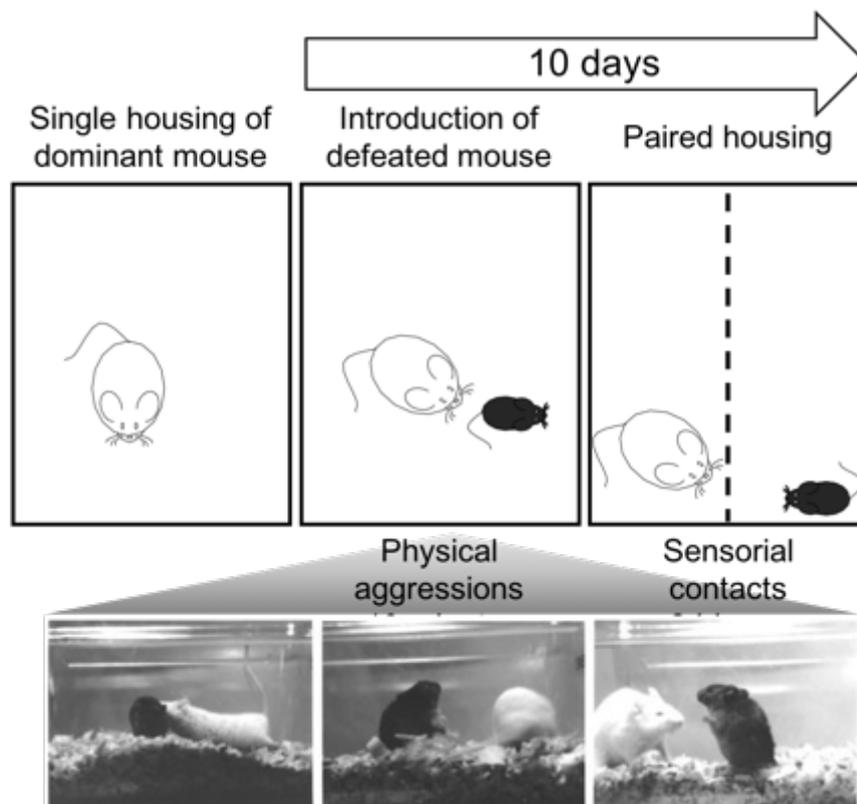


Figure 2. Procedure of the chronic stress induced by social defeat. Photographs at the bottom are from <http://schaechter.asmblog.org>

recombination system (Reichardt, Kellendonk, Tronche, & Schutz, 1999), leading to viable adult mice with loss of GR function restricted to the nervous system. The resultant animals exhibit increased levels of glucocorticoids, reduced anxiety-like behavior, and an altered behavioral stress response (Tronche et al., 1999).

Another mouse model was created, by knocking down GR gene expression using a GR antisense RNA, leading to a decline in its expression in several parts of the body, including in the brain (Pepin, Pothier, & Barden, 1992). These transgenic mice, called GRi mice, may exhibit depressive- and anxiety-like behavior, memory deficits and altered response to fear, that can be reversed by antidepressant medication (Boulle et al., 2016; Montkowski et al., 1995). In addition, GRi mice show neurobiological alterations (Froger et al., 2004), such as decreased neurogenesis and brain-derived neurotrophic factor (BDNF) expression, that are both presumed to be altered in depressed subjects (Paizanis et al., 2010) (see section 4). Hence, because this transgenic model of MDD meets the criteria of construct, face and predictive validity, and since it was widely used and characterized in our lab, it was chosen in the present study.

MDD rodent models can also be based on environmental manipulation. Among them, the learned helplessness model, in which rodents are subjected to an uncontrollable and inescapable stress such as electrical shocks, was developed in the 70's. When animals are exposed back to the same shocks but with an easy getaway, they fail to escape or the latency to escape is increased. This behavior is characterized as a state of helplessness (Seligman, Rosellini, & Kozak, 1975). In this model, rodents exhibit persistent changes such as alterations in HPA axis, weight loss, sleep pattern disturbance that may be reversed by antidepressant treatment (Haddjeri, Blier, & De Montigny, 1998; Leshner, Remler, Biegon, & Samuel, 1979; Martin, Laporte, Soubrie, El Mestikawy, & Hamon, 1990). However, in this model, there is a high variability in the expression of the helplessness phenotype, since from 10 to 80% of animals fail to exhibit escape deficits (Krishnan & Nestler, 2011). Therefore, genetically vulnerable strains have been generated (Henn & Vollmayr, 2005), and this animal model finally encompasses both genetic and environmental manipulations.

Models of chronic stress are also widely used in the depression field. Based on the clinical evidence that stressful life events may significantly increase the risk of depressive episodes, their strength is mostly due to their ability to report the

neuroplasticity changes accompanying chronic stress or antidepressant exposures (Krishnan & Nestler, 2011). One of them is the chronic mild stress (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987), also called chronic unpredictable stress, in which animals are submitted to varied intermittent physical stresses applied during 1 to 7 weeks. The resultant phenotype involves among other increased anhedonia and reduced evoked grooming (see section 1.2.2), that could be reversed by chronic antidepressants given during or after the stress period (Strekalova, Gorenkova, Schunk, Dolgov, & Bartsch, 2006). Probably because of its sensitivity to subtle variations in design, this model is reported to have a relatively low reproducibility rate, hence failing to satisfy the construct validity (discussed in Krishnan & Nestler, 2011).

Models of psychosocial stress are based on innate behavior because of a natural social hierarchy between male rodents. The social defeat model is regarded as one of the strongest paradigms of chronic stress-induced mood-related illnesses (Miczek, Yap, & Covington III, 2008), and that is the reason we used this animal model in the present study. Briefly, defeated animals repeatedly undergo subordination to unfamiliar dominant in its own territory, through physical aggression and sensory contacts (Figure 2) (Golden, Covington III, Berton, & Russo, 2011). The dominant animal often comes from an extremely aggressive strain. The resultant phenotype observed in the subordinate animal encompasses behavioral alterations comparable to symptoms observed in MDD patients (Krishnan et al., 2007), that could be explained by neurobiological changes. In addition, defeated animals exhibit memory deficits, anhedonia, decreased locomotor activity, sexual, aggressive, and exploratory behaviors (Henriques-Alves & Queiroz, 2016; Martin et al., 2017; Weber et al., 2013). In addition, these symptoms can be reversed by pharmacotherapy used in depression (Der-Avakian, Mazei-Robison, Kesby, Nestler, & Markou, 2014). Hence, this stress-induced model of MDD also meets the criteria of construct, face and predictive validity.

1.2.2. Assessment of depressive-like behavior in rodents

Historically described as a tool for the screening of new antidepressants, the forced swim test and the tail suspension test are now mostly used to assess depressive like behaviors. In the forced swim test initially described by Porsolt (Porsolt, Le Pichon, & Jalfre, 1977), animals are placed in an inescapable cylinder filled with water, while

in the tail suspension test, they are suspended from their tails. Following an initial period during which animals attempt to escape, they eventually display immobility. This immobility was first interpreted by Porsolt as despair behavior, but this is subjected to controversies since immobility can also refer to a coping strategy to maintain energy (Cryan & Mombereau, 2004). Importantly, this behavior is reversed by acute administration of antidepressant compounds. These tests are hence useful for pharmacological validation of novel molecules. In the present work, we focused on tests that assess the rodent “normal” behavior described as persistently altered in animal models of depression, including anhedonia, social behavior, and grooming-related behavior.

Anhedonia is often assessed with the sucrose preference test that is based on the natural affinity of rodents for sweet substances (Paul Willner, Muscat, & Papp, 1992). After a baseline assessment of water consumption, two different solutions are made available for animals. The first is the regular water rodents are used to drink, the second is a sucrose preparation. Preference is expressed as the percentage of volume and/or weight of consumed sucrose solution, compared to those of consumed water. Healthy animals display percentage of sucrose preference around 90%, whereas it is reduced following chronic exposure to stressors (Eagle, Mazei-Robison, & Robison, 2016).

The social interaction test is based on the fact that rodents are social animals. In this test, an unknown counterpart is placed in a removable enclosure located in a virtual interaction area. The tested animal is free to explore the field and to interact with the other rodent. The social interaction index is expressed as the time spent in the interaction area in presence of the unknown fellow, compared to this time when the enclosure is empty (Kim, Call, Carotenuto, Johnson, & Ferguson, 2017). Healthy animals have a social interaction ratio higher than 100%, whereas that of stressed rodents is lower (Golden et al., 2011).

Besides the primary purpose of hygiene, grooming also serves to thermoregulation, chemo-communication, social interaction, and stress reduction, among other functions (Smolinsky, Bergner, LaPorte, & Kalueff, 2009). The splash test is used to induce a grooming behavior, in order to evaluate a depressive-like behavior. This is different from measuring the spontaneous grooming of the animal. In this test, a sucrose solution is sprayed on the back of the animal and the time during which the

animal is grooming is measured. Regarding the expected phenotype, data are divergent since chronic mild stress has been shown to increase both spontaneous and induced grooming in mice (Isingrini et al., 2010; Kompagne et al., 2008; Mutlu et al., 2012; Yalcin, Belzung, & Surget, 2008), whereas depressive-like behavior is often related to a decrease in grooming (Shiota, Narikiyo, Masuda, & Aou, 2016).

2. Etiology of MDD: historical hypotheses

Different hypotheses have been proposed for MDD etiology, involving alterations in the monoamine neurotransmission, the HPA axis, neuroplasticity and neuro-inflammatory processes (Jentsch et al., 2015). Biological interactions between these systems, and probably others, are suspected to be responsible for the complex pathophysiology of MDD. Since these theories constitute the starting point of the MDD pathophysiology investigations, they will be briefly discussed in the following sections. The neurotrophic hypothesis, encompassing neuroplasticity impairments reported in MDD will be detailed in the section 4.5, because a large part of the present thesis is based on this hypothesis.

2.1. The monoamine hypothesis

2.1.1. Monoamines as neurotransmitters

The monoamine hypothesis was historically the first to be proposed to explain some features of MDD etiology. Monoamines, which derived from aromatic amino-acid containing a single amine group, are neuromodulators of synaptic transmission. They include serotonin (5-hydroxytryptamine, 5-HT), dopamine and noradrenaline, which all are produced in the central nervous system (CNS). Once synthesized into neurons, these monoamine neurotransmitters are transported and stored into synaptic vesicles thanks to the vesicular monoamine transporter 2 (VMAT2) (Erickson & Eiden, 1993). When the presynaptic neuron is activated, neurotransmitters are released into the synaptic cleft where they bind to their specific receptors. These targets are mainly G protein-coupled receptors (GPCRs), except for some of them such as the 5-HT₃ receptor (see below), located at the plasma membrane of the post- or presynaptic cell, triggering various signaling pathways described in section 4.3. The amount of

extracellular monoamines is highly regulated by two mechanisms. The first, and predominant under physiological conditions, involves a presynaptic recapture through plasma membrane by monoamine transporters (PMATs) (Wang, 2016) or specific transporters, leading to a recycling of monoamines into synaptic vesicles with the help of VMAT2 activity. The second is the enzymatic degradation in the synaptic cleft by the monoamine oxidase (MAO, type A or B), a mitochondrial enzyme located in neurons or glial cells (Kuhn et al., 1986). The rate of expression of MAO is correlated with its own activity, and high MAO activity is associated with low brain monoamine levels (Ramsay, 2016).

2.1.2. Serotonergic neurotransmission

The principal source of serotonin in the brain is located in the brainstem, and more precisely in neurons of the raphe nuclei (Dahlström & Fuxe, 1964). The precursor of serotonin is the dietary L-tryptophan. Serotonin biosynthesis path first involves the conversion of L-tryptophan into 5-hydroxytryptophan by tryptophan hydroxylase 2, a rate-limiting enzyme. Then, the action of the enzyme aromatic amino acid decarboxylase, leads to the production of serotonin (Kuhn et al., 1986). When released, serotonin can activate more than 15 distinct receptor subtypes, which most

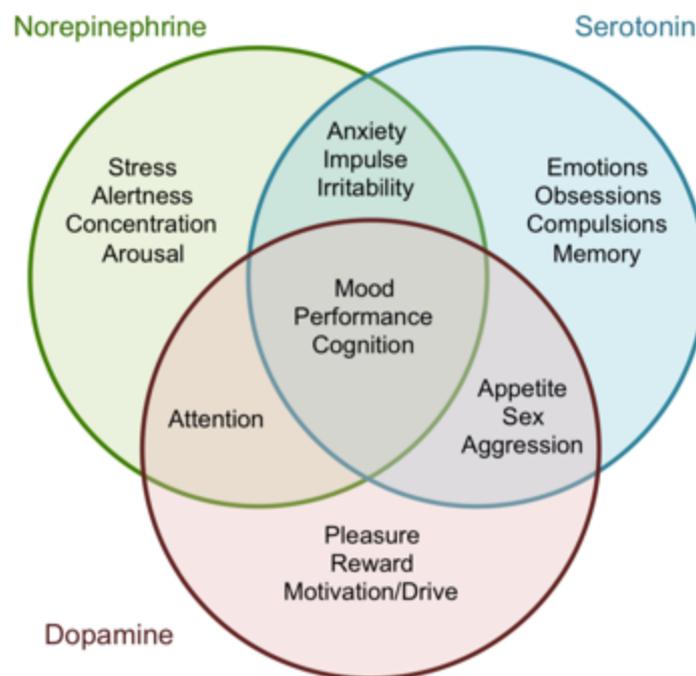


Figure 3. Monoamine involvement in behavior. From tylerlab.com

of them are GPCRs, and one is ligand-gated cation channel (5-HT₃ family; leading to plasma membrane depolarization) (Hoyer et al., 1994). Some serotonin receptors are located at the presynaptic plasma membrane, exerting a negative feedback leading to an autoregulation of serotonin release (Bramwell, 1974; Hamon et al., 1990). After activation of its targets, serotonin is recaptured by either PMAT or the serotonin transporter (SERT or 5-HTT) located at the presynaptic plasma membrane (Ruddick et al., 2006).

Serotonergic neurons have projections in almost every parts of the CNS (Figure 4A), forming the serotonergic neurotransmission network involved in several functions, including emotions, obsessions, compulsions and memory (Figure 3).

2.1.3. Dopaminergic neurotransmission

Dopamine and noradrenaline belong to the catecholamine family, in which members are all derived from the essential amino acid L-phenylalanine, converted into L-tyrosine by the enzyme phenylalanine hydroxylase. L-tyrosine can also be provided by diet. After reuptake, catecholamines can be degraded by catechol-O-methyl transferase (COMT) besides MAO-A.

The production of dopamine occurs mainly in neurons of the ventral tegmental area and the substantia nigra, both located in the brainstem (Dahlström & Fuxe, 1964). The action of the tyrosine hydroxylase on L-tyrosine leads to L-DOPA (L-3,4-dihydroxyphenylalanine) formation. Then, the activity of the DOPA decarboxylase (also known as aromatic L-amino acid decarboxylase) transforms L-DOPA into dopamine. After being released into the synaptic cleft, dopamine mediates its actions through the activation of two families of GPCRs: D1-like (including D1 and D5) and D2-like (including D2, D3 and D4) receptors. Then, dopamine is recaptured into the presynaptic cytoplasm through the action of the dopamine active transporter.

The major dopaminergic pathways include the mesocorticolimbic and the nigrostriatal projections. The mesocorticolimbic pathways convey dopamine from the ventral tegmental area to either the ventral striatum or the prefrontal cortex (Koob, Riley, Smith, & Robbins, 1978). These circuits are respectively involved in reward- and aversion-related cognition, and in executive functions (Grace, 2016). The nigrostriatal pathway connects the pars compacta, a region of the substantia nigra, with the dorsal

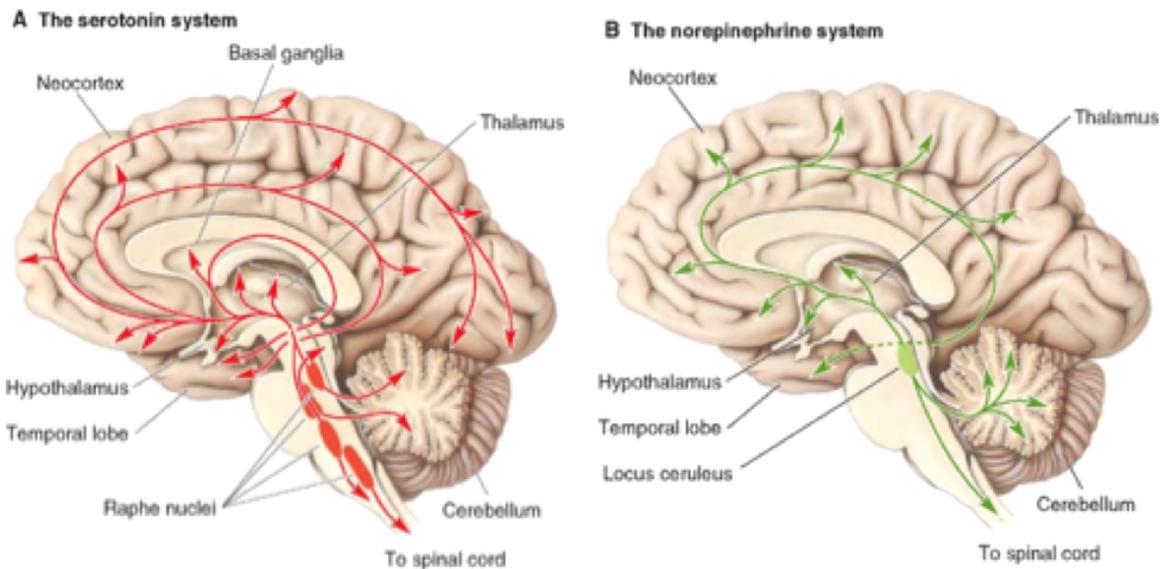


Figure 4. Serotonergic (A) and noradrenergic (B) anatomical pathways in human brain. From what-when-how.com

striatum (Bédard, Larochelle, Parent, & Poirier, 1969), and mainly regulates motor function, reward-related cognition and associative learning (Grace, 2016) (Figure 3).

2.1.4. Noradrenergic neurotransmission

Noradrenaline is directly metabolized from dopamine by the action of the dopamine β -hydroxylase, mainly in the locus coeruleus, also located in the brainstem (Dahlström & Fuxe, 1964). Once released, noradrenaline mediates its actions through activation of three families of adrenergic receptors: α_1 , α_2 and β (3 subtypes) (U'Prichard & Snyder, 1978). Similarly to the serotonergic system, adrenergic receptors can be present at the presynaptic plasma membrane, thus called autoreceptors (Adler-Graschinsky & Langer, 1975). Noradrenaline is then absorbed back into the cytoplasm of the presynaptic cell, via reuptake supported by the noradrenaline transporter (NET) (Iversen, 1974).

The functional neuroanatomy of the noradrenergic system in the brain (Figure 4B) has been largely studied and reviewed, revealing that noradrenergic neurotransmission increases arousal, alertness and vigilance (Szabadi, 2013). In addition, noradrenergic projections can connect the locus coeruleus to the limbic system that includes the prefrontal cortex, the amygdala and the hippocampus (see section 4.1), where α (1 and 2) and β subunits of the adrenergic receptors are expressed. Acting on these structures, noradrenaline promotes the formation and

retrieval of memory, but also restlessness and anxiety (Figure 3) (Szabadi, 2013; Uematsu et al., 2015).

Overall, serotonin, dopamine, and noradrenaline combined actions lead to regulation of mood and cognitive processes (Figure 3), two altered features of MDD.

2.1.5. Monoamines in MDD patients

Historically, research about MDD etiology was initiated in the 1950's, when clinical studies reported that isoniazid, an inhibitor of MAO, and imipramine, belonging to the class of tricyclic antidepressants, were both shown to improve mood in MDD patients (Kiloh & Ball, 1961; Pletscher, 1991; Sainz, 1960; Salzer & Lurie, 1953). MAO inhibitors reduce the rate of monoamine deamination and tricyclic antidepressants block the reuptake of these molecules within the presynaptic neuron, all resulting in a surge in monoamine extracellular levels. Hence, these pharmacological treatments likely lead to a reinforcement of monoamine neurotransmission, which probably triggers their therapeutic effects (see section 3.1). Then it has been proposed that the lack of extracellular monoamines, leading to a reduced monoamine neurotransmission, could strongly contribute to MDD onset (Schildkraut, 1965; Woolley & Shaw, 1954).

As illustrated in the Figure 5, the monoamine deficiency in the synaptic cleft can be the consequence of various processes: an increase in their recapture or degradation, and/or a decrease in their synthesis or release. An impressive number of clinical studies have been published regarding the involvement of the monoamine actors (synthesis, receptors...) in the pathophysiology of mood disorders at biochemical, epigenetic and genetic levels. However, since these processes are not the real purpose of the present work, the complex and extensive literature in this field will be summarized and extremely simplified. Regarding serotonin and noradrenaline reuptake, no direct link between monoamine transporters and etiology of MDD has been demonstrated in patients, although polymorphisms in genes encoding for SERT and NET do exist (Buttenschøn et al., 2013; Cao et al., 2018; Juhasz et al., 2015; Lesch et al., 1992). However, in depressed patients, MAO-A levels have been shown to be increased in the prefrontal cortex and the anterior cingulate cortex compared to healthy subjects (Johnson et al., 2011; Meyer et al., 2006). In addition, tryptophan

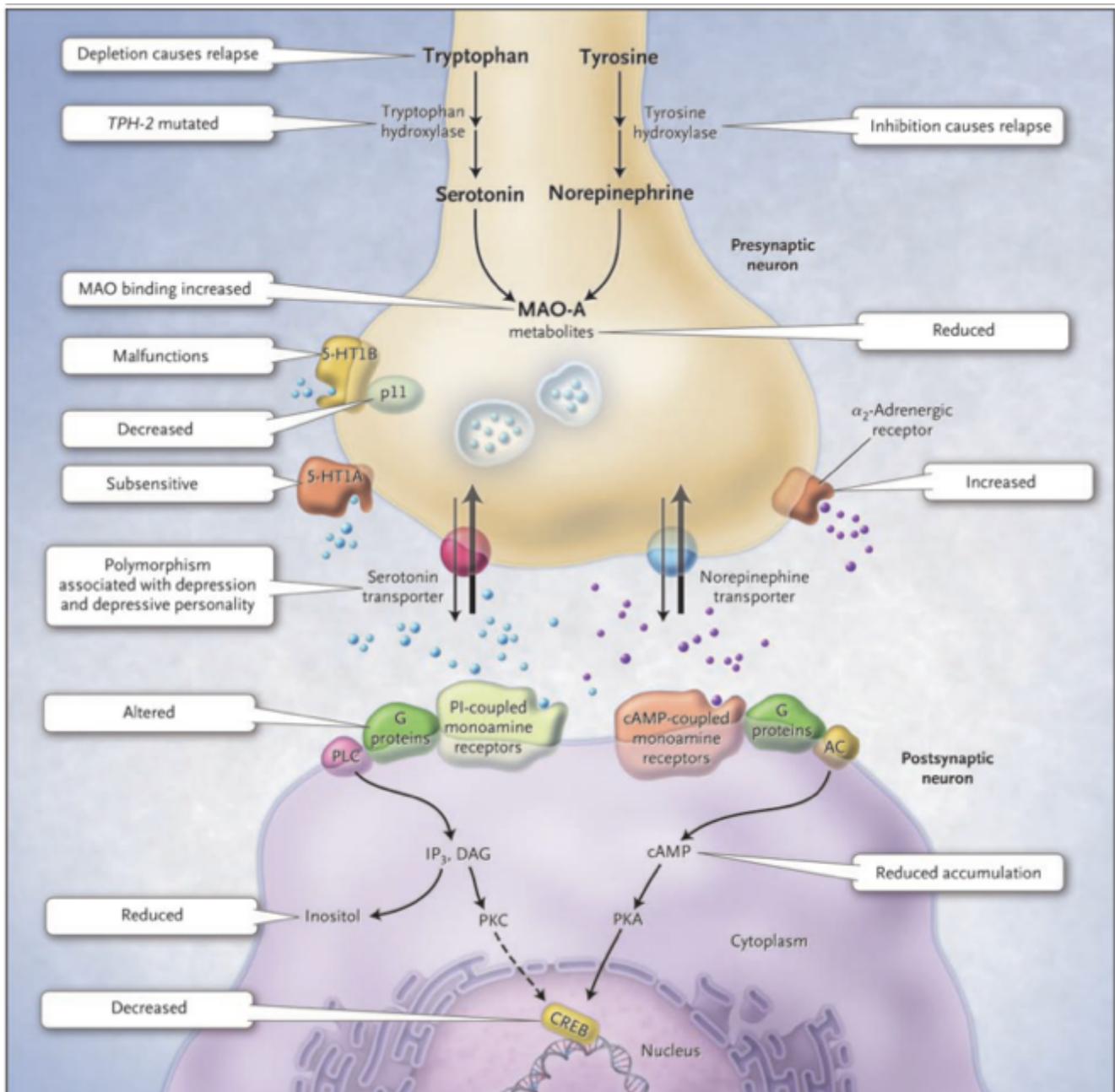


Figure 5. The monoamine hypothesis: alterations in serotonin and noradrenaline neurotransmission. From Belmaker & Agam, 2008.

depletion and tyrosine hydroxylase inhibition cause relapse in patients under antidepressant treatment (Ruhé et al., 2007), and a mutation in the gene encoding for tryptophan hydroxylase 2 has been associated to depression (Xiaodong Zhang et al., 2005). The last support of the monoamine hypothesis is that autoreceptors 5-HT_{1B} and α_2 adrenoceptors, regulating monoamine release by negative feedback, are also down-regulated in MDD (Ordway et al., 2003; Svenningsson et al., 2006).

Monoamine depletion studies were conducted in humans to elucidate the role of serotonin, norepinephrine and dopamine in MDD (Delgado et al., 1991). Ruhé et al. performed a meta-analysis in order to review the evidence for mood lowering properties after monoamine depletion (Ruhé et al., 2007). The most striking finding was that the depletion of monoamines did not induce a lower mood in healthy subjects with no family history of MDD, showing that monoamine hypothesis seems to be necessary but not sufficient to explain MDD etiology (Ruhé et al., 2007).

2.2. The glucocorticoid hypothesis

The HPA axis is a major neuroendocrine system that elicits cortisol production and is designed to help the organism coping with adversity through stress response mediation. It is involved in several biological processes, such as cardiovascular function, inflammatory reactions, arousal, learning and memory. Hence, when HPA axis is activated following a stress, all these systems are affected.

2.2.1. Anatomy of the HPA axis

The HPA axis is composed of the hypothalamus, the pituitary gland and the adrenals (Figure 6). The hypothalamus, a part of the limbic system, is a clustering of several nuclei that have distinct functions. One of them, the paraventricular nucleus, contains neurons that synthesize vasopressin and corticotropin-releasing hormone (CRH). These neurons are considered to be the principal effector of the stress response. Vasopressin is transported to the posterior pituitary gland via an axonal transport, and reaches the anterior part of the pituitary gland through blood capillary network. In contrast, CRH is hormonally secreted directly in the anterior pituitary via a portal blood vessel system. By binding to their specific receptors, they both stimulate the production of adrenocorticotrophic hormone (ACTH). The action of vasopressin is not sufficient to elicit ACTH release, but it complements that of CRH. When neurons in

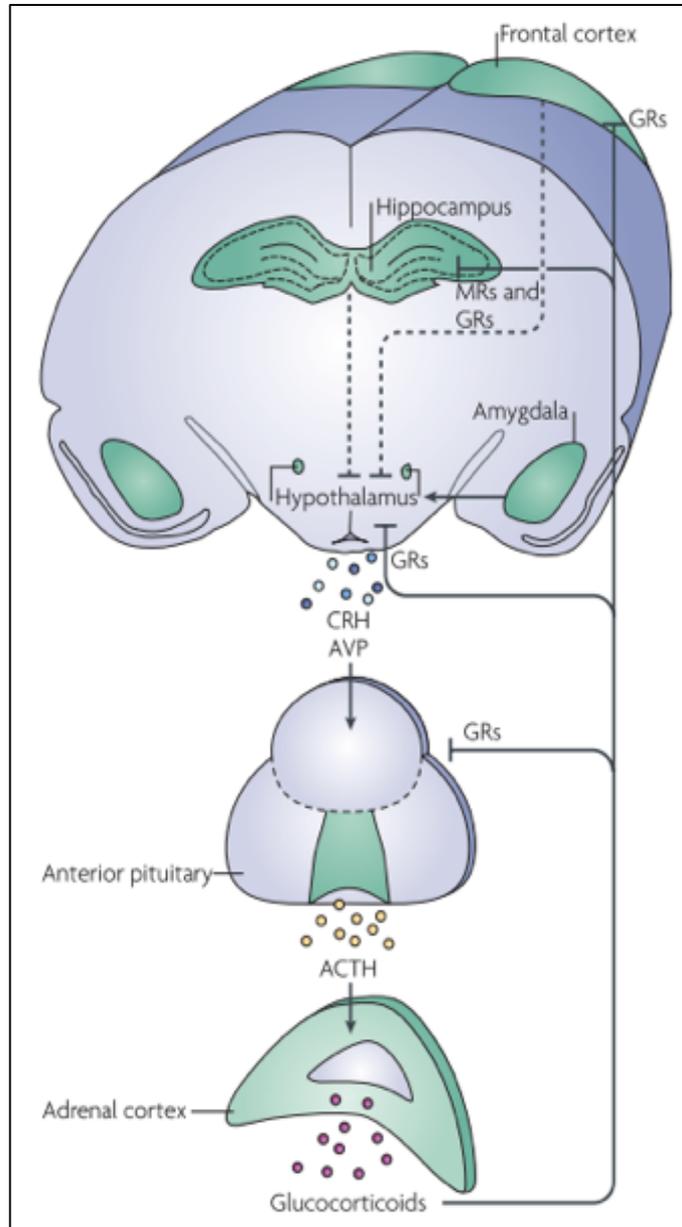


Figure 6. The stress response is mainly supported by neuroendocrine activity in the HPA axis. From Lupien et al., 2009

the anterior pituitary gland are stimulated by CRH binding to its receptor, the precursor proopiomelanocortin is cleaved into ACTH and β -lipotrophin. Proopiomelanocortin is also upregulated at the transcriptional level, where CRH promotes its production (Smith & Vale, 2006). The hormonal secretion of ACTH leads to stimulation of melanocortin receptors located in the cortical part of the adrenals, which in turn produces and secretes glucocorticoids, such as cortisol in human or corticosterone in rodents. Glucocorticoids can promote the production of adrenaline and noradrenaline in the medullar portion of the adrenals. When the HPA axis is activated, several parasympathetic effects occur, such as elevated arterial pressure, suppression of the immune response and inflammation, inhibition of reproductive physiology and behavior (Sapolsky et al., 2000).

In the brain, glucocorticoids act on both high affinity mineralocorticoid receptor (MR) and low affinity GR that both belong to the nuclear receptor family. They are present in the cytoplasm and nucleus. When cortisol binds to its receptor, the complex translocates and/or accumulates in the nucleus where it acts as transcription factor (Akner et al., 1995; Parker, 1993). GRs and MRs are expressed throughout the brain. The highest density of GRs is found in the cerebral cortex, hippocampus and paraventricular nucleus of the hypothalamus, while MRs are mostly located in the hippocampus and dorsolateral septum (Reul et al., 2000; Usuku et al., 2005). Affinity of MRs for cortisol or corticosterone is more than 10-fold higher than that of GRs (Reul & De Kloet, 1985). Hence, MRs are occupied at low glucocorticoid levels, like under basal state, whereas GRs are activated following an increase in glucocorticoid levels, such as under stressful conditions (De Kloet, Vreugdenhil, Oitzl, & Joëls, 1998).

2.2.2. Neuronal and hormonal regulation

The energy and arousal state needed to cope with a stressful event are provided by glucocorticoids and noradrenaline released following the stress-induced activation of the HPA axis. This axis is regulated by a plethora of mechanisms, including those that are hormonal and neuronal, as illustrated in the Figure 7.

Neuronal regulation of the HPA axis mostly occurs at CRH-releasing neurons. Indeed, as mentioned above, these neurons are considered as the sensor of the stress response. Under basal conditions, the neurotransmitter γ -aminobutyric acid (GABA)

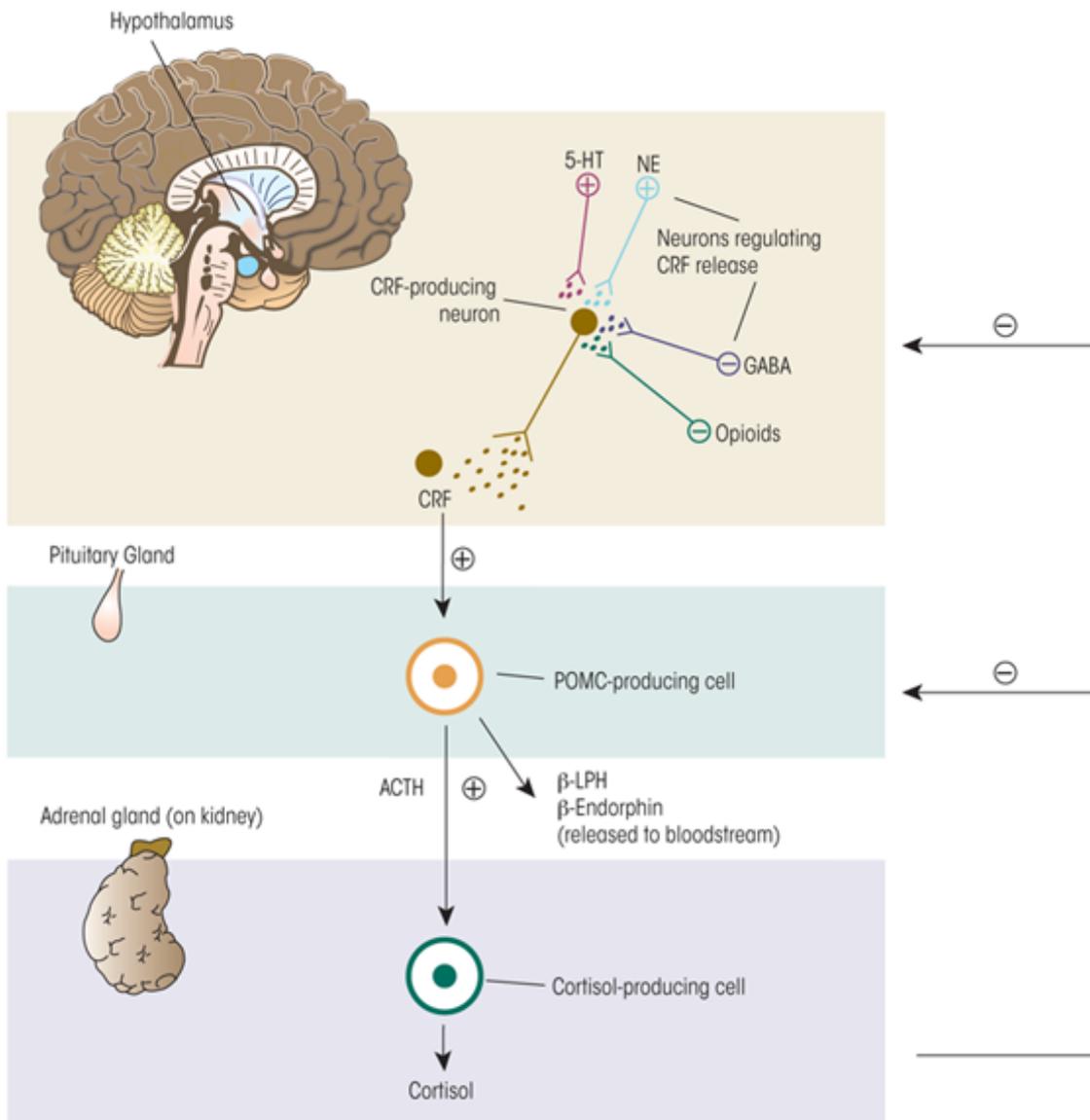


Figure 7. Neuronal and hormonal regulation of the HPA axis. From Stephens & Wand, 2012.

exerts an inhibitory tonus on CRH-releasing neurons, primarily via GABA_A receptor binding, and this maintains low CRH neuronal activity (Cole & Sawchenko, 2002; Radley, Gosselink, & Sawchenko, 2009; Radley & Sawchenko, 2011). The GABAergic inhibition can be potentiated by noradrenaline acting through α_1 adrenergic receptor activation. Under stressful conditions, the GABAergic inhibition is suppressed, and this disinhibition is believed to be the principal source of HPA axis activation (Hewitt et al., 2009). Noradrenaline contributes to suppress GABA release, acting on α_2 adrenoceptors located at presynaptic terminals (Han et al., 2011), and potentiates glutamatergic inputs through α_1 adrenoceptor activation (Plotsky et al., 1987). L-glutamate, besides participating in cell metabolism, is the major mediator of excitatory neurotransmission in the CNS. CRH neurons of the paraventricular nucleus express two kinds of glutamate receptors: ionotropic (N-methyl-D-aspartate, NMDA; α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA; and kainate receptors) and metabotropic (mGluR) (Durand et al., 2008; Herman et al., 2000). At the beginning of a stressful event, glutamatergic neurotransmission is activated and prevents the inhibitory action of GABA, hence participating to the disinhibition of CRH neurons and the activation of the stress response (Feldman & Weidenfeld, 1997; Johnson et al., 2001; Lang & Ajmal, 1995). In addition, serotonergic fibers projecting from the raphe nuclei are known to stimulate ACTH and corticosterone secretion through 5-HT_{1A} and 5-HT_{2A} receptors (Pan & Gilbert, 1992; Van de Kar et al., 2001).

The paraventricular nucleus also receives synaptic innervation from ascending stress-regulatory neurons of the caudal medulla and locus coeruleus (Cunningham & Sawchenko, 1988). These inputs mainly come from viscerosensory relays and are activated in response to disturbed homeostasis that may occur following visceral illness or fluid/electrolyte balance for example (Herman et al., 2016). Neuropeptides, cytokines and inflammatory factors released by ascending stress-regulatory neurons can hence participate to HPA axis activation in the stress response since they activate CRH-releasing neurons of the paraventricular nucleus (Herman et al., 2016).

After the stressor subsides, released glucocorticoids bind to GRs, and terminate the HPA axis response through various feedback mechanisms, including a “rapid” and a “delayed” feedback (Keller-Wood & Dallman, 1984). The rapid feedback is operated by non-genomic actions and mainly consists in CRH and ACTH return to basal levels. In the paraventricular nucleus, glucocorticoids inhibit CRH secretion, via retrograde

endocannabinoid release, that rapidly lessens the activity of glutamatergic inputs in the paraventricular nucleus (Di et al., 2003). Glucocorticoids also inhibit ACTH outputs of the anterior pituitary gland and repress the transcription of its precursor proopiomelanocortin. The delayed feedback, supported by genomic effects, mainly occurs at GRs expressed in the hippocampus and prefrontal cortex (Lupien et al., 2009) (Figure 6), and allows storage of information in memory for future use, and recovery from the stressful experience (de Kloet et al., 2008).

When individuals are exposed to chronic stress, glucocorticoids, stress peptides and proinflammatory cytokines levels may remain high. Because of their catabolic properties, this could have deleterious consequences on the organism, possibly causing depressive symptoms.

2.2.3. HPA axis in MDD patients

Hyperactive HPA axis has been largely reported in MDD (Figure 8). Indeed, while depressed patients display similar levels of plasmatic cortisol than healthy subjects under basal conditions, these levels are much higher in patients during the recovery phase after a psychological stressor (Burke et al., 2005). In addition, CRH levels are increased in the hypothalamus, prefrontal cortex, and cerebrospinal fluid of patients with MDD (Merali et al., 2004; Raadsheer et al., 1994) (Figure 8).

In depressed patients, HPA axis hyperactivity is not reduced by pharmacological GR stimulation by dexamethasone, a synthetic glucocorticoid, whereas this is the case in healthy subjects (Carroll et al., 1981; Menke et al., 2012; Pariante & Lightman, 2008). This strongly suggests that HPA axis chronic activation found in MDD patients could be related to dysfunction in glucocorticoid negative feedback occurring at GR level (Holsboer, 2000; Zunszain et al., 2011). Impairment in GR signaling can come from a decrease in their expression or function. For example, a reduction in GR mRNA expression in both the frontal cortex and the dentate *gyrus* of the hippocampus has been reported in patients with MDD (Webster et al., 2002). Moreover, genetic variant within the gene encoding for FK506 binding protein 5 modifies its interaction with GR, leading to a decreased affinity of the receptor for cortisol and this could influence MDD predisposition (Szczepankiewicz et al., 2014).

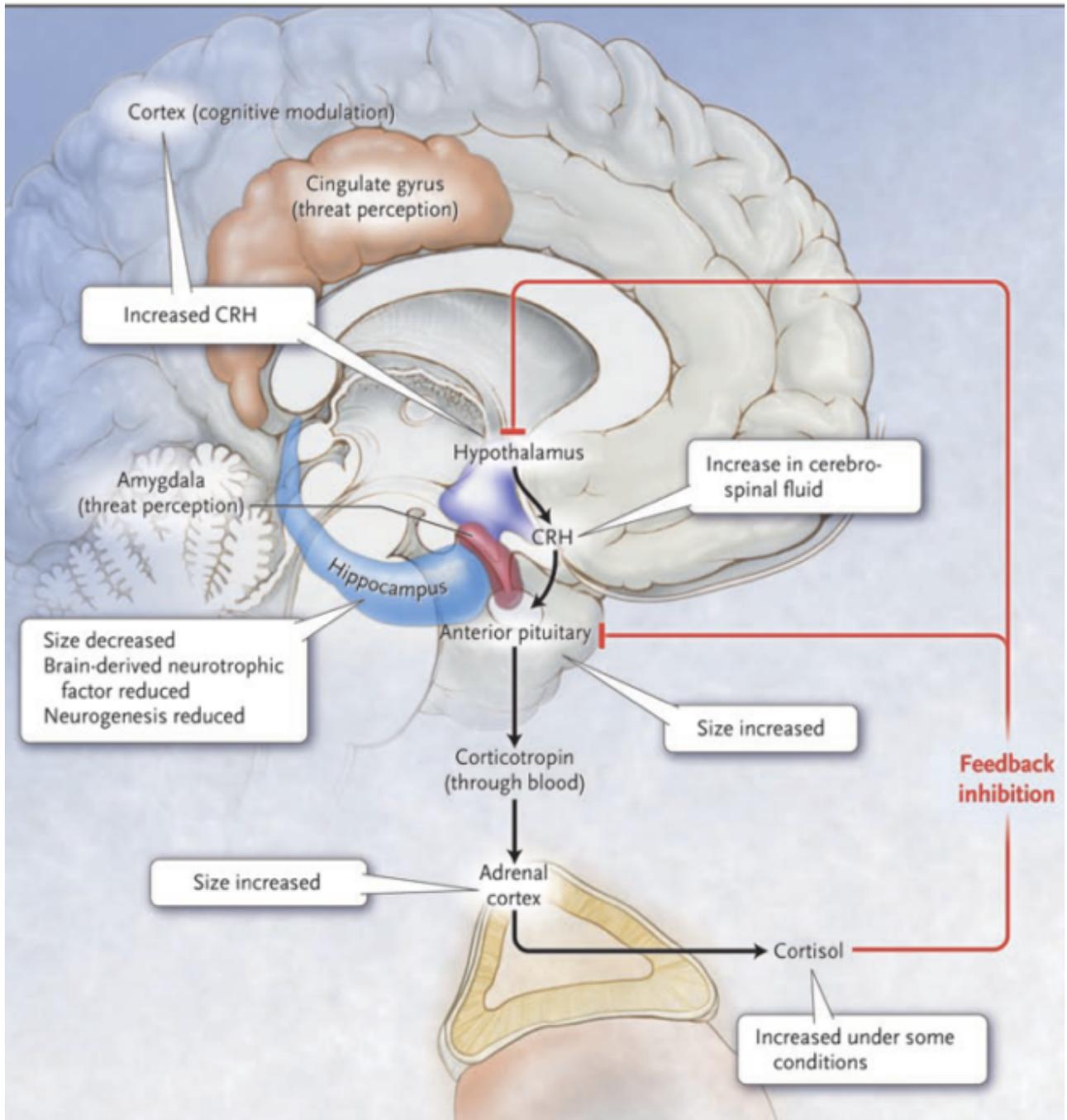


Figure 8. The glucocorticoid hypothesis for MDD etiology. From Belmaker & Agam, 2008.

2.3. The inflammation hypothesis

The inflammation hypothesis relies on the fact that predominantly pro-inflammatory cytokines are over-expressed in the blood of depressed subjects, even in the absence of systemic inflammation (Dowlati et al., 2010; Haapakoski et al., 2015). In addition, subjects with medical illness or treated with cytokines have a higher risk to develop MDD. Conversely, depressive patients have a higher risk of inflammatory illness (Krishnadas & Cavanagh, 2012).

2.3.1. Cytokines in the CNS

When a tissue is damaged, inflammation process occurs as a protective response from the body to injury or infection. This arise from the recruitment of monocytes, lymphocytes and neutrophils, along with an activation of several resident inflammatory cells, including macrophages, lymphocytes, mast cells, fibroblasts and endothelial cells. Immune cells can produce and release cytokines, which constitute a large family of soluble proteins that are major mediators of the inflammatory response. Cytokines interleukin 1 β , interleukin 6, interferon γ , tumor necrosis factor α are able to stimulate their own production (Chrousos, 1995). They can act locally or systemically via autocrine and paracrine pathways. Their actions are mediated by binding to cytokine receptors, which can be soluble or membrane-anchored.

The CNS and the immune/inflammatory system are strongly connected, and cytokines are considered as a stressor (Anisman et al., 2002). They also inhibit GR function, probably leading to glucocorticoid resistance (Chrousos, 1995; Pace & Miller, 2009). Interestingly, interleukin 6 concentration has been shown to be increased in blood during stress and independently to inflammation activation, and noradrenaline, acting through β 2 adrenoceptors, may be responsible for this action (Chrousos, 1995). In contrast, glucocorticoids are the most potent anti-inflammatory hormones in the body (Zunszain et al., 2011). They can suppress the immune activation of leukocytes and inhibit the production of cytokines, eventually resulting in a resistance to cytokines (Boumpas et al., 1993; Chrousos, 1995).

2.3.2. Cytokines in MDD patients

MDD patients have higher levels of immune cytokines interleukin 6 and tumor necrosis factor α and leukocytes in blood than healthy subjects (Dowlati et al., 2010;

Liu, Ho, & Mak, 2012; Maes et al., 1992). Proinflammatory cytokines activate the indoleamine-2,3-dioxygenase (IDO), an enzyme that metabolizes tryptophan along the kynurenine pathway (O'Connor, Andre, et al., 2009; O'Connor, Lawson, et al., 2009). IDO activation is required for both the down-regulation of the immune response and the development of inflammation-induced depression-like behavior (Dantzer & Walker, 2014). Since tryptophan is also the precursor of serotonin (see section 2.1.2.), an increase in IDO activity could lead to a decrease in tryptophan levels and serotonin production, that may explain some symptoms characterizing MDD. In addition, proinflammatory cytokines increase the expression of SERT, possibly leading to a decrease in serotonin levels in the synaptic cleft (Dantzer & Walker, 2014). This association between reduced levels of tryptophan and MDD has been confirmed in several clinical studies (Raison et al., 2010; Swardfager et al., 2009).

An increase in IDO activation triggers a greater production of kyurenine and metabolites that are cytotoxic. Kyurenine is further transformed into quinolinic acid, a NMDA receptor agonist, which has been associated to depression. Indeed, in MDD patients, quinolinic acid is increased in the prefrontal cortex and decreased in the hippocampus (Busse et al., 2015; Steiner et al., 2011). Hence, dysfunction of the immune system in depression could be responsible for impairments in NMDA neurotransmission.

In repeated social defeat stress, chronic treatment with antidepressants could reverse the depressive-like phenotype, but failed to normalize cytokine levels in periphery (Hodes et al., 2014). Also, circulating interleukin 6 could be a predictive marker for susceptibility versus resilience to stress, evidenced in this model (Hodes et al., 2014). Given the fact that some subjects with high cytokine concentration will not develop MDD, it may exist a subtype of depression related to a greater inflammation. Hence, inflammation hypothesis only partially explains to pathophysiology of MDD.

3. Pharmacotherapy in MDD

3.1. Inhibitors of monoamine reuptake or degradation

Since the 1950's, monoamine neurotransmission remains the major target of antidepressant pharmacological treatment. They cause the heightening of serotonin, noradrenaline and dopamine levels in the synaptic cleft. First-generation antidepressant drugs include MAO inhibitors and tricyclic antidepressants (see section 2.1.5.) (Langer & Schoemaker, 1988). Although a comparable efficacy, second-generation antidepressants are preferentially prescribed for MDD. Indeed, their higher specificity leads to reduced risk for fatality in case of overdose and less severe side effects (Hindmarch, 2002). These molecules include selective serotonin reuptake inhibitors (SSRI), norepinephrine reuptake inhibitors and reuptake inhibitors of both serotonin and norepinephrine. They all selectively block SERT and/or NET, and some of them also act on some serotonin and/or noradrenaline receptors (Immadisetty et al., 2013).

These antidepressants can also act on other parameters characterizing MDD pathophysiology. They exert their clinical action via up-regulation of GR expression and signaling in the brain, hence leading to normalization of HPA axis activity (Brady et al., 1991; Reul et al., 1994; Seckl & Fink, 1992). In addition, antidepressants could decrease inflammatory response by lessening blood concentration of cytokines (Kakeda et al., 2018), and increase BDNF levels in several brain regions, including the hippocampus and the prefrontal cortex (Levy et al., 2018).

3.2. Atypical antidepressants

To prevent suicide attempts, the NMDA receptor antagonist ketamine is acutely administered in patients, including those who are resistant to other antidepressants. Indeed, a single intravenous administration of ketamine induces robust and rapid antidepressant effects that remain significant for two weeks (Murrough, Iosifescu, et al., 2013; Murrough, Perez, et al., 2013; Zarate et al., 2006). These therapeutic effects are supported by findings showing alterations in the glutamatergic neurotransmission in MDD. Indeed, metabotropic glutamate receptor 5 density is reduced in depressed patients compared to healthy subjects (Deschwenden et al., 2011). In addition,

reduced glutamate levels were reported in the prefrontal cortex (Hasler et al., 2007). Regarding glutamate levels in the occipital cortex, data are divergent since some studies found them elevated, while others fail to evidence any difference compared to control subjects (Godlewska, Near, & Cowen, 2015). Nevertheless, the exact effect of ketamine on depression-related features is still unknown.

Agomelatine is an agonist of type 1 and 2 melatonin receptors, and a 5-HT_{2C} receptor antagonist (De Bodinat et al., 2010). Melatonin (N-acetyl-5-methoxy tryptamine) is a hormone produced in the pineal gland that regulates sleep and wakefulness and is involved in the circadian rhythms (Claustrat & Leston, 2015). Acting on both the melatonergic and serotonergic system, agomelatine can reduce anxiety symptoms of MDD patients (Stein, Picarel-Blanchot, & Kennedy, 2013), and possibly improve cognition in schizophrenic patients with comorbid depression (Englisch et al., 2018).

Treatment with non-steroidal anti-inflammatory drugs in MDD patients are suspected to decrease depressive symptoms (Köhler et al., 2014), even if no clear therapeutic effect has been evidenced yet (Colpo, Leboyer, Dantzer, Trivedi, & Teixeira, 2018; Raison et al., 2013).

Antidepressants targeting the stress axis can also be prescribed. For example, mifepristone, a GR antagonist, improves mood and cognitive deficits in depressed patients (Blasey, Block, Belanoff, & Roe, 2011; Roat-Shumway, Wroolie, Watson, Schatzberg, & Rasgon, 2018).

3.3. Limitations of current treatments

Current antidepressant pharmacotherapy has several limitations. First, their efficacy is relatively low since only one half of patients responds to the first prescription of SSRI antidepressant, while one third presented remission (Trivedi et al., 2006). A more recent study estimated the remission rate fewer than 50% after 12 weeks of treatment under monotherapy or combined therapy in MDD patients (Bobo et al., 2011). In fact, 30% to 40% of depressed subjects do not achieve remission to any classical treatment, which render them patients with treatment-resistant depression. This is defined by a failure to respond to adequate doses and duration of

pharmacotherapy of two different antidepressants from two distinct classes (Bentley et al., 2014).

This low remission rate can be related to the delayed onset of action of agents targeting monoamines. Indeed, their potential therapeutic effects are observed within at least two or three weeks, which can lead to treatment discontinuation (Trivedi et al., 2006). This can also be due to inconvenient side effects associated to this kind of treatment, such as sleep disruption, gastrointestinal transit disturbance, sex dysfunction or sedation (Bentley et al., 2014).

Importantly, to date, efficacy of pharmacotherapy to cure the cognitive deficits associated to MDD is limited. Indeed, they are still present even in remitted patients from depression (Soria et al., 2018). Hence, new strategies are needed to attempt to cure depression, and especially the cognitive alteration reported in MDD patients. In the following sections, a statement in neuroplasticity processes underlying cognition will be exposed.

4. Neuroplasticity mechanisms in cognition

According to the Oxford dictionaries, cognition is defined as “the mental action or process of acquiring knowledge and understanding through thought, experience, and the senses”. In humans, it concerns all biological processes that spearhead attention, learning, memory, reasoning, judgment, decision making, problem solving, understanding, eventually leading to knowledge formation or use. In rodents, cognitive-like behavior is essentially assessed by measuring learning, memory and attention (Wallace, Ballard, & Glavis-Bloom, 2015). Cognitive impairments in MDD patients are related to neuroplasticity alterations in some brain regions, including in the limbic system.

4.1. Anatomy and functions of the limbic system

The limbic system is a set of brain structures encompassing subcortical regions, comprising the olfactory bulb, hypothalamus, amygdala, mammillary bodies, nucleus accumbens, septum, and some thalamic nuclei, such as the anterior dorsomedial nuclei. Some cortical regions also belong to the limbic system, this is the case for the

hippocampal formation, insular cortex, orbitofrontal cortex, cingulate *gyrus*, and parahippocampal *gyrus* (Figure 9). This system is involved in planning complex cognitive behaviors, personality expression, decision-making and moderating social behavior. Here, the focus will be made on the hippocampus, the nucleus accumbens, the amygdala and the prefrontal cortex that all play a pivotal role in MDD pathophysiology, since changes in these structures are reported in depressed patients.

The hippocampus is a brain structure present in each hemisphere and located in the temporal lobe under the cerebral cortex. It is composed of the dentate *gyrus* and the *Cornu Ammonis* 3, 2 and 1 (CA3, CA2 and CA1) (Schultz & Engelhardt, 2014). Most of afferences coming from the entorhinal cortex constitute the perforant pathway (Witter & Amaral, 1991). The granule cells of the dentate *gyrus* connect to CA3 via the mossy fibers. The projections of CA3 pyramidal cells are called Schaffer collaterals and connect to CA1 neurons which send their axons to the entorhinal cortex (Schultz & Engelhardt, 2014). Among other connections, the hippocampus projects glutamatergic fibers to the ventral tegmental area and prefrontal cortex; and receives serotonergic and glutamatergic afferences from the raphe nuclei and the prefrontal cortex respectively (Morgane, Galler, & Mokler, 2005). This brain structure encompasses a dorsal and ventral regions proposed to be functionally distinct; the

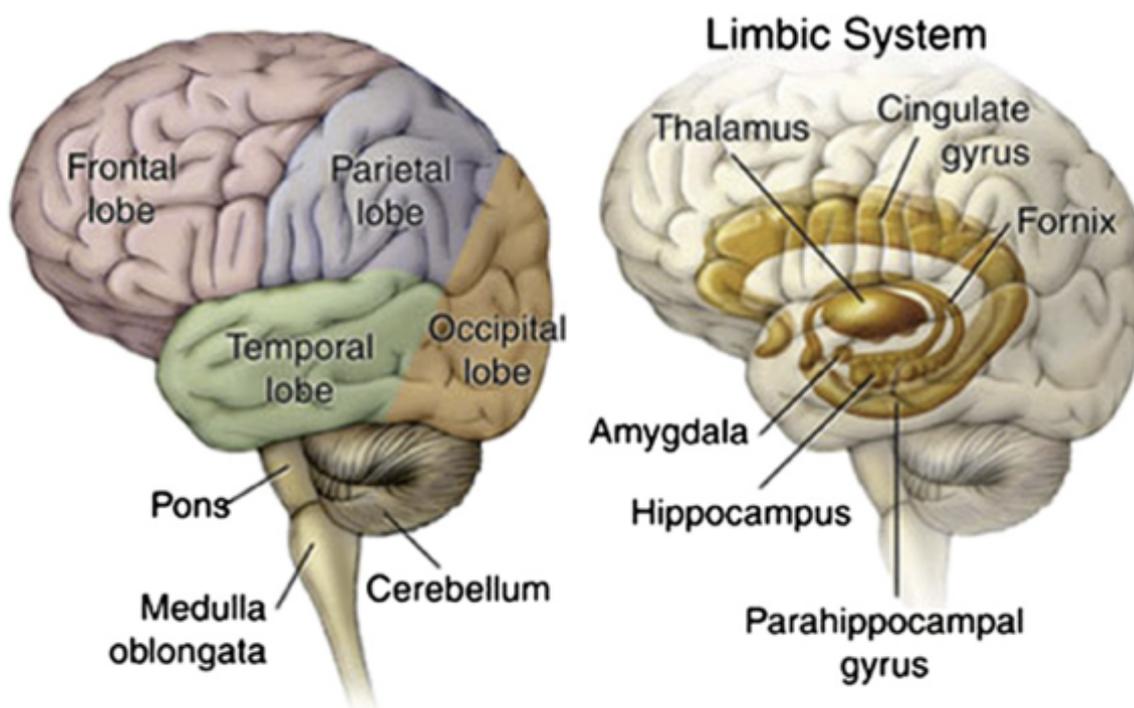


Figure 9. Anatomic location of the limbic system components in humans. From Braun, 2011.

dorsal part being mostly involved in cognitive processes, whereas mood-related behaviors primarily engage the ventral hippocampus (Fanselow & Dong, 2010).

The nucleus accumbens is located in the ventral part of the striatum, present in the forebrain of both hemispheres. The ventral striatum, receiving dopaminergic afferences from the ventral tegmental area (see section 2.1.3.), contains medium spiny neurons that express dopamine receptors. These neurons are GABAergic and regulated by several kinds of inhibitory interneurons. Other inputs from several regions connect to the ventral striatum, including from the limbic system itself. The nucleus accumbens is primarily involved in decision making and goal-oriented behavior.

The amygdala is a cluster of nuclei located in each side of the brain, within the temporal lobe. It is usually divided into three parts: the basolateral, the medial and the central amygdala (Sah et al., 2003). The central amygdala receives inputs from intercalated cell clusters and from the basal amygdala. The activity of this structure is highly inhibited under resting state by GABA-mediated mechanisms. When a stressor is perceived by the organism, the amygdala is disinhibited by inputs coming from the prefrontal cortex (Zhang et al., 2018). The amygdala also receives serotonergic afferences from the raphe nuclei that participate in processing stress stimuli. The amygdala plays a major role in processing physiologic and behavioral responses to stress and is widely involved in fear- and aggressive-related behaviors.

The prefrontal cortex is located in the front part of the frontal lobe. It can be divided into the medial, dorsolateral, ventrolateral and orbitofrontal parts. Pyramidal neurons of the cortical layer V receive inputs from the thalamus and other cortical areas. Their apical dendrites express 5-HT_{2A} receptors, and serotonin coming from the raphe nuclei induces an excitatory effect on these neurons (Azmitia & Segal, 1978; Blue, Yagaloff, Mamounas, Hartig, & Molliver, 1988). Afferences of the prefrontal cortex are also dopaminergic, emerging from the substantia nigra *pars compacta* and the ventral tegmental area and mostly acting on D1-like receptors (Puig, Rose, Schmidt, & Freund, 2014). Outputs from the prefrontal cortex connects to subcortical areas, including the other structures of the limbic system. The prefrontal cortex sends glutamatergic projections to the basal amygdala (McGarry & Carter, 2017). The medial prefrontal cortex also controls the serotonergic system (Celada, Puig, Martin-Ruiz, Casanovas, & Artigas, 2002). The prefrontal cortex is considered as the conductor of cognition and other executive functions.

Besides the individual role of each component of the limbic system, the connections between regions are of great significance in behavioral adaptation. Indeed, because of their high susceptibility to neuroplasticity processes, neuronal networks between the hippocampus, amygdala, nucleus accumbens and prefrontal cortex are essential for several emotion-related behaviors; acquisition, storage, and retrieval of aversive memories; planning of behavioral response.

4.2. Evidences for neuroplasticity in adulthood

The brain is constantly remodeled in response to experience through neuroplasticity. The pioneer work in neuroplasticity was conducted by Hebb, who postulated that synaptic plasticity involves both structural and functional changes, occurring in excitatory and inhibitory networks (Hebb, 1949). In the next sections, we will see that although structural and functional plasticity depend on distinct mechanisms, they are closely associated and both lead to more integrated plasticity processes, such as neurogenesis.

4.2.1. Structural plasticity

Structural plasticity refers to the complexity of the neuronal arbor, including the number of ramifications and the dendritic length. These parameters are under control of actin and microtubule cytoskeletons. In neurons, microtubules are cylindrical associations of 13 protofilaments, themselves composed of α - and β -tubulin heterodimers (Figure 10). They are formed by polymerization of tubulin heterodimers. At their ends, cycles of polymerization/depolymerization occur, leading to a dynamic structure (Walker et al., 1988). Microtubules are polarized since they have a slow-growing minus-end, generally located near the cell body. α -tubulin can undergo post-translational modifications such as acetylation and tyrosination/detyrosination. Tubulin acetylation is the addition of an acetyl group to a lysine located in the luminal surface of the microtubules (Soppina, Herbstman, Skiniotis, & Verhey, 2012). Tyrosination or detyrosination refers respectively to the addition or the removal of the tyrosine located at the C-terminal of α -tubulin (Ersfeld et al., 1993; Gundersen, Khawaja, & Bulinski, 1987). Even if no correlation has been demonstrated, acetylation is primarily observed at the stable minus-end, and tyrosination/detyrosination cycles mostly occur at the dynamic plus-end.

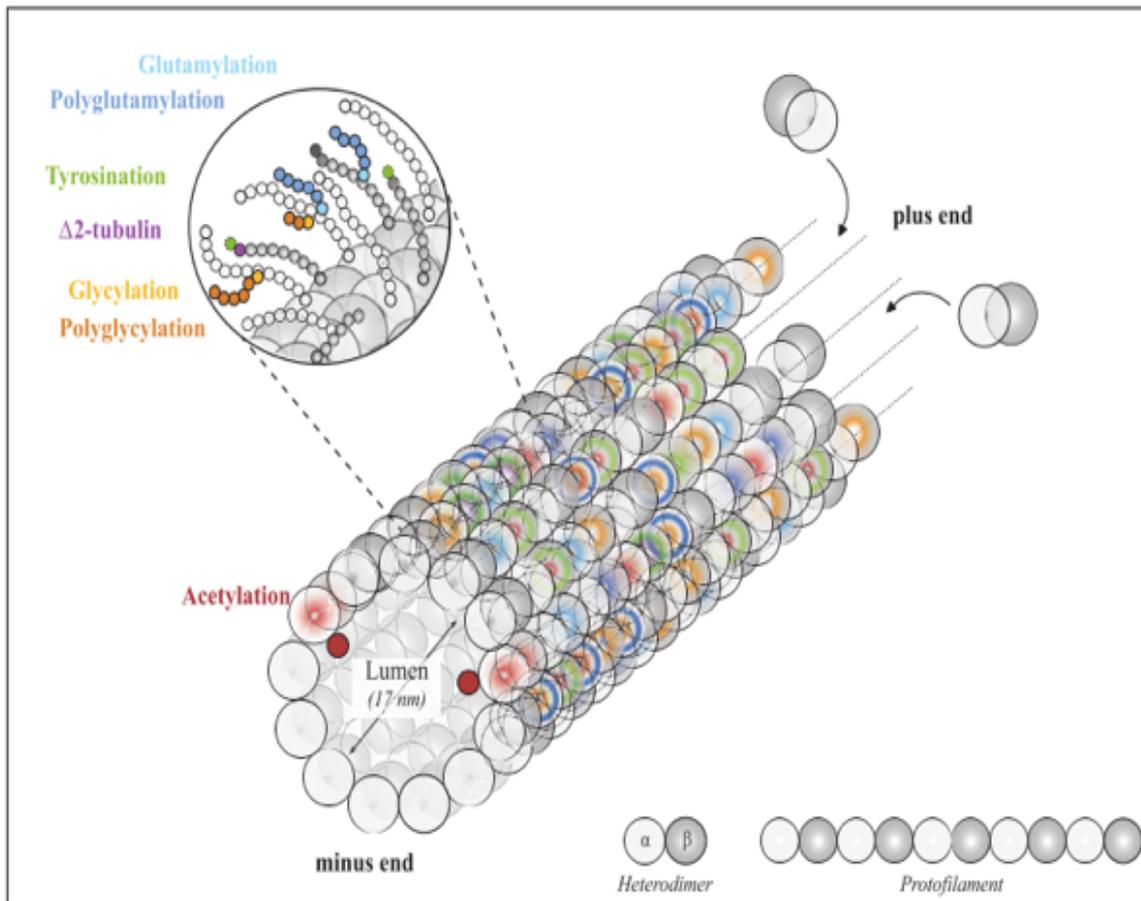


Figure 10. Microtubule formation and post-translational modifications. From Penazzi et al., 2016

At the fast-growing plus-end, polymerization and depolymerization rates dominate during respectively growth or retraction of neuronal processes (Penazzi, Bakota, & Brandt, 2016). Under certain conditions, microtubule can undergo sudden transitions between phases of fast shortening (catastrophe) and elongation (rescue). This phenomenon called dynamic instability is fundamental to proper function of microtubule cytoskeleton (Goodson & Jonasson, 2018; Mitchison & Kirschner, 1984). Microtubules are regulated by microtubule binding proteins, which include microtubule associated proteins (MAPs), motors, and cytoplasmic linker proteins (CLIPs). MAPs directly regulate the dynamic of microtubules, by modulating their stability, polymerization or depolymerization. For example, it is well accepted that MAP6, could be a central regulator of structural plasticity, since its *in vivo* deletion leads to anatomical abnormalities in the brain (Powell et al., 2007). On the other hand, MAP2 (described in the section 5.2.1) phosphorylation state is positively correlated with dendrite branching *in vitro* (Audesirk, Cabell, & Kern, 1997), further demonstrating the

critical role of MAPs in microtubule-related structural plasticity. Motors are attached to microtubules and allow the transport of different features involved in the synapse. CLIPs are primarily critical for cell organization since they anchor organelles to microtubules (Goodson & Jonasson, 2018). Overall, microtubules play a pivotal role in structural plasticity, since they can regulate neuronal shape, and they are particularly essential for neurite outgrowth and guidance (Penazzi et al., 2016).

At the synaptic scale, structural plasticity refers to the shape and density of dendritic spines (Figure 11), which are small protrusions that serve as postsynaptic biochemical compartments and receive excitatory inputs (Sala & Segal, 2014). They are formed and eliminated in an activity-dependent manner (Nägerl et al., 2004), and become mature with the help of glutamate-induced activation of NMDA and AMPA receptors (Mattison et al., 2014). In addition, GABA controls the competitive selection of dendritic spines via local calcium signaling inhibition (Hayama et al., 2013). These protrusions are found mostly in glutamatergic neurons, such as pyramidal neurons of

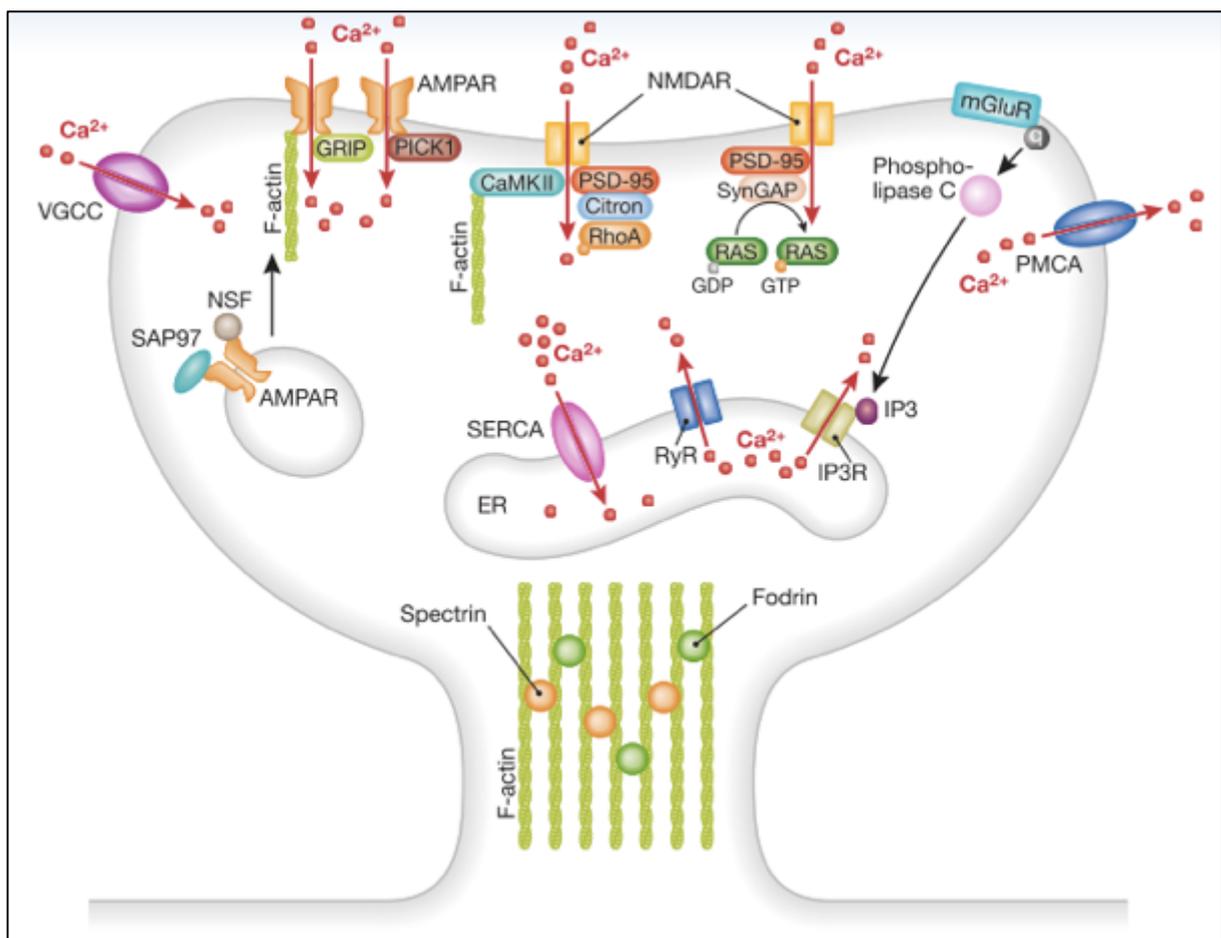


Figure 11. Structural and molecular organization of dendritic spines.
From Rochefort & Konnerth, 2012.

the neocortex and CA3 neurons of the hippocampus (Hering & Sheng, 2001); but also in a small population of GABAergic interneurons, mostly located in the hippocampus (Scheuss & Bonhoeffer, 2014).

4.2.2. Functional plasticity

Functional plasticity can be observed when repetitive electrical activity induces a long-term potentiation (LTP) or depression (LTD) of synaptic efficacy in brain regions (Bliss & Lomo, 1973; Linden & Connor, 1995). These phenomena occur at both excitatory and inhibitory synapses (Castillo, 2012). Excitatory signals are mainly mediated by glutamate, acting on mGluRs or ionotropic receptors (AMPA and NMDA receptors). The mGluR superfamily encompasses 13 members that are GPCRs. They trigger various intracellular pathways (described in the section 4.3) to modulate neuroplasticity (Willard & Koochekpour, 2013). AMPA receptors are responsible for rapid excitatory neurotransmission, since they are the first targets being activated by glutamate. AMPA receptors are heterotetrameric proteins composed of GluA1-4 subunits and found in neurons and glial cells. When a synapse is stimulated, the released glutamate binds to AMPA receptors, and their subsequent activation leads to postsynaptic membrane depolarization which eventually activates NMDA receptors (Henley & Wilkinson, 2013). NMDA receptors are composed of NR1 subunits and three other among NR2 and/or NR3 entities (Stephenson, 2006). Under normal resting membrane potential, the NMDA channel is blocked by Mg^{2+} ions that are then chased by membrane depolarization. Activation of NMDA receptors needs the simultaneous binding of glycine and glutamate at specific sites, along with a prior post-synaptic depolarization induced by AMPA receptor activation, leading to several synaptic changes including redistribution of AMPA receptors (Shi et al., 1999; Stephenson, 2006). NMDA receptor activity can also be stimulated by $\sigma 1$ receptor, an endoplasmic reticulum resident chaperone protein having a wide range of ligands, including pregnenolone (Alonso et al., 2000; Hayashi, 2015). When activated, $\sigma 1$ receptor directly upregulates the expression of NMDA receptor subunits and carries them to the plasma membrane (Pabba et al., 2014). LTP and LTD are mostly sustained by NMDA and AMPA receptors, and can be modulated by GABAergic neurotransmission (Malenka & Bear, 2004; Wu et al., 2014).

4.2.3. Interaction between structural and functional plasticity

Structural and functional plasticity are closely associated: dendritic spine morphology and motility can be regulated by increased calcium levels occurring after glutamate receptor activation, and this relies on actin-dependent mechanisms (Brüning et al., 2004). In addition, LTP induces formation and growth of dendritic spines (Alvarez & Sabatini, 2007; Toni, Buchs, Nikonenko, Bron, & Muller, 1999), whose head size is positively correlated with AMPA receptor expression (Kasai et al., 2010). In contrast, LTD causes spine retraction and elimination in the adult mammalian brain (Alvarez & Sabatini, 2007). Moreover, it could induce morphological changes in presynaptic elements and reduce their contact with spines (Becker et al., 2008).

Adult neurogenesis consists in the formation of new mature neurons, using structural and functional clues. It is reported to occur in the subgranular zone of the hippocampal dentate *gyrus* and in the subventricular zone, involving proliferation of neural stem cells and neural progenitor cells, neuronal migration, differentiation and integration into a network of functional synapses within the brain (Lledo et al., 2006). This process is highly regulated, including by neurotransmitter-related mechanisms. Indeed, GABA released by hippocampal interneurons activates GABA_A receptors located in progenitor plasma membrane, leading to their depolarization and inhibition of proliferation (Song et al., 2012). NMDA receptor activation leads to decreased neurogenesis in hippocampal dentate *gyrus* (Kitayama et al., 2003; Cameron et al., 1995). Adult neurogenesis is suspected to play a role in cognitive function (Zunszain et al., 2011), but also in pathological conditions such as depression (see section 4.5.1). It is noteworthy that adult hippocampal neurogenesis in human is still controversial. Indeed, several *post-mortem* studies could evidence this process (Dennis, Suh, Rodriguez, Kril, & Sutherland, 2016; Eriksson et al., 1998; Spalding et al., 2013), whereas a recent study suggested that it could be decreased throughout life, leading to undetectable levels in adulthood (Sorrells et al., 2018). Nevertheless, it has been confirmed that in human dentate *gyrus*, about 700 new neurons were generated per day whatever the age (Boldrini et al., 2018).

4.3. Intracellular signals of neuroplasticity

4.3.1. Main signal transduction pathways

Neuroplasticity involves both structural and functional events, which are majorly sustained by well-described intracellular pathways, including some that are altered in MDD. These pathways can be triggered by binding of specific ligands to either GPCRs or receptors with a tyrosine-kinase activity (RTKs), both located at the plasma membrane. GPCRs are composed of seven transmembrane domains and change their conformation following the binding of a specific ligand. This provokes the activation of the associated G protein, leading to the dissociation of its α subunit (4 subtypes). RTKs are composed of one transmembrane domain. The binding of a specific ligand causes or stabilizes the receptor homodimerization and transphosphorylation on tyrosine residues, thanks to kinase activity. Once activated, these receptors are able to act on downstream targets, mainly represented by enzymes responsible for production of second messengers, referring to small molecules that spread information from the outside and/or the inside of neurons. Signal amplification can occur following the activation of different pathways by a single membrane receptor, leading to production of several second messengers; and following the activation of several enzymes by only one second messenger molecule (Newton, Bootman, & Scott, 2016). For terminating the signal, these second messengers are rapidly inactivated. There is a plethora of well-described intracellular signaling pathways, and in the present work, the focus will be done on those shown to be implicated in MDD (see section 4.3). Here, their transduction cascade leading to neuroplasticity modulation will be mentioned, in addition to some regulatory processes relevant for the study of depression.

The cyclic adenosine monophosphate (cAMP) pathway starts with the activation of the adenylyl cyclase that metabolizes adenosine triphosphate into cAMP (Figure 12). 5-HT₄, 5-HT₆ and 5-HT₇, D1-like, β -adrenergic, and CRH receptors are GPCRs coupled with α_s that stimulates adenylyl cyclase activity, whereas 5-HT₁ and 5-HT₅, D2-like, and α_2 adrenergic receptors inhibit it (Andersen et al., 1990; Beaulieu & Gainetdinov, 2011; Genuit et al., 2009; Lovejoy, Chang, Lovejoy, & Del Castillo, 2014; McCorvy & Roth, 2015; U'Prichard & Snyder, 1978). Increasing levels of cAMP change the conformation of protein kinase A (PKA) that becomes active (Castro, Guiot, Polito, Paupardin-Tritsch, & Vincent, 2014). PKA can phosphorylate a wide range of proteins, including glycogen phosphorylase, an enzyme strongly involved in the stress response elicited by adrenaline, since it catalyzes the formation of glucose-1-phosphate, essential for example for adenosine triphosphate production (Hardie, 2012). PKA

promotes LTP by increasing the phosphorylation of glutamate receptors (Woolfrey & Dell'Acqua, 2015). This kinase also regulates dendritic spine density, and promotes the growth of the synapse (Cho et al., 2015; Maiti, Manna, Ilavazhagan, Rossignol, & Dunbar, 2015). Overall, due to these action on neuroplasticity, PKA activity can promote long-term memory (Bollen et al., 2014).

The phosphatidylinositol pathway begins with the activation of phosphoinositide 3-kinase (PI3K), which catalyzes the metabolism of phosphatidylinositol 4,5-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate (Figure 12). The major target of phosphatidylinositol 3,4,5-trisphosphate is Akt, also called protein kinase B, located in the cytoplasm. Akt translocates to the plasma membrane for activation, and once activated, it is released within the cell (Manning & Cantley, 2007). This kinase has a large range of substrates, and is mainly involved in cell survival and proliferation by acting on proteins that regulate cell cycle. In addition, Akt can directly activate mTORC1 (mechanistic target of rapamycin complex 1); and indirectly, via the inhibition of glycogen synthase kinase-3 (GSK3), itself inhibiting mTORC1. These actions lead to neuronal growth, hence Akt has a significant impact on neuroplasticity (Manning & Cantley, 2007). In addition, Akt modulates basal synaptic transmission and participates

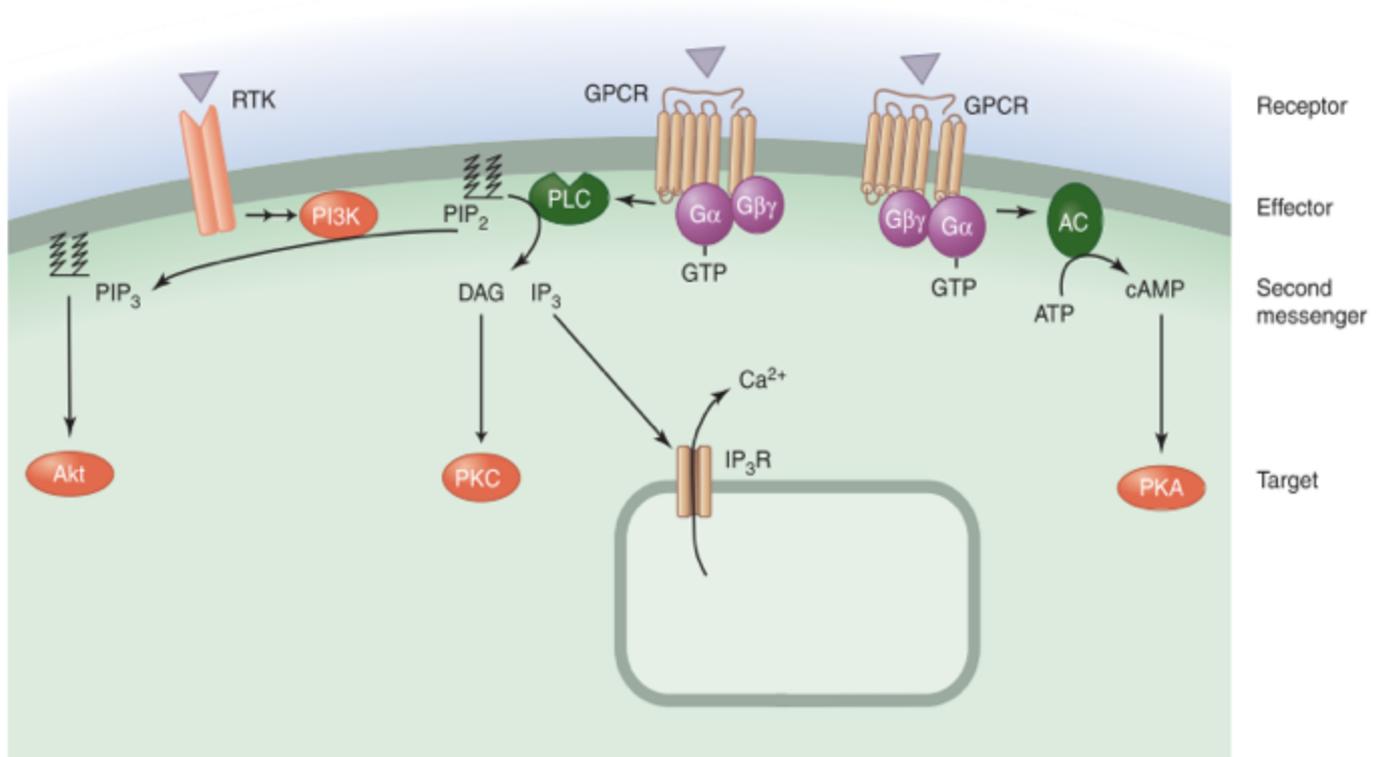


Figure 12. cAMP, phosphatidylinositol and IP₃/DAG pathways. From Newton et al., 2016.

to LTP via increasing the activation of post-synaptic AMPA receptors (Pen, Borovok, Reichenstein, Sheinin, & Michaelievski, 2016).

Signals from GPCRs coupled with α_q subunit, such as 5-HT₂, α_1 adrenergic, and vasopressin (AVP1B) receptors, lead to phospholipase C activation (Figure 12) (Herman et al., 2016; McCorvy & Roth, 2015; U'Prichard & Snyder, 1978). This leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate into two lipids, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), whose combined actions trigger the activation of protein kinase C (PKC). Indeed, following IP₃ binding to its specific receptor located at the reticulum endoplasmic membrane, cytoplasmic levels of calcium are increased. Calcium and DAG can interact with a C2 and C1 domain of PKC, respectively, leading to PKC activation and translocation to the plasma membrane (Newton et al., 2016). PKC has been described as a neuroplasticity enhancer, acting on both short- and long-term plasticity (Figure 13) (Chu, Fioravante, Leitges, & Regehr, 2014; Hasegawa et al., 2015; Wang, Sheng, Ren, Tian, & Lu, 2016). In dendritic spines, it phosphorylates AMPA receptors, and that contributes to the spine growth observed in LTP (Sanderson & Dell'Acqua, 2011).

Calcium comes from the extracellular compartment, or from the endoplasmic reticulum that represents an intracellular store. It has a plethora of effects, including the mediation of synaptic vesicle fusion at the plasma membrane to release neurotransmitters (Südhof, 2013). When calcium increases in the cytoplasm, it binds to calmodulin, hence forming a complex interacting with calcium/calmodulin-dependent protein kinase II (CaMKII), that undergo autophosphorylation (Hudmon & Schulman, 2002). In the hippocampus, CaMKII potentiates AMPA receptor function by phosphorylation (Figure 13), promoting LTP, and probably learning and memory (Derkach, Barria, & Soderling, 1999). In addition, CaMKII promotes structural plasticity, since it participates to the maintenance of dendritic spine enlargement following LTP (Bosch et al., 2014).

Calcium influx can also activate a signaling pathway resulting in the phosphorylation of extracellular signal-regulated kinases (ERKs) that belongs to the mitogen-activated protein kinase family. The ERK cascade is complex and is reported to have a critical role in long-term synaptic plasticity in the amygdala and the hippocampus (English & Sweatt, 1997; Huang, Martin, & Kandel, 2000). Among various targets, ERK is able to phosphorylate cAMP-responsive element (CREB), a

transcription factor known to promote neuroplasticity through the production of plasticity-related proteins. Indeed, CREB activation enhances neuronal excitability in several parts of the brain, and this possibly results in LTP (Dong et al., 2006; Lopez de Armentia et al., 2007; Viosca, De Armentia, Jancic, & Barco, 2009; Zhou et al., 2009), and dendritic spine formation (Sargin et al., 2013). On the other hand, ERK phosphorylates another transcription factor called Elk-1 (Gille, Strahl, & Shaw, 1995). Once activated, Elk-1 can interact with microtubule through a direct binding to tubulin (Demir, Korulu, Yildiz, Karabay, & Kurnaz, 2009), hence participating to neuroplasticity. Elk-1 phosphorylation is also involved in synaptic plasticity, since it participates to LTD mechanisms in the hippocampus (Thiels, Kanterewicz, Norman, Trzaskos, & Klann, 2002).

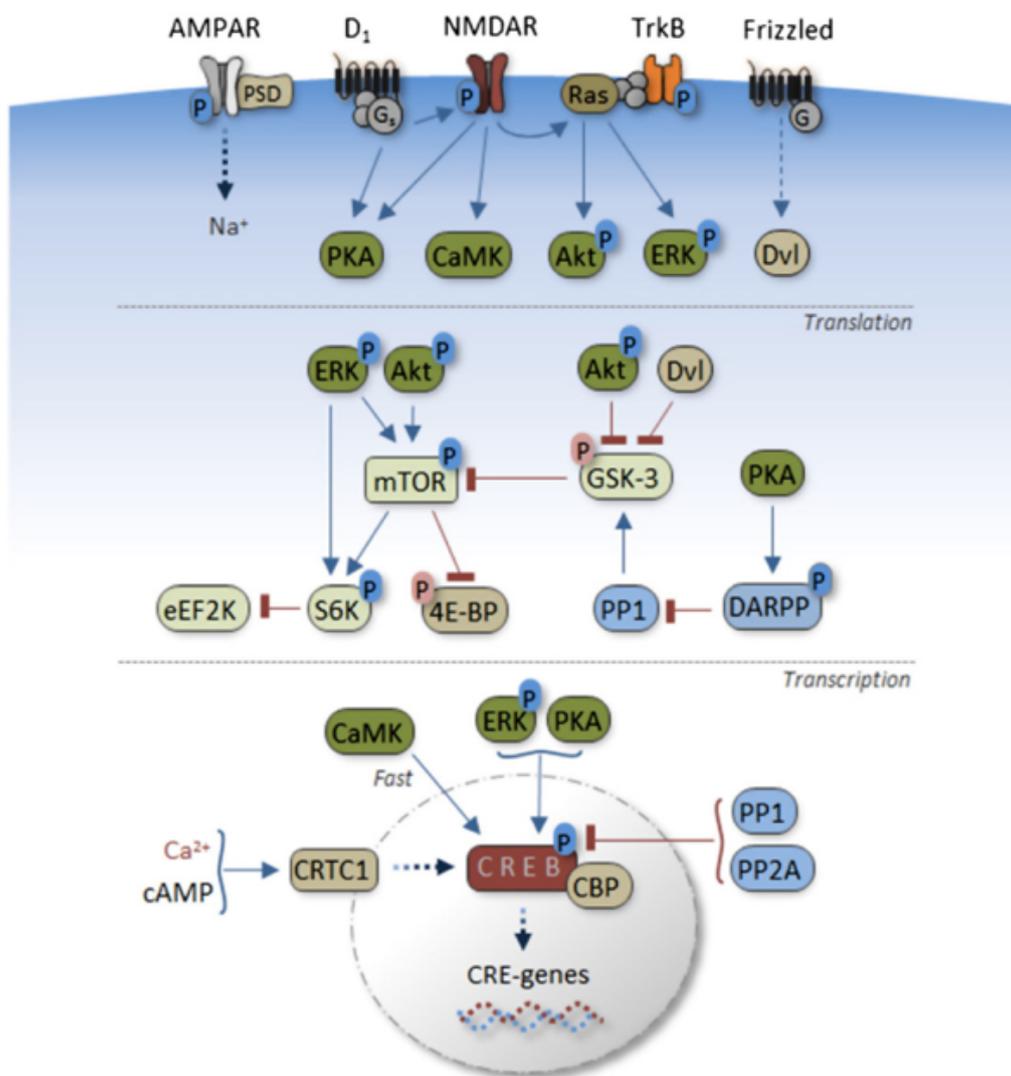


Figure 13. Regulation of translation and transcription during hippocampal synaptic plasticity. From Marsden, 2013.

The cyclic guanosine monophosphate (cGMP) pathway is also involved in neuroplasticity. This cyclic second messenger is produced by the cytosolic soluble guanylyl cyclase that can be activated by nitric oxide, a gas that rapidly diffuses in both aqueous and lipid environments; or by other mechanisms such as natriuretic peptides (Feil & Kleppisch, 2008). This second messenger can then activate protein kinase G, its major target. The cGMP/protein kinase G signaling is able to potentiate neurotransmitter release (Feil & Kleppisch, 2008), regulate AMPA and NMDA receptor function (Incontro et al., 2013), and control gene expression following LTP (Schafe & Ping, 2010). In addition, prevention of cyclic nucleotide degradation may result in improvements in synaptic plasticity, by promoting LTP and inducing the production of synaptic proteins (Sanderson & Sher, 2013).

CREB is a transcription factor modulated by all second messengers previously cited, since it can be phosphorylated by PKA, PKC, Akt, CaMKII, and ERK (Du & Montminy, 1998). CREB activation promotes the transcription of genes regulating several events including plasticity, and is necessary for LTP. However, this transcription factor is deactivated during LTD, possibly by the action of GSK3 pathway (Peineau et al., 2007). As mentioned above, GSK3 is able to inhibit mTORC1, and this effect leads to the transcription of factors that promote cell cycle arrest and prevent neuroplasticity and their inhibition seems to enhance cognitive processes. (O'Leary & Nolan, 2015).

4.3.2. The example of BDNF/TrkB signaling

Neurotrophins, which belong to the neurotrophic factor family, are proteins that play a critical role in survival, growth, and function of both developmental and mature neurons, and that all trigger signaling pathways involved in neuroplasticity. They include BDNF, a secreted protein belonging to neurotrophic family of growth factors (Leibrock et al., 1989). It is widely expressed throughout the brain, its highest concentration being found in the cortex and hippocampus (Ernfors et al., 1990).

The *Bdnf* gene has a complex structure that has been extensively studied in both human and rodents (Boulle et al., 2012). In human, the gene encoding for BDNF is composed of several untranslated exons with independent promoters. These promoters are differentially targeted depending on the nature of the stimuli or signal

events (Dias, Banerjee, Duman, & Vaidya, 2003; Tian, Marini, & Lipsky, 2010; Wong et al., 2010). At the posttranslational level, all BDNF mRNA are translated into pro-BDNF protein, which is cleaved to give mature BDNF, and the balance between these two proteins in the synaptic cleft is necessary for plasticity in the hippocampus (Pang et al., 2004).

Two receptors show an affinity for pro-BDNF and BDNF, the p75 neurotrophin receptor and the tropomyosin-related kinase receptor B (TrkB) (Chao & Hempstead, 1995). p75 receptor has high affinity for pro-BDNF, leading to mostly cell death and decreased synaptic plasticity (Egashira et al., 2010), whereas TrkB has high affinity for BDNF, resulting in the opposite effects than pro-BDNF/p75 interaction. BDNF is involved in a plethora of biological processes, including neuronal survival, growth and differentiation, making it a critical actor in neuroplasticity (Levy et al., 2018). TrkB is a membrane receptor with a tyrosine kinase activity (Chao & Hempstead, 1995). Following BDNF binding, the receptor undergoes transphosphorylation, that triggers various biological actions through activation of mainly three different intracellular pathways, leading to gene transcription regulation and protein translation (Figure 14) (Boulle et al., 2012). The first pathway triggered by BDNF binding to TrkB is the

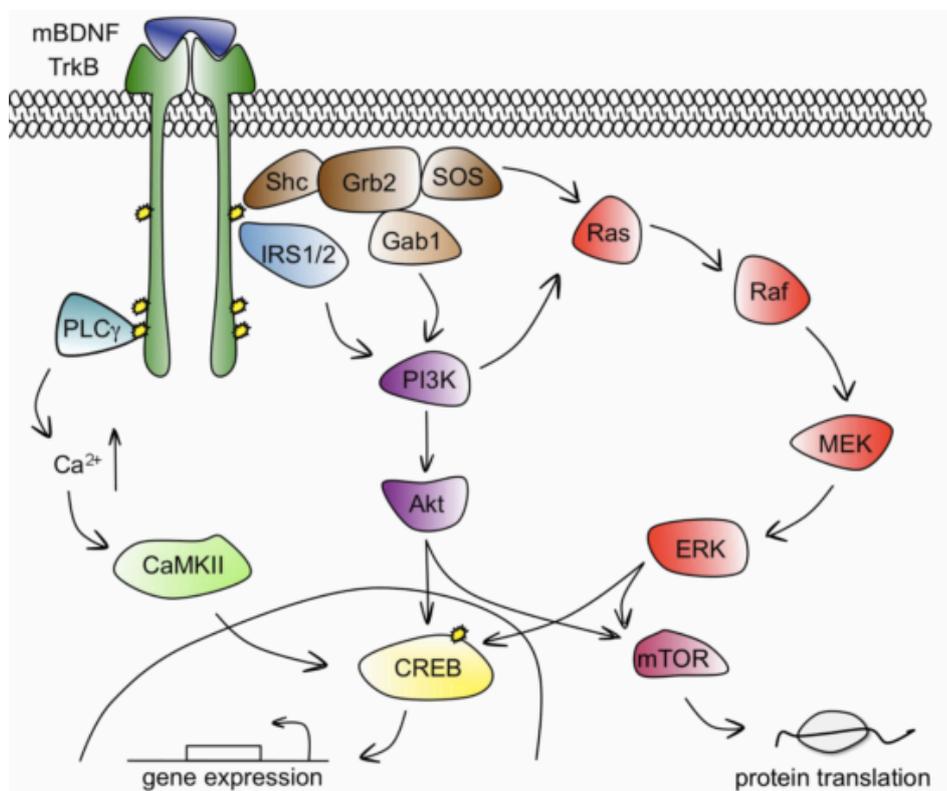


Figure 14. Intracellular pathways triggered by activation of TrkB by BDNF. From Cunha et al., 2010.

phosphatidylinositol pathway (section 4.3). Once activated, PI3K phosphorylates protein 3-phosphoinositide-dependent protein kinase-1, which in turn activates Akt (Figure 14). BDNF/TrkB interaction can also lead to ERK activation, via the action of Ras, a small GTPase protein. Ras can be phosphorylated by PI3K-dependent or -independent mechanisms. Finally, BDNF binding to TrkB acts on IP3/DAG pathway that results in the activation of both CaMKII and PKC. Overall, intracellular effects caused by BDNF/TrkB binding are mostly supported by CREB activity (Figure 14), promoting the production of proteins involved in survival, growth and plasticity (Cunha, Brambilla, & Thomas, 2010).

The role of BDNF/TrkB signaling in neuroplasticity has been largely studied. Since TrkB is located at pre- and postsynaptic sites, BDNF acts on both compartments to exert its proplastic actions. Acting through TrkB/ERK signaling, BDNF phosphorylates synapsin, a presynaptic protein involved in the release of neurotransmitter-containing vesicles, inducing presynaptic release of glutamate and GABA (Jovanovic, Czernik, Fienberg, Greengard, & Sihra, 2000). BDNF is also able to potentiate a wide range of plasma membrane postsynaptic channels involved in synaptic neurotransmission (Figure 15) (Cunha et al., 2010). Overall, BDNF can cause rapid membrane depolarization of neurons, leading to action potential (Kafitz, Rose,

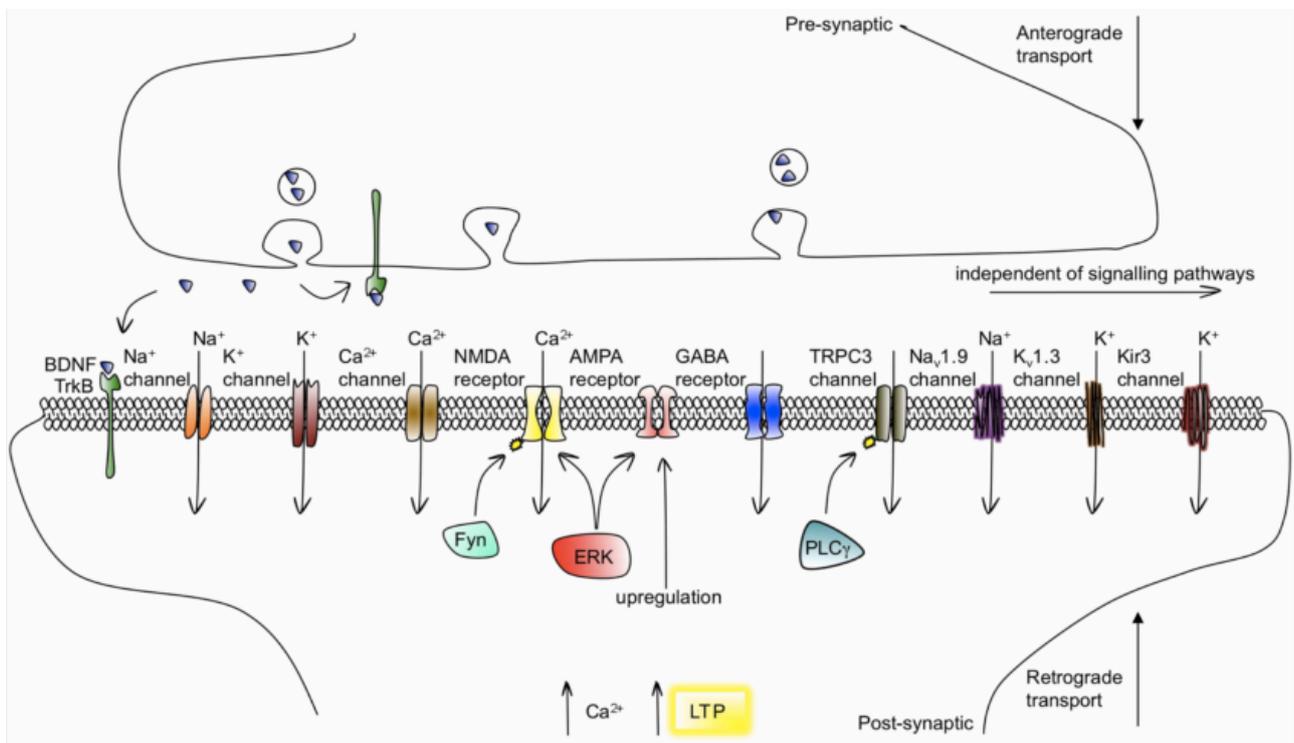


Figure 15. BDNF/TrkB signaling promotes the activity of ligand-gated, voltage-gated and second-messenger-gated ion channels involved in synaptic neurotransmission. From Cunha et al., 2010.

Thoenen, & Konnerth, 1999). Hence, giving its effects on synaptic plasticity, BDNF is able to enhance LTP in CA1 and dentate *gyrus* (Kang & Schuman, 1995; Messaoudi, Ying, Kanhema, Croll, & Bramham, 2002).

4.4. Neuroplasticity integration in the limbic system

Neuroplasticity allows the brain and the organism to adapt to changing environment, via learning and memory processes, both supported by the limbic system. In particular neuroplasticity-related signaling pathways in the hippocampus are critically involved in these cognitive functions (Figure 13).

Learning is a cognitive function that consists in gain or modification of knowledge, understanding, or skills. It primarily follows experience or instructions. Learning can be non-associative, meaning that it is triggered by repeated exposure to a single event; active, when it is conscious and willed; or associative, when learning is an association between two stimuli. The reward system plays a key role in cognitive processes such as associative learning (Berridge, Robinson, & Aldridge, 2009). This system is mainly composed of reciprocal connections within the limbic system between the nucleus accumbens, hippocampus, and prefrontal cortex (Abela, Duan, & Chudasama, 2015). Dopamine neurons of the nucleus accumbens project to the prefrontal cortex. In return, both the prefrontal cortex and hippocampus send glutamatergic fibers to the nucleus accumbens (Burton, Nakamura, & Roesch, 2015). The activity of striatal dopamine neurons is related to a quantitative prediction error during learning: they are activated, silenced, or depressed when the reward is better, same, or worse than predicted, respectively (Schultz, 1998). The reward system is regulated by monoamine, glutamatergic and GABAergic neurotransmission (Kehagia, Murray, & Robbins, 2010; Laviolette & Van der Kooy, 2001; Luo, Zhou, & Liu, 2015; Poschel & Ninteman, 1964; van Huijstee & Mansvelder, 2015). Preclinical data have shown that learning induces synaptic modifications, including either weakening of synaptic connections, or potentiation in synaptic strength in various brain area (Bear, 1996; Cohen, Reuveni, Barkai, & Maroun, 2008; McKernan & Shinnick-Gallagher, 1997; Schroeder & Shinnick-Gallagher, 2005), such as the hippocampus, the amygdala or the prefrontal cortex (Reuveni, Lin, & Barkai, 2018). These synaptic changes could be mediated by glutamate since glutamate receptor activation, possibly via ERK signaling activation (Wang, Fibuch, & Mao, 2007), affects both hippocampal-

dependent associative learning and amygdala-dependent emotional processing during and after a stressful event (Figure 13) (Bermudo-Soriano et al., 2012).

Memory is the brain ability to encode, store and retrieve information. LTP and LTD are thought to be the functional substrates of memory in the hippocampus (Bliss & Collingridge, 1993). Memory formation is associated with time-dependent development of dendritic spines in this structure (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009). Activation of NMDA receptors, requiring combined action of both calcium influx mediated by AMPA receptors and glutamate binding, is essential for memory formation (Lynch et al., 1983; Morris et al., 1986). After memory formation, the rapid trafficking of AMPA receptors to the postsynaptic element is a critical step that eventually leads to the strengthening of synaptic transmission, especially for GluA1-containing AMPA receptors (Henley & Wilkinson, 2013). Within a few hours, they are gradually substituted by GluA2-containing AMPA receptors at the synapse, which form more stable AMPA receptors, resistant to disruption (Figure 13) (Shi et al., 2001). In both the hippocampus and amygdala, this could be caused by the recruitment of PI3K/Akt pathway following BDNF/TrkB interaction, reported to correlate with memory formation (Lin et al., 2001; Mizuno et al., 2003). GABA_A receptors are also implicated in memory processes, since their pharmacological blockade by picrotoxin leads to learning enhancement (McGaugh & Roozendaal, 2009). In addition, a genetic mutation on GABA_A receptors leading to an impossible modulation of GABA currents triggers memory impairments (Tretter et al., 2009). This GABAergic control of memory could be related to CaMKII activity. Indeed, activation of this kinase leads to learning enhancement mediated by GABAergic neurotransmission in the cortex (Ghosh, Reuveni, Lamprecht, & Barkai, 2015).

4.5. Neuroplasticity in MDD: the neurotrophic hypothesis

In MDD, neuroplasticity processes are disrupted in some brain regions, leading to structural and functional changes in neuronal networks. The neurotrophic hypothesis postulates that impairments in neurotrophin signaling could participate to the pathophysiology of MDD (Pittenger & Duman, 2008).

4.5.1. Neurobiological changes in neuronal networks

A marked hypometabolism in the medial prefrontal cortex and a significant hypermetabolism in the right hippocampus and parahippocampal *gyrus* were reported in MDD patients (Aihara et al., 2007). Studies in animal models of depression show that chronic stress reduces hippocampal neurogenesis producing behaviors that parallel those observed in depression (Drew & Hen, 2007; Warner-Schmidt & Duman, 2006). Also, this neuroplasticity process could be a target for antidepressant treatment (Santarelli et al., 2003). However, *post-mortem* histological studies in depressed patients revealed no difference in the expression of Ki-67, a cell proliferation marker, compared to healthy subjects (Reif et al., 2006). Hence, the rate of adult hippocampal neurogenesis in depressed subjects could be involved in depression, although no clear evidence is reported yet.

In humans with MDD, there are similar alterations in the hippocampus and the prefrontal cortex. Indeed, the volume of these two structures is reduced (Rajkowska et al., 1999; Sheline, Wang, Gado, Csernansky, & Vannier, 1996). In the hippocampus, dendritic arborization shrinkage and alteration in dendritic spine integrity are reported (Leuner & Shors, 2013; Dias et al., 2014; Duman & Duman, 2015). In the prefrontal cortex, reduced cortical thickness is associated with a decrease in density and size of neurons and glial cells (Rajkowska et al., 1999). In both structures, glutamatergic and GABAergic neurons are affected, as well as LTP (Khundakar, Morris, Oakley, McMeekin, & Thomas, 2009; Oh, Son, Hwang, & Kim, 2012; Pittenger, 2013; Price & Drevets, 2010). In addition, the expression of synaptic proteins and AMPA and NMDA receptor changes in the hippocampus and prefrontal cortex of depressed patients (Marsden, 2013). These alterations could be explained by several mechanisms. First, a reduction in BDNF and TrkB expression has been reported (see section 4.5.2) (Dwivedi et al., 2003; Pandey et al., 2008), in particular in CA3 and in dentate *gyrus*, where low BDNF and TrkB levels are directly linked to a reduced expression of synaptic proteins, and AMPA and NMDA receptors (Duric et al., 2013; Law & Deakin, 2001). Long-term exposure to glucocorticoids could also explain these anatomical and functional changes. Preclinical studies showed that chronic stress triggering a depressive-like phenotype prolongs the elevation of glucocorticoid amounts that can result in cell death, and suppression of neurogenesis (Mirescu & Gould, 2006; Sapolsky, Krey, & McEwen, 1985). In addition, in these chronically stressed animals,

neuroplasticity impairments have also been evidenced, since an increase in LTD together with a decrease in LTP have been reported in the prefrontal cortex and hippocampus (Kim & Diamond, 2002). Early data showed that both glucocorticoids and stress could impair microtubule dynamics in the hippocampus, including a reduction in MAP2 expression (Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994). Studies further conducted in this field, showed that microtubule alteration in stress or depressive-like animal models could be reversed by chronic antidepressant treatment (Bianchi et al., 2009; Ladurelle et al., 2012), but no direct evidence has been reported in patients so far. Nevertheless, these data were also supported by the fact that *in vivo* deletion of MAP6 lead to impaired cognitive performances together with altered mood (Fournet et al., 2012).

In the amygdala, the pathophysiological processes occurring in MDD may vary depending on the cell type and/or the anatomical part of this structure. Indeed, synaptic proteins and NMDA receptors are upregulated in the lateral amygdala, while they decline in the basal amygdala from *post-mortem* samples of depressed patients (Karolewicz et al., 2009; Varea et al., 2012). Moreover, in unmedicated patients, the amygdala volume was found smaller than in healthy subjects, probably due to a loss of glia and interneuron atrophy (Hamilton, Siemer, & Gotlib, 2008; Savitz et al., 2010). GABA_A receptor function also declines in the amygdala and the hippocampus (Möhler, 2012). Considering the fact that in this structure, BDNF levels are low in depression, this neurotrophic lack could at least in part sustain neuroplasticity impairments observed in the amygdala (see section 4.5.2) (Guilloux et al., 2012).

Post-mortem studies also reported increased hippocampal expression of nitric oxide synthase, the enzyme responsible for nitric oxide production in MDD subjects (Oliveira, Guimarães, & Deakin, 2008), suggesting elevated levels of the second messenger cGMP, which can promote LTP (see section 4.3.1). Hence, elevation of nitric oxide are often accompanied with antidepressant-like properties (Hua et al., 2008). However, it seems that nitric oxide role in the pathophysiology of MDD can also be related to other mechanisms than cGMP increase, since cGMP reduction is thought to be related to therapeutic actions of antidepressants (Marsden, 2013).

PKA and PKC expressions are lower in the prefrontal cortex of depressed patients, and also in the hippocampus of MDD animal models (Dwivedi & Pandey, 2011; Marsden, 2013), while acute treatment with antidepressant tends to reverse

these changes (Réus et al., 2011). In addition, in *post-mortem* studies, ERK- and Akt-mediated signaling declines in the prefrontal cortex and hippocampus of MDD patients and animal models (Karege et al., 2007, 2011; Marsden, 2013). Consistently with reduced Akt activity, GSK3 expression is found increased in hippocampus of patients with MDD (Oh, Park, & Kim, 2010).

It is noteworthy that cytokines, which are elevated in MDD (see section 2.3), are also neuroplasticity modulators. Indeed, interleukin 6 is suspected to be negatively correlated with prefrontal cortex size in MDD (Kakeda et al., 2018). In the brain of depressed patients, cytokines can cause neuronal debranching and LTP inhibition (Hayley, Poulter, Merali, & Anisman, 2005; Pickering & O'Connor, 2007).

4.5.2. The role of BDNF/TrkB signaling

BDNF/TrkB signaling (described in the section 4.3.2) is suspected to have a critical role in MDD pathophysiology processes. In particular, several studies report lower peripheral BDNF levels in MDD. However, because of its high variability between individuals, serum BDNF concentration could not be considered as a reliable biomarker for MDD diagnostic (Levy et al., 2018). In the brain of depressed patients that committed suicide, a reduction in both BDNF and TrkB expression is reported in the hippocampus and prefrontal cortex (Dwivedi et al., 2003; Pandey et al., 2008). In animals, direct infusion of BDNF in these regions leads to antidepressant-like effects, whereas its depletion have the opposite outcome (Levy et al., 2018). In *post-mortem* studies, mRNA expression of Elk-1, a transcription factor phosphorylated by ERK, is increased in the hippocampal dentate *gyrus* of depressed patients (Apazoglou et al., 2018). In addition, Elk-1 inhibition could result in antidepressant-like actions in mice (Apazoglou et al., 2018). On the other hand, the long-lasting behavioral response to stress may involve ERK/Elk-1 direct interaction with GR (Gutiérrez-mecinas et al., 2011; Revest et al., 2005). Furthermore, GR can repress the expression of BDNF *in vitro* possibly through this pathway, further comforting the neurotrophic hypothesis of MDD (Chen, Lombès, & Le Menuet, 2017).

The activity-dependent secretion of BDNF can be altered by a single nucleotide polymorphism occurring in the gene encoding for BDNF, consisting in a valine substitution to methionine at the codon 66 (val66met) (Egan et al., 2003). This

polymorphism is thought to be related to susceptibility to depression (Youssef et al., 2018).

Finally, all these neuroplasticity alterations could explain the cognitive deficits observed in MDD, since, as we have exposed earlier, stress, cytokines and cortisol exposure are able to alter learning and memory in animal models (Stephens & Wand, 2012).

5. Neurosteroids in MDD: focus on pregnenolone

Baulieu and colleagues showed in 1981 (Baulieu, 1981) that some steroids were present in the brain even when the steroidogenic glands adrenals and gonads were ablated (Corpéchet et al., 1983; Corpéchet, Robel, Axelson, Sjøvall, & Baulieu, 1981). It was the first evidence that the brain could synthesize some steroids, then called “neurosteroids”. On the other hand, neuroactive steroids are steroids that can modulate the central nervous system function, independently of their synthesis origin, that can be either central or peripheral (Melcangi, Panzica, & Garcia-Segura, 2011). Hence, due to their central localization, their targets are different from those found in periphery, leading to their original properties.

In periphery, some steroids were considered only as precursor compounds, since they have no biological activity by themselves, except being metabolized into other steroids. However, some of these precursors were found neuroactive (Schumacher et al., 2003; Schumacher et al., 2000). These molecules, including pregnenolone, dehydroepiandrosterone, their sulfate esters and allopregnanolone, have been identified as neuroplasticity enhancers, and their functions were the subject of a review article written during this thesis (Schverer, Lanfumey, Baulieu, Froger, & Villey, 2018). This publication represents a large review of the literature, and will follow the present introduction. Here, we aimed to focus on pregnenolone and its sulfate ester, mainly because the pharmacological compound I have studied in the present work was a pregnenolone derivative (see section 5.3).

5.1. Biosynthesis

5.1.1. Pregnenolone

Steroidogenic enzymes are expressed in both neurons and glial cells in the vertebrate brain (Do Rego et al., 2009). Neurosteroids all derive from a unique precursor, cholesterol. In mitochondria, cholesterol is metabolized into pregnenolone by an enzymatic system composed of cytochrome P450 side-chain cleavage, ferredoxin and ferredoxin reductase. This step is the rate-limiting reaction in the steroidogenesis (Miller, 2013), and pregnenolone can then be converted into all other steroids, including progestogens, estrogens, androgens, mineralocorticoids and glucocorticoids (Figure 16) (Häggström, Stannared, Hoffmeier, Settersr, & Richfield, 2014). Cytochrome P450 side-chain cleavage mRNA is expressed in the limbic system of male and female animals (Compagnone, Bulfone, Rubenstein, & Mellon, 1995; Mellon & Deschepper, 1993; Ukena, Usui, Kohchi, & Tsutsui, 1998). Pregnenolone biosynthesis can be regulated by several mechanisms. Indeed, cytochrome P450 side-

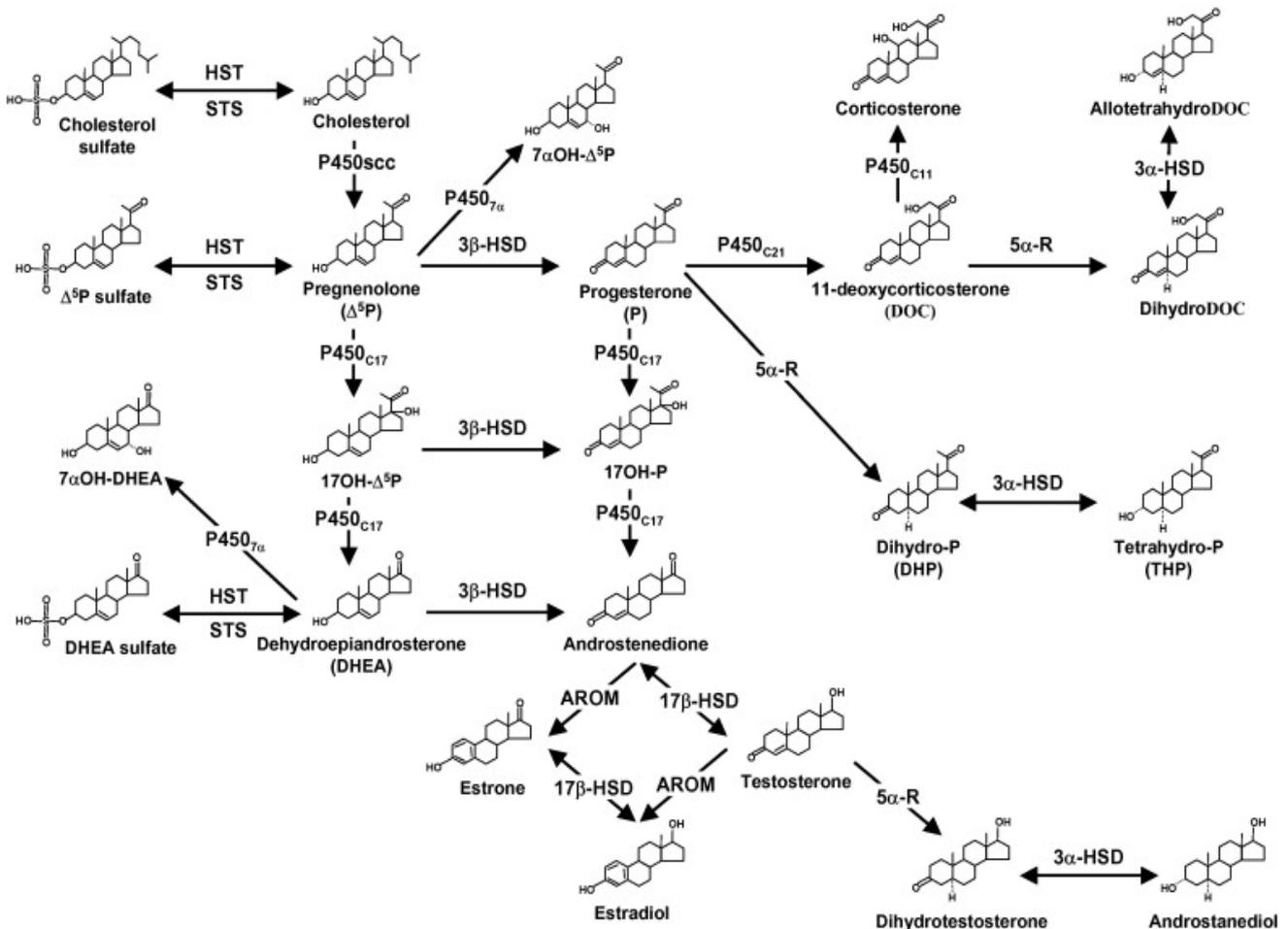


Figure 16. Steroidogenesis in the vertebrate brain. From Do Rego et al., 2009.

chain cleavage brain expression depends on hormonal status, since it can vary in lactating or pregnant rats (Warner, Tollet, Stromstedt, Carlstrom, & Gustafsson, 1989). In addition, the GABA synthesis blocker isoniazid, and the type 1 cannabinoid receptor ligand Δ^9 -tetrahydrocannabinol, are both able to increase pregnenolone levels in the rat brain (Barbaccia et al., 1996; Vallée et al., 2014).

The enzymatic action of the cytosolic hydroxysteroid sulfotransferase on pregnenolone leads to the soluble and circulating compound pregnenolone sulfate (Do Rego et al., 2009). This enzyme belongs to the sulfotransferase 2 family and is mostly expressed in the hippocampus, the thalamus and the *globus pallidus* (Do Rego et al., 2009; Schumacher et al., 2008). The expression of hydroxysteroid sulfotransferase can be increased by gonadotrophin-releasing hormone (Burel et al., 2013) and repressed by neuropeptide Y (Beaujean et al., 2002). Sulfation is a reversible reaction, and desulfation is performed by the steroid sulfatase primarily in the endoplasmic reticulum, but also in the plasma membrane, showing distinct location between these two enzymatic activities (Mueller, Gilligan, Idkowiak, Arlt, & Foster, 2015).

Pregnenolone sulfate is probably the most abundant steroid in the brain, even if its presence has not been observed in all mammals. Although it seems to be detected in the human cerebellum and frontal cortex, it was not found in the rat brain (Liere et al., 2004). In addition, neurosteroid levels can be measured in human in blood or cerebrospinal fluid, or in *post-mortem* studies. The blood-brain barrier is believed to be permeable to steroids, but impermeable to sulfated forms since they are hydrophilic. However, an active transport allowing sulfated steroids to cross the blood-brain barrier has been evidenced (Wang, Wahlström, & Bäckström, 1997; Wood, Gridley, & Keller-Wood, 2003), and pregnenolone sulfate is reported to undergo massive desulfation before being rapidly metabolized (Kaiser et al., 2017). Hence, it seems that pregnenolone sulfate present in the brain is locally produced, suggesting that peripheral levels of steroids may not reflect those in the brain.

5.2. Effects on neuroplasticity

5.2.1. Mechanism of action

Neurosteroids can have genomic and/or non-genomic actions. Genomic actions are quite delayed and consist in neurosteroid binding to its nuclear receptor, leading

to gene transcription regulation. Neurosteroids pregnenolone and pregnenolone sulfate do not have any nuclear receptor identified so far, but act through non-genomic pathways (Weng & Chung, 2016). Pregnenolone rather modulates structural plasticity, since it interacts with microtubule cytoskeleton (Hsu, Liang, Chen, & Chung, 2006) through its binding to microtubule binding proteins MAP2 and CLIP-170, whereas pregnenolone sulfate mostly acts on functional plasticity by directly interacting with amino acid ionotropic receptors (see below).

MAP2 is a somatodendritic protein that is 1827 amino acid long, weighing around 200 kDa (Figure 17). It is produced from a single gene that can lead to three isoforms, MAP2a, MAP2b and MAP2c, all expressed in a time dependent manner. Indeed, MAP2a is expressed from 10 days after birth, MAP2b during development and in adulthood and MAP2c until 10 days after birth in rats (Chung, Kindler, Seidenbecher, & Garner, 1996; Kindler, Schulz, Goerdert, & Garner, 1990). MAP2 contains binding sites for both microtubules and F-actin (Dehmelt et al., 2004). Its amino-terminal end is negatively charged, and encompasses a neurosteroid interaction domain (Fontaine-Lenoir et al., 2006; Laurine et al., 2003; Mizota & Ueda, 2008). The carboxy-terminal end of MAP2 contains a conserved KXGS motif which can be phosphorylated (Illenberger et al., 1996; Sánchez, Díaz-Nido, & Avila, 2000). When phosphorylated, this protein dissociates from microtubules leading to reduced stability and increased dynamic instability, and an increase in dendrite branching, as mentioned in the section 4.2.1. The activity of this protein can be related to cAMP pathway (see section 4.3.1), because of the amino-terminal domain that could interact with protein kinase A (Vallee, DiBartolomeis, & Theurkauf, 1981). The main function of MAP2 is to promote

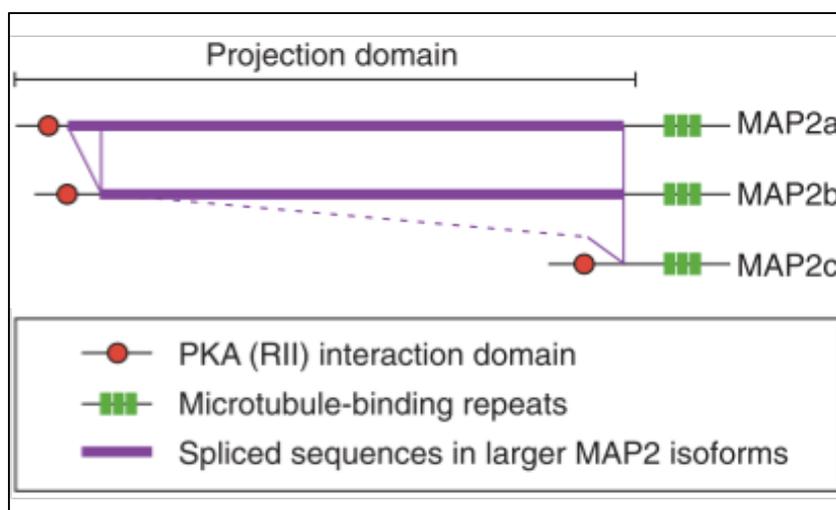


Figure 17. Structure of MAP2. Adapted from Dehmelt et al., 2004.

microtubule stabilization by binding to their lattice (Goodson & Jonasson, 2018). Its deletion results in a reduction of dendritic length associated to reduced microtubule density (Harada, Teng, Takei, Oguchi, & Hirokawa, 2002). Interestingly, pregnenolone is able to potentiate the activity of MAP2, leading to a in tubulin polymerization and microtubule growing (Fontaine-Lenoir et al., 2006).

CLIP-170 (170 is the molecular weight in kDa) is also a microtubule binding protein (Figure 18). It is composed of an amino-terminal microtubule-binding domain, a central coiled-coil domain that allows homodimerization, and a carboxy-terminal metal-binding domain (Pierre, Pepperkok, & Kreis, 1994; Scheel et al., 1999). Located at the growing end of microtubules, CLIP-170 potentiates their elongation (Goodson & Jonasson, 2018). Pregnenolone binds to CLIP-170 with high affinity, changes its conformation rendering it able to interact with microtubules, and eventually potentiates its activity (Weng et al., 2013).

Pregnenolone sulfate is described as an allosteric modulator of NMDA and AMPA receptors (Wu, Gibbs, & Farb, 1991). Its activity on NMDA receptors depends on their subunit composition: pregnenolone sulfate potentiates NR2A- and NR2B-containing NMDA receptors, and inhibits NR2C- and NR2D-containing NMDA receptors (Gibbs, Russek, & Farb, 2006; Horak, Vlcek, Chodounska, & Vyklicky, 2006; Malayev, Gibbs, & Farb, 2002). In the adult brain, and especially in very plastic structures such as the hippocampus or the cortex, NR2A and NR2B are the predominant subunits (Paoletti, Bellone, & Zhou, 2013), hence pregnenolone sulfate majorly potentiates NMDA receptors. In addition to this direct interaction, pregnenolone sulfate also activates σ 1 receptors, that eventually enhances the activity of NMDA receptors (Monnet & Maurice, 2006). Pregnenolone sulfate also inhibits AMPA



Figure 18. Structure of CLIP-170. From Niccoli et al., 2004.

receptor activity through its binding to the amino terminal domain of the GluA2 subunit AMPA receptors (Cameron et al., 2012; Wu et al., 1991).

Regarding the inhibitory neurotransmission, pregnenolone sulfate binds to GABA_A receptors at an unknown site, and inhibits its activity (Majewska, Mienville, & Vicini, 1988). More precisely, it reduces chloride currents and accelerates the receptor desensitization (Le Foll, Louiset, Castel, Vaudry, & Cazin, 1997; Park-Chung, Malayev, Purdy, Gibbs, & Farb, 1999).

5.2.2. Effects on cognition

The effects of pregnenolone and its sulfate ester on cognition have predominantly been evidenced through testing memory in animals. When administered through the intranasal pathway or in the third ventricle, pregnenolone enhances recognition and spatial memory in rats (Abdel-Hafiz et al., 2016). Using the same administration path, it also promotes fear memory in the footshock active avoidance task (Flood, Morley, & Roberts, 1992). These potentiating effects of pregnenolone could be related to its capacity to promote structural plasticity (see section 5.2.1), but no evidence demonstrates that it is actually pregnenolone itself that acts, rather than its metabolites.

Pregnenolone sulfate has divergent effects on memory. When infused into the dorsal region of CA1, this sulfated neurosteroid restores memory of cirrhotic rats (Dastgheib, Dehpour, Heidari, & Moezi, 2015). Similarly, pregnenolone sulfate infusion into cerebral ventricles also increases recognition in mice and rats (Akwa, Ladurelle, Covey, & Baulieu, 2001). This memory enhancement action could be due to pregnenolone sulfate ability to promote functional plasticity through potentiation of glutamatergic and reduction of GABAergic neurotransmissions (see section 5.2.1). However, it is possible that pregnenolone sulfate could interfere with learning processes, since *in vivo* infusion of this neurosteroid in the lateral septum of rats before the training phase of the novel-object recognition task leads to memory impairments (Nanfarò, Cabrera, Bazzocchini, Laconi, & Yunes, 2010).

5.2.3. Effects in MDD

As mentioned in the section 2.2.3, the steroid cortisol (or corticosterone) has a critical role in MDD pathogenesis. Nevertheless, it is not the only steroid that has such

effect. Pregnenolone concentration is reported to be decreased in plasma or cerebrospinal fluid in MDD (George et al., 1994; Rupprecht et al., 1998). However, in *post-mortem* studies, pregnenolone levels would tend to be increased in the cerebral cortex of depressed patients (Marx et al., 2006). Besides, evidences show that neurosteroidogenesis could also participate to MDD pathophysiology, because it is reported to be altered in stress-related behaviors, including depression (Girdler & Klatzkin, 2007; Schüle et al., 2011).

In vivo administration of pregnenolone or pregnenolone sulfate leads to antidepressant-like effects in animals (Melchior & Ritzmann, 1994; Reddy, Kaur, & Kulkarni, 1998). To date, only pregnenolone has been tested in human and resulted in a therapeutic activity during the depressive phase of bipolar subjects (Brown et al., 2014).

5.3. MAP4343: an innovative treatment for MDD

Mapreg was founded by Professor Etienne-Emile Baulieu, Paul Robel (PhD), Arlette Fellous (PhD) and Koichi Murakami (PhD) in 2000, following their discovery of new potential mechanisms of action to treat neurological disorders. MAP4343, also called 3 β -methoxypregnenolone, is the lead compound engineered by Mapreg. It is a pregnenolone derivative that cannot be metabolized, meaning that it is unresponsive to enzymatic systems that transform pregnenolone into metabolites. The advantage of this approach is that the activity of MAP4343 reflects that of pregnenolone itself.

MAP4343 was obtained by changing the hydroxyl into a methoxyl group, rendering the molecule non-metabolizable (Figure 19). MAP4343 has no *in vitro* affinity for any neurotransmitter receptor (see the following table). Nevertheless, MAP4343 is

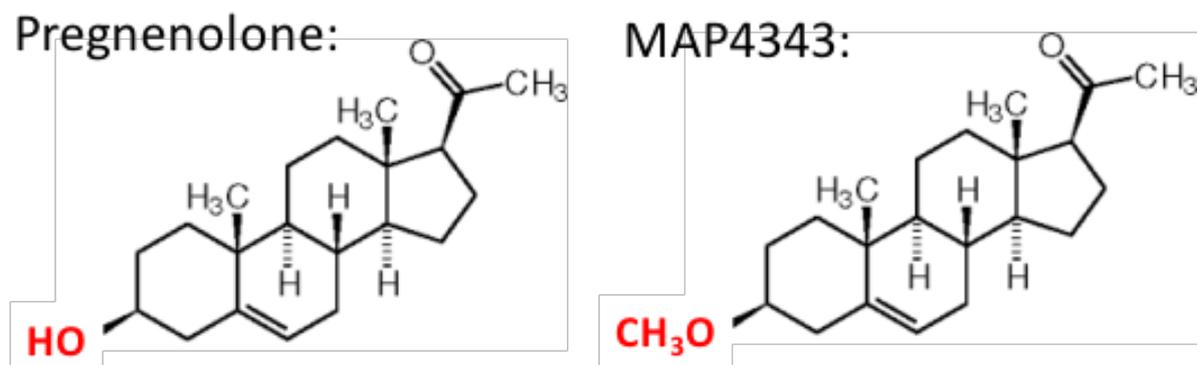


Figure 19. Chemical structure of pregnenolone and MAP4343.

able to interact with the microtubule cytoskeleton, in the same way than pregnenolone (see section 5.2.1). Indeed, it promotes outgrowth of cell processes and increases MAP2 expression *in vitro*, probably through its direct binding to this protein (Fontaine-Lenoir et al., 2006). Interestingly, pregnenolone and MAP4343 have a protective effect since they prevent the depolymerization of microtubules induced by nocodazole (Fontaine-Lenoir et al., 2006). Overall, the action of MAP4343 leads to microtubule polymerization, hence influencing structural plasticity.

5.3.1. MAP4343 effects on neuroplasticity

Similarly than pregnenolone, MAP4343 binds to MAP2 with affinity in the nanomolar range. This results in an increased outgrowth of cell processes, together with an increase in MAP2 expression *in vitro* (Fontaine-Lenoir et al., 2006). By interacting with the microtubule cytoskeleton, MAP4343 can enhance structural plasticity. Indeed, it could promote locomotor recovery after spinal cord injury in rats, possibly by increasing the expression of MAP2 and total dendritic arbor of lumbar spinal cord motoneurons in injured rats (Duchossoy, David, Baulieu, & Robel, 2011).

It has also been reported that MAP4343 could induce changes in tubulin isoforms at the posttranslational level (see section 4.2.1). Indeed, in rats, a single injection of MAP4343 increases the tyrosinated and decreases the acetylated forms of tubulin (Bianchi & Baulieu, 2012), possibly rendering the microtubules more dynamic. However, in the three shrew model, MAP4343 rather caused an increase in acetylated tubulin in hippocampus (Parésys et al., 2016). Hence, MAP4343 effects on tubulin posttranslational modifications is still controversial, even if they are supposed to be mediated by the potentiation effect of MAP4343 on MAP2 activity (Fontaine-Lenoir et al., 2006).

5.3.2. MAP4343 antidepressant-like activity

As mentioned above, pregnenolone has antidepressant activity in patients (see section 5.2.3). However, even if it is well tolerated, this precursor can be metabolized into steroids that will act throughout the body, hence questioning the specificity of pregnenolone effects.

Receptor assay	[H3]Ligand (concentration)	% inhibition of control specific binding
Serotonergic Receptors		
5-HT _{1A}	[³ H]8-OH-DPAT (0.3 nM)	2
5-HT _{1B}	[³ H]CYP (0.1 nM)	4
5-HT _{2A}	[³ H]Ketanserin (0.5 nM)	1
5-HT _{2B}	[³ H]DOI (0.2 nM)	-4
5-HT _{2C}	[³ H]Mesulergine (1 nM)	7
5-HT ₃	[³ H]BRL 43694 (0.5 nM)	10
5-HT _{5A}	[³ H]LSD (1 nM)	-5
5-HT ₆	[³ H]LSD (2 nM)	12
5-HT ₇	[³ H]LSD (4 nM)	-11
Adrenergic Receptors		
α ₁	[³ H]prazosin (0.25 nM)	4
α ₂	[³ H]RX 821002 (0.5 nM)	7
β ₁	[³ H](-)CGP (0.15 nM)	1
β ₂	[³ H](-)CGP (0.20 nM)	-4
Dopaminergic Receptors		
D ₁	[³ H]SCH 23390 (0.3 nM)	2
D ₂	[³ H]spiperone (0.3 nM)	6
D ₃	[³ H]spiperone (0.3 nM)	8
D ₄	[³ H]spiperone (0.3 nM)	6
D ₅	[³ H]SCH 23390 (0.3 nM)	-7
Monoamine transporters		
5-HT Transporter	[³ H]imipramine (0.3 nM)	3
Noradrenaline transporter	[³ H]nisoxetine (1 nM)	3
Dopamine Transporter	[³ H]BTCP (4 nM)	9
Muscarinic Receptors		
M ₁	[³ H]pirenzepine (2 nM)	-3
M ₂	[³ H]AF-DX-384 (2 nM)	20
M ₃	[³ H]4-DAMP (0.2 nM)	3
M ₄	[³ H]4-DAMP (0.2 nM)	20
M ₅	[³ H]4-DAMP (0.2 nM)	12
Histaminic receptors		
H ₁	[³ H]pyrilamine (3 nM)	9
H ₂	[³ H]APT (0.075 nM)	-21
GABA receptors		
GABA _A and GABA _B	[³ H]GABA (10 nM)	2
Glutamate receptors		
PCP (NMDA site)	[³ H]TCP (5 nM)	-10
Cannabinoid receptors		
CB ₁	[³ H]CP 55940 (0.5 nM)	12
Opioid receptors		
δ ₂	[³ H]DADLE (0.5 nM)	-3
κ	[³ H]CP 55940 (0.5 nM)	19
μ	[³ H]CP 55940 (0.5 nM)	0
Neuropeptide receptors		
Bradykinin (B ₂)	[³ H]Bradykinin (0.2 nM)	8
Urolecystokinine (UCLK1)	[¹²⁵ I]UCLK (0.08 nM)	6,3
Endothelin (ETA / ETB)	[¹²⁵ I]endothelin-1 (0.03 nM)	0
Galanin (GLA1 / GAL2)	[¹²⁵ I]galanin (0.1 / 0.05 nM)	2
Neurokinin 1 (NK1)	[³ H]BH-SP (0.15 nM)	-15
Neurokinin 2 (NK2)	[³ H]NKA (0.1 nM)	1
Neurokinin 3 (NK3)	[³ H]SR 142801 (0.4 nM)	4
Neurotensin	[¹²⁵ I]Tyr ³ -neurotensin (0.05)	0
PACAP (PAC1)	[¹²⁵ I]PACAP (0.015 nM)	-15
Steroid nuclear receptors		
GR	[³ H]dexamethasone (1.5 nM)	31
MR	[³ H]Aldosterone (4.5 nM)	25
AR	[³ H]Mibolerone (1.5 nM)	18
ER (α / β)	[³ H]Estradiol (0.5 nM)	-0,5
Others receptors		
Melatonin (MT ₁)	[¹²⁵ I]2-iodomelatonin (0.025)	-10
Sigma (σ)	[³ H]DGT (8 nM)	3

Figure 20. Pharamcological profile of MAP4343 (10 μM). Unpublished data from Mapreg.

Subchronic administration of MAP4343 in the isolation-rearing model of depression rescues anxiety- and depressive-like behavior in rats (Bianchi & Baulieu, 2012). This model of depression induces memory deficits in the novel object recognition task, in which the animal has to discriminate a novel object from a familiar one (Bianchi et al., 2006). Both acute and subchronic MAP4343 administrations persistently rescue these memory deficits in isolated rats (Bianchi & Baulieu, 2012). Even if this has not been demonstrated, these effects on memory further suggest the ability of MAP4343 to promote neuroplasticity.

In the MDD model of chronic psychosocial stress applied to tree shrew, a close-to-primate species, MAP4343 could additionally restore locomotor and exploratory activity, urinary concentrations of cortisol and noradrenaline and body temperature, all being increased in stressed animals compared to controls (Parésys et al., 2016). Following these remarkable findings, MAP4343 has successfully passed the parts A and B of phase 1 clinical trial in healthy volunteers. It is currently tested in patients with treatment-resistant depression. In parallel, investigations are conducted in Mapreg group in order to better understand the mechanism of action of this molecule.

Thesis Project

Regarding the promising therapeutic potential of MAP4343, this thesis project has been designed to further characterize its antidepressant-like activity, and to investigate its mechanism of action.

MDD is associated with cognitive deficits that are poorly reversed by current pharmacotherapy. The first working hypothesis was that the antidepressant-like activity of MAP4343 should reverse cognitive deficits, additionally to reverse the classical depressive-like behavior measured in animal models. In this study, we have chosen the model of chronic stress induced by social defeat, since it induces memory alterations.

Neuroplasticity impairments are reported in MDD; hence the second working hypothesis was that MAP4343 could exert its potential therapeutic effects through neuroplasticity enhancement. This hypothesis was first investigated *in vivo*, in the chronic social defeat stress model, and transgenic mice in which the stress-response is altered, by investigating cell proliferation and neuroplasticity-related gene expression in the hippocampus of mice receiving repeated MAP4343 administration. A cellular model was also used to elucidate the effects of MAP4343 on neuronal plasticity *in vitro*.

Further investigations were conducted *in vitro* to characterize the impact of MAP4343 in the expression of several intracellular kinases, and the involvement of the BDNF/TrkB signaling in its mechanism of action.

Review article

Brain plasticity is one of the most striking ability the brain has to adapt its structure and function in response to external or internal stimuli which might be disrupted in various neurological diseases, including Alzheimer's disease, schizophrenia, stress-related disorders or epilepsy. These pathologies are more and more widespread in our societies, and need for urgent identification of new targets and pharmacological treatments. Actually, while current therapeutics that attempts to reinforce brain plasticity fails most of time to reverse clinical symptoms, neurosteroids, which have been characterized as neuroplasticity modulators, could constitute an innovative strategy to care patients.

Neurosteroids can modulate neuroplasticity through two kinds of activities. They can act through either nuclear receptors and regulate transcription, or via mechanisms that are independent from gene activity. The genomic actions of neurosteroids have been largely reviewed during the past decades. Although increasing evidences showed that some neurosteroids only involve non-genomic targets to impact neuroplasticity, their potential involvement in neurological disease needed to be compiled and discussed. Indeed, the following review was the first to address this issue.

We focused on pregnenolone, dehydroepiandrosterone, their sulfate esters, and allopregnanolone, since they seem to act on neuroplasticity exclusively through non-genomic actions, and because they are suspected to influence some neurological disorders, affecting both their pathophysiology and treatment.

This article, entitled "Neurosteroids: non-genomic pathways in neuroplasticity and involvement in neurological diseases" was peer-reviewed and published in June, 2018, in the journal *Pharmacology & Therapeutics*.



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Neurosteroids: non-genomic pathways in neuroplasticity and involvement in neurological diseases

Marina Schverer^a, Laurence Lanfumey^{a,*}, Etienne-Emile Baulieu^{b,c}, Nicolas Froger^b, Isabelle Villey^b

^a Inserm U894, Centre de Psychiatrie et Neurosciences, Université Paris Descartes, 75014 Paris, France

^b MAPREG SAS, Le Kremlin-Bicêtre, France

^c Inserm UMR 1195, Université Paris-Saclay, Le Kremlin Bicêtre, France

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ABSTRACT

Neurosteroids are neuroactive brain-born steroids. They can act through non-genomic and/or through genomic pathways. Genomic pathways are largely described for steroid hormones: the binding to nuclear receptors leads to transcription regulation. Pregnenolone, Dehydroepiandrosterone, their respective sulfate esters and Allopregnanolone have no corresponding nuclear receptor identified so far whereas some of their non-genomic targets have been identified. Neuroplasticity is the capacity that neuronal networks have to change their structure and function in response to biological and/or environmental signals; it is regulated by several mechanisms, including those that involve neurosteroids.

In this review, after a description of their biosynthesis, the effects of Pregnenolone, Dehydroepiandrosterone, their respective sulfate esters and Allopregnanolone on their targets will be exposed. We then shall highlight that neurosteroids, by acting on these targets, can regulate neurogenesis, structural and functional plasticity. Finally, we will discuss the therapeutic potential of neurosteroids in the pathophysiology of neurological diseases in which alterations of neuroplasticity are associated with changes in neurosteroid levels.

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1. Introduction

Neurosteroids refer to steroids that are produced *de novo* in the central nervous system and display neuronal activity (Baulieu, 1981; Baulieu & Robel, 1990). Steroidogenic glands were thought to be the only sources of hormonal steroids, until Baulieu and colleagues showed the presence in brain of steroids Pregnenolone (PREG) and Dehydroepiandrosterone (DHEA) in higher concentrations than in plasma, even when both adrenals and gonads were ablated (Corpéchet et al., 1983; Corpéchet, Robel, Axelson, Sjoval, & Baulieu, 1981). These neurosteroids are the same molecules as steroids found in the rest of the body, but due to their localization, they display activities in the brain that they do not have in periphery. Peripheral steroids mostly act via genomic pathways, binding to nuclear receptors and regulating

Abbreviations: σ 1 receptor, sigma-1 receptor; AD, Alzheimer's disease; ALLO, Allopregnanolone; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CLIP-170, cytoplasmic linker protein 1; CA1, Cornu Ammonis 1; CA3, Cornu Ammonis 3; CSF, Cerebrospinal fluid; DHEA, Dehydroepiandrosterone; DHEA-S, Dehydroepiandrosterone sulfate; HST, Hydroxysteroid sulfotransferase; GABA, γ -aminobutyric acid; GluA, AMPA receptor subunit; LTD, Long-Term Depression; LTP, Long-Term Potentiation; MAP, Microtubule-Associated Protein; NMDA, N-methyl-D-aspartate; NR, NMDA receptor subunit; P450_{c17}, steroid 17 α -hydroxylase/17,20 lyase; P450_{scc}, mitochondrial enzyme cholesterol side-chain cleavage; PFC, Prefrontal cortex; PREG, Pregnenolone; PREG-S, Pregnenolone sulfate.

* Corresponding author at: Centre de Psychiatrie et Neurosciences, Inserm U894, 102-108 Rue de la Santé, 75014 Paris, France.

E-mail address: laurence.lanfumey@inserm.fr (L. Lanfumey).

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gene transcription. PREG, DHEA, their sulfate esters (PREG-S and DHEA-S) and Allopregnanolone ($3\alpha,5\alpha$ -tetrahydroprogesterone; ALLO) are essentially known as precursor and intermediate compounds in the hormonal steroid biosynthetic pathways, while they are also neuroactive neurosteroids (Schumacher et al., 2000; Schumacher et al., 2003). However, they have no nuclear receptor identified and act on non-genomic targets (Weng & Chung, 2016). At the plasma membrane level, there are non-genomic actions of neurosteroids, regulating inhibitory and excitatory neurotransmission through amino acid ionotropic receptors, similarly to classical neurotransmitters (Rudolph et al., 2016). In the cytoplasm, non-genomic pathways triggered by neurosteroids mainly involve Sigma 1 receptor ($\sigma 1$ receptor; Monnet & Maurice, 2006) and some microtubule-associated proteins (MAPs) (Laurine et al., 2003; Mizota & Ueda, 2008; Murakami, Fellous, Baulieu, & Robel, 2000; Weng et al., 2013).

Neuroplasticity is the property of neurons to adapt in response to multiple internal and external stimuli. In adulthood, it involves newly formed neurons located in few parts of the brain. This process called neurogenesis occurs in the subgranular zone of the hippocampal dentate gyrus and in the subventricular zone (Lledo, Alonso, & Grubb, 2006). Neuroplasticity also implicates the remodeling of mature neurons at different levels, from the cell shape (structural plasticity) to the synapse function (functional plasticity). Structural plasticity refers to the complexity of neuronal arbor, and to the variation in morphology or density of dendritic spines, which are major post-synaptic sites of excitatory synapses (Sala & Segal, 2014). Functional plasticity is related to synaptic changes in response to electrical activity. In summary, neuroplasticity is highly regulated by numerous mechanisms, including several that involve neurosteroids.

Here, we will focus on neurosteroids that act exclusively via non-genomic pathways: PREG, PREG-S, DHEA, DHEA-S and ALLO, and that are able to trigger three levels of neuroplasticity (i.e. structural plasticity, functional plasticity and neurogenesis). After a description of their biosynthesis and mechanisms of action, the role of these neurosteroids in neuroplasticity will be discussed. Finally, we will highlight their

involvement in the pathophysiology of some neurological pathologies in which alterations of neuroplasticity are associated with changes in neurosteroid levels, opening original therapeutic potentialities.

2. Neurosteroids: biosynthesis and non-genomic pathways

2.1. Biosynthesis of PREG, DHEA, PREG-S, DHEA-S and ALLO

Steroidogenesis is under the dependency of specific enzymes largely present in adrenal and genital glands (Do Rego et al., 2009). Genes encoding these enzymes are also expressed in both neurons and glial cells of human and other mammalian brains (Do Rego et al., 2009; Melcangi, Celotti, Castano, & Martini, 1993; Mellon & Deschepper, 1993; Pelletier, Luu-The, & Labrie, 1994). Steroid biosynthetic pathways in brain and in peripheral glands are very similar. Steps leading to formation of PREG, DHEA, ALLO, PREG-S and DHEA-S are described below, and illustrated in Fig. 1.

Cholesterol is the unique precursor of all neurosteroids. It is converted into PREG in mitochondria thanks to an enzymatic system composed of the mitochondrial enzyme cholesterol side-chain cleavage (P450_{sc}, Fig. 1), ferredoxin and ferredoxin reductase. P450_{sc} mRNA is abundantly expressed in the cerebral cortex, and in lower amounts in the amygdala, hippocampus, midbrain and cerebellum of male and female individuals (Compagnone, Bulfone, Rubenstein, & Mellon, 1995; Mellon & Deschepper, 1993; Ukena, Usui, Kohchi, & Tsutsui, 1998). This first step is a rate-limiting reaction which determines the net steroidogenic capacity (Miller, 2013). Some regulatory mechanisms of PREG biosynthesis have been identified. For instance, P450_{sc} levels in the brain may be modified with hormonal changes occurring in pregnant or lactating females (Warner, Tollet, Stromstedt, Carlstrom, & Gustafsson, 1989). Moreover, some compounds can modulate PREG synthesis: isoniazid, a GABA (γ -aminobutyric Acid) synthesis blocker, and $\Delta 9$ -tetrahydrocannabinol, a type 1 cannabinoid receptor ligand, increase PREG content in the rat brain (Barbaccia et al., 1996; Vallée et al., 2014).

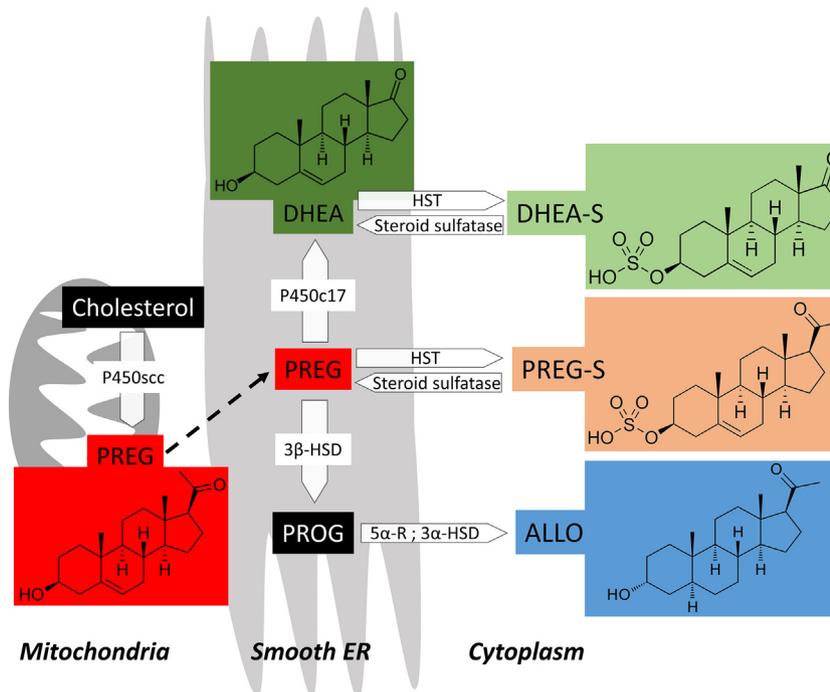


Fig. 1. Biosynthesis of neurosteroids PREG, DHEA, PREG-S, DHEA-S and ALLO from a common precursor: cholesterol. Cholesterol is transformed into PREG inside the mitochondria by the cytochrome P450 side-chain cleavage (P450_{sc}). In the smooth endoplasmic reticulum (ER), PREG is metabolized into DHEA or Progesterone (PROG) thanks to cytochrome P450 17 α -hydroxylase/C17,20-lyase (P450c17) or 3 β -hydroxysteroid dehydrogenase (3 β -HSD) activities respectively. In the cytoplasm, PREG and DHEA are sulfated by a hydroxysteroid sulfotransferase (HST) activity, desulfation is performed by a steroid sulfatase; and PROG is metabolized into ALLO by the successive actions of 5 α -reductase (5 α -R) and 3 α -hydroxysteroid dehydrogenase (3 α -HSD).

In the endoplasmic reticulum, PREG can be transformed either into DHEA or into Progesterone (Fig. 1). The production of DHEA results from two successive modifications catalyzed by cytochrome P450_{C17} (steroid 17 alpha-hydroxylase/17,20 lyase) enzymatic activities: a 17- α -hydroxylation followed by the scission of the C17-20 bond (Akwa, Morfin, Robel, & Baulieu, 1992; Compagnone & Mellon, 1998). This enzyme is found in the striatum, the preoptic area, the optic tectum, the thalamus and the hippocampal dentate gyrus (Do Rego et al., 2009), suggesting high content of DHEA in these structures. However, despite the large amount of DHEA measured, the presence of P450_{C17} in the adult brain is still controversial (Le Goascogne et al., 1991; Liu, Pociavsek, & Papadopoulos, 2009) and cells which do not express P450_{C17} gene still contain DHEA, possibly suggesting another unknown DHEA biosynthesis pathway, that differ from those in periphery (Cascio, Prasad, Lin, Lieberman, & Papadopoulos, 1998). Another hypothesis that could explain this can be proposed: it is possible that DHEA present in the brain could come from the periphery or from the desulfation of DHEA-S (see below). Formation of Progesterone results from the action on PREG of 3 β -hydroxysteroid dehydrogenase (Fig. 1), a membrane bound mitochondrial enzyme widely expressed in the brain (Do Rego et al., 2009). The successive actions of 5 α -reductase and 3 α -hydroxysteroid dehydrogenase II/III on Progesterone leads to the production of ALLO (Fig. 1). 5 α -reductase is abundantly present in the cerebral cortex, the hippocampus, the hypothalamus, the amygdala, the olfactory bulb and in the Purkinje cell layer of the cerebellum. 3 α -hydroxysteroid dehydrogenase II/III is found in the same structures, but in lower amounts than 5 α -reductase (Do Rego et al., 2009; Furukawa, Miyatake, Ohnishi, & Ichikawa, 1998), suggesting that ALLO may be less expressed than its precursors.

Several compounds, that partly could be associated with different physiological conditions, can affect the neurosteroid biosynthesis downstream PREG biosynthesis. In frog diencephalic neurons, neurohypophysial neurohormones (i.e. vasotocin and mesotocin) as well as endozepines, that are endogenous compounds with benzodiazepine-like effects, can simulate the production of DHEA and Progesterone (Do Rego et al., 1998; Do Rego et al., 2001; Do Rego et al., 2006), that may result in a rise in ALLO production. In contrast, in frog hypothalamic neurons, GABA, by interacting on GABA_A receptors, can decrease the levels of DHEA and Progesterone (Do Rego et al., 2000), and possibly ALLO, likely through downregulation of P450_{C17} and 3 β -hydroxysteroid dehydrogenase enzymatic activity. The production of these neurosteroids can also be stimulated by drugs, including etiofexine, a non-benzodiazepine anxiolytic drug (Do Rego, Vaudry, & Vaudry, 2015).

PREG and DHEA can be converted into their sulfate ester forms PREG-S and DHEA-S that are the soluble circulating forms, and the possibly most abundant neurosteroids in the brain (Corpéchet et al., 1981; Corpéchet et al., 1983; Rajkowski, Robel, & Baulieu, 1997). Conjugation is catalyzed by cytosolic hydroxysteroid sulfotransferases (HST) belonging to the sulfotransferase 2 family (Fig. 1; Mellon & Vaudry, 2001; Strott, 2002). HST activity is present in various parts of the brain, including the hippocampus, the globus pallidus and the thalamus (Do Rego et al., 2009; Schumacher et al., 2008). The production of sulfated neurosteroids is repressed by the Neuropeptide Y (Beaujean et al., 2002), whereas it is enhanced by the gonadotrophin-releasing hormone (Burel et al., 2013). The reverse reaction is performed by the steroid sulfatase (Fig. 1), located primarily in the endoplasmic reticulum, but also found in the plasma membrane (Mueller, Gilligan, Idkowiak, Arlt, & Foster, 2015).

When it comes to studying the possible implication of neurosteroids in neurophysiology and in neuropathology, one needs to question the local brain levels of these compounds. Whereas the peripheral compartment is readily accessible and neurosteroids can be measured in blood and cerebrospinal fluid (CSF), actual measures of the local cerebral steroids are far more complicated to ensure, especially in humans, where only post-mortem studies could allow assessing this parameter

(Schumacher et al., 2008). Hence, in physiological conditions, no clear data is available regarding PREG, DHEA, their sulfate esters and ALLO levels in the brain of humans. Controversial data are available about the detection of PREG-S in brain. Studies using direct method for detection of PREG-S and DHEA-S concluded to undetectable amounts in various structures from rat brain, while it was detectable in cerebellum and frontal cortex from humans (Liere et al., 2004). There are rare systematic studies performed in mice that attempt to address this issue, also showing that it depends on the strain (Tagawa, Sugimoto, Yamada, & Kobayashi, 2006). The few data available regarding neurosteroid levels in pathological conditions are reported in the Section 3.2. By contrast, several studies report the blood levels of PREG, PREG-S, DHEA, DHEA-S and ALLO, both in physiological and pathological conditions. To what extent do peripheral measures account for brain levels is therefore a real issue, since peripheral and central compartments are separated by the blood-brain barrier. This barrier is considered to be permeable to PREG, DHEA and ALLO, and impermeable to conjugated sulfates due to their hydrophilicity. Several studies mention the existence of an active transport process allowing the passage of sulfated neurosteroids from the blood to the brain tissue (Wang, Wahlström, & Bäckström, 1997; Wood, Gridley, & Keller-Wood, 2003), accounting for the relevance of using such compounds via the peripheral route to achieve a central action. In addition, PREG-S enters more rapidly in the brain than DHEA-S, and both undergo massive desulfation. Then, PREG itself is not metabolized, contrarily to DHEA. This suggests that PREG is a stable form while DHEA is not (Qaiser et al., 2017). The transport of circulating sulfated steroids inside the cells is done by a variety of organic anion transporter polypeptides with different affinities for the different steroids. Conversely, the efflux of sulfated steroids is performed by ATP-binding cassette transporters (Mueller et al., 2015). Hence, it is possible that similar machinery allows the passage of sulfated steroids across the blood-brain barrier, but this remains an open question.

Overall, little is known about the cerebral distribution of neurosteroids, both in physiological and in pathological states. Relying on peripheral measures when studying central properties and activities of neurosteroids can thus be strongly misleading. To achieve central actions, modifying neurosteroids levels in situ and administering neurosteroids in periphery are both relevant paths.

2.2. Amino acid ionotropic receptors as direct targets of neurosteroids

Whether neurosteroids could interfere with neurotransmission is a hypothesis that has long been proposed. The two main classes of neurotransmission, glutamatergic and GABAergic systems, are indeed modulated by neurosteroids, as described below and illustrated in Table 1.

2.2.1. Modulation of ionotropic glutamate receptors

NMDA (N-methyl-D-aspartate) and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors are subtypes of ionotropic glutamate receptors that mediate a part of the excitatory transmission in the brain. NMDA receptors are heterotetrameric proteins always composed of the NR1 subunit, yielding eight isoforms from splicing and containing the glycine binding site, plus NR2 subunits, that contain the glutamate binding site, and/or NR3 subunits (Stephenson, 2006). Under normal resting membrane potential, the NMDA channel is blocked by Mg²⁺ ions which are then chased by membrane depolarization. Activation of NMDA receptors requires the simultaneous binding of glutamate and glycine at their specific sites, together with a prior post-synaptic depolarization induced by AMPA receptor activation, leading to several synaptic changes including redistribution of AMPA receptors (Shi et al., 1999; Stephenson, 2006) (Fig. 2A). AMPA receptors are responsible for rapid excitatory neurotransmission. They are heterotetrameric proteins composed of GluA1-4 subunits (Fig. 2B) and found in neurons and glial cells. When the synapse is stimulated, the released glutamate binds to AMPA receptors, and their subsequent

Table 1
Neurosteroid actions on their receptors. The values represent the concentrations that potentiates or inhibits NMDA, AMPA, GABA_A, σ 1 receptors or MAPs. “~” means “in the range of”. *Depends on NMDA receptor subunit composition.

Neurosteroids	NMDA receptors	AMPA receptors	GABA _A receptors	σ 1 receptors	MAPs
PREG	Unknown	Unknown	Inactive Gibbs et al., 2006	Inactive Monnet & Maurice, 2006	Binding to MAP2 and CLIP-170 (30–60 nM) Murakami et al., 2000 Weng et al., 2013 Unknown
PREG-S	Stimulation or inhibition* (300 μ M) Malayev et al., 2002 Horak et al., 2006	Inhibition (100 μ M) Wu et al., 1991 Cameron et al., 2012	Inhibition (60 μ M) Majewska et al., 1988	Stimulation (~ μ M) Monnet & Maurice, 2006	Unknown
DHEA	Unknown	Unknown	Inhibition (~ μ M) Park-Chung et al., 1999 Inhibition (~ μ M) Majewska et al., 1990	Stimulation Bergeron et al., 1996 Stimulation (~ μ M) Monnet & Maurice, 2006	Binding to MAP2 (~nM) Laurine et al., 2003 Inactive on MAP2 Murakami et al., 2000
DHEA-S	Unknown	Unknown	Stimulation (~nM to μ M) Carver & Reddy, 2013	Unknown	Unknown
ALLO	Inactive Mellon, 2007	Inactive Mellon, 2007	Inactive	Unknown	Unknown

activation leads to postsynaptic membrane depolarization which eventually activates NMDA receptors (Henley & Wilkinson, 2013).

PREG actions on ionotropic glutamate receptors remain unknown. PREG-S is known as an allosteric modulator of NMDA and AMPA receptors (Wu, Gibbs, & Farb, 1991). Its effects on NMDA receptors depend on their subunit composition: it potentiates NR2A- and NR2B-containing NMDA receptors by increasing channel opening probability, and inhibits NR2C- and NR2D-containing NMDA receptors (Fig. 2A) (Gibbs, Russek, & Farb, 2006; Horak, Vlcek, Chodounska, & Vyklicky, 2006; Malayev, Gibbs, & Farb, 2002). Given the affinity for NMDA receptor subunits (Table 1), it seems that PREG-S preferentially binds to NR2D subunit ($K_d = 290 \mu\text{M}$) rather than NR2B subunit ($K_d = 1.47 \text{ mM}$) (Cameron et al., 2012). However, in the adult brain, NR2A and NR2B are the predominant subunits, especially in very plastic brain structures such as the hippocampus and cortex (Paoletti, Bellone, & Zhou, 2013), therefore the positive modulation of NMDA-evoked excitatory currents could be the main action of PREG-S on NMDA receptors. By increasing

the delayed excitatory neurotransmission, PREG-S enhances several processes including NMDA-gated currents in spinal cord neurons (Wu et al., 1991), convulsant potency of NMDA in mice (Maione, Berrino, Vitagliano, Leyva, & Rossi, 1992), and NMDA-induced phasic firing of vasopressin neurons in the rat supraoptic nucleus (Wakerley & Richardson, 1997). These effects cannot be only attributed to a direct modulation of NMDA receptors since PREG-S can also indirectly potentiate NMDA receptors, through its binding to σ 1 receptors (Monnet & Maurice, 2006; see Section 2.3.1.).

PREG-S, at the same concentration in vitro (100 μM), inhibits AMPA receptors by binding to the amino-terminal domain of the GluA2 subunit (Fig. 2B and Table 1) (Cameron et al., 2012; Wu et al., 1991). GluA2 subunit is believed to be required for long-term depression processes (LTD; see below), so it is possible that PREG-S down-regulates this phenomenon (Huganir & Nicoll, 2013). When an excitatory synapse is activated, GluA1-containing AMPA receptors are the first activated, followed by GluA2-containing AMPA receptors. It hence can be

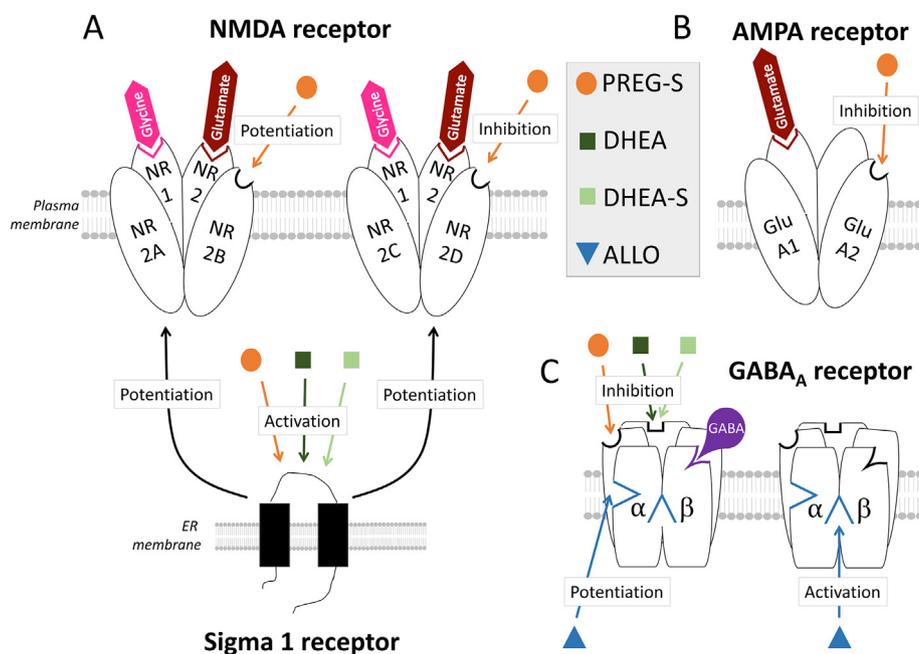


Fig. 2. Neurosteroid actions on membrane receptors. A. NMDA receptors are heterotetrameric and composed of NR1 and NR2 subunits. NR1 contains the glycine binding site and NR2 contains the glutamate and probably the PREG-S binding sites. PREG-S (orange dot) potentiates NR2A- and NR2B- and inhibits NR2C- and NR2D-containing NMDA receptors activated by glycine and glutamate binding. PREG-S, DHEA (dark green dot) and DHEA-S (light green dot) bind to σ 1 receptors (located in the membrane of the endoplasmic reticulum) at unknown binding sites, leading to its activation. In turn, σ 1 receptors upregulate the NMDA receptor function. B. GluA1 and GluA2 are subunits of the AMPA receptor which is heterotetrameric. PREG-S inhibits AMPA receptors through a binding to the N-terminus of GluA2. C. α and β are subunits of the GABA_A receptor which is heteropentameric. In the presence of GABA, PREG-S, DHEA and DHEA-S inhibit GABA_A receptors at unknown binding sites. ALLO (blue triangle) binds to a potentiation site located in the transmembrane part of the α subunit and potentiates GABA_A receptor function. In the absence of GABA, ALLO binds to direct activation site located between the transmembrane parts of α and β subunits and directly activates GABA_A receptor currents.

hypothesized that AMPA receptor inhibition by PREG-S is a regulatory process to avoid too much excitation leading to glutamate toxicity (Huganir & Nicoll, 2013). PREG-S induces a presynaptic Ca^{2+} influx by increasing presynaptic glutamate release and promoting the insertion of AMPA receptors to the postsynaptic membrane, and this could result in activation of silent synapses (Valenzuela, Partridge, Mamei, & Meyer, 2008). The insertion of AMPA receptors is mediated by NR2B-containing NMDA receptors, therefore potentiated by PREG-S (Ferreira et al., 2015). To sum up, regarding its role on ionotropic glutamate receptors, it seems that PREG-S does not activate the rapid glutamatergic response, but allows the maintaining of this response via NMDA receptor potentiation. Since AMPA receptors are activated prior to NMDA receptors, it is possible that PREG-S differential effects on them lead to a sustained glutamatergic response rather than trigger it.

DHEA is able to protect the retina against AMPA-induced toxicity, but without acting directly on the AMPA receptors, suggesting a distinct mode of action from PREG-S (Kokona, Charalampopoulos, Pediaditakis, Gravanis, & Thermos, 2012) (Fig. 2B). Intravenous administration of DHEA induces an increase in the response of dorsal hippocampal CA3 pyramidal neurons to NMDA (Bergeron, de Montigny, & Debonnel, 1996). DHEA-S is known to potentiate NMDA receptor activity in the rat hippocampus and spinal cord where it enhances NMDA-induced pain (Fig. 2A) (Yoon et al., 2010). However, no direct binding of DHEA or DHEA-S at NMDA receptor has been identified so far, and studies suggest that these effects could be mediated by the activation of $\sigma 1$ receptors (see Section 2.3.1. and Table 1).

2.2.2. Modulation of GABA_A receptors

Steroids such as Progesterone or Desoxycorticosterone acetate have been empirically used for their rapid sedative and anesthetic properties (Selye, 1941), indicating their easy passage through the blood-brain barrier. The fact that these effects occurred on a time scale incompatible with classical modulation of gene expression strongly suggested that steroids could modulate brain excitability through non-genomic pathways. The anesthetic effect of neurosteroids has been proposed to be related to their capacity to increase inhibitory neurotransmission within the mammalian central nervous system, through the neurotransmitter GABA and its type A ionotropic receptor (GABA_A receptor) (Scholfield, 1980), whose main endogenous modulators are molecules belonging to the endozepine family (Steyaert et al., 1991).

GABA_A receptors mediate fast inhibitory neurotransmission in the central nervous system. They are pentameric channels composed of several subunit isoforms (6 isoforms of α subunit, 3 isoforms of β subunit, 3 isoforms of γ subunit and only 1 isoform of θ , ϵ , δ and π subunits). Early studies showed that the synthetic pregnane steroid alfaxalone (3 α -hydroxy-5 α -pregnane-11,20-dione), which displays anesthetic properties, enhances GABA inhibitory activity through its binding to GABA_A receptors, in a similar way than barbiturates, but at another binding site that remains unknown (Harrison & Simmonds, 1984; Turner, Ransom, Yang, & Olsen, 1989). It was the first evidence that, besides endozepines, neurosteroids are endogenous modulators of GABA_A receptors: they can either potentiate (i.e. reinforce the inhibitory action), or antagonize (i.e. reduce the inhibitory action) the GABAergic neurotransmission (Akk et al., 2007; Mellon, 2007; Seljeset, Laverty, & Smart, 2015).

ALLO can potentiate the natural affinity of GABA for GABA_A receptors and/or directly activate the channel conductance (Carver & Reddy, 2013). Accordingly, two binding sites for ALLO are characterized in the transmembrane domain of the receptor: a potentiation site located into α -subunit and a direct activation site located between α - and β -subunits (Fig. 2C) (Hosie, Wilkins, da Silva, & Smart, 2006). At nanomolar concentrations, ALLO produces an enhancement of GABA-evoked chloride currents, whereas at micromolar concentrations, it directly activates GABA_A receptors, in the absence of GABA (Table 1) (Puia et al., 1990). Thanks to these effects on GABA_A receptor activity, ALLO produces sedative, anxiolytic, and anticonvulsant behavioral

effects (Carver & Reddy, 2013). The action of ALLO seem to be specific to GABA_A receptors since this compound has slight or no affinity for glutamate ionotropic receptors (Mellon, 2007).

PREG is inactive on GABA_A receptors (Gibbs et al., 2006), whereas DHEA blocks them at an unknown binding site with micromolar affinity (Table 1) (Le Foll, Louiset, Castel, Vaudry, & Cazin, 1997; Park-Chung, Malayev, Purdy, Gibbs, & Farb, 1999). Sulfated neurosteroids PREG-S and DHEA-S also inhibit GABAergic activity (Fig. 2C) (Majewska, Demirgören, Spivak, & London, 1990; Majewska, Mienville, & Vicini, 1988). No binding sites for PREG-S and DHEA-S on GABA_A receptors has been identified yet, although they act through distinct sites from those of ALLO (Park-Chung et al., 1999). PREG-S reduces chloride currents and accelerates the receptor desensitization, with affinity for GABA_A receptors estimated in the low micromolar range (Table 1) (Le Foll et al., 1997, Park-Chung et al., 1999, Akk et al., 2007). These inhibitory properties of PREG-S lead to pro-convulsant effects, inducing seizures when administered directly into the brain (Williamson, Mtchedlishvili, & Kapur, 2004). With similar affinity, DHEA-S inhibits these currents by affecting their amplitude in frog melanotrope cells (Table 1) (Le Foll et al., 1997; Park-Chung et al., 1999).

All these data suggest that glutamatergic and GABAergic neurotransmissions are both modulated by sulfated neurosteroids while the action of ALLO seems to be restricted to a facilitation of the inhibitory neurotransmission. ALLO is therefore considered as inhibitory neurosteroid. To date, PREG-S is described as the most active neurosteroid. Its affinity for GABA_A receptors seems to be slightly higher than its affinity for NMDA receptors, although these two pathways probably coexist. Therefore, PREG-S effects may converge toward a potentiation of neuronal excitation. DHEA and DHEA-S bind to $\sigma 1$ receptor, resulting in an indirect activation of NMDA receptors, and inhibit GABA_A currents, eventually leading to excitatory effects.

2.3. Cytoplasmic targets of neurosteroids

Two main cytoplasmic targets for neurosteroids have been described so far. $\sigma 1$ receptors play probably a critical role in the neurosteroid modulation of NMDA receptors, while MAPs, largely modulated by some neurosteroids, are essential for structural plasticity regulation.

2.3.1. Sigma 1 receptor

$\sigma 1$ receptors are endoplasmic reticulum resident chaperone proteins distributed in neurons, ependymocytes and oligodendrocytes (Alonso et al., 2000; Palacios et al., 2003). Several chemically unrelated drugs, including cytochrome P450 inhibitors and neuroactive steroids, are able to bind to $\sigma 1$ receptors (Hayashi, 2015). Among them, PREG-S, DHEA and DHEA-S are described as $\sigma 1$ receptor agonists (Maurice, Phan, Urani, & Guillemain, 2001). Indeed, they dose-dependently attenuate the amnesia induced by the β -amyloid-related peptide β_{25-35} , and this is prevented by haloperidol, a $\sigma 1$ receptor antagonist (Maurice, Su, & Privat, 1998). $\sigma 1$ receptors are characterized as voltage- and ligand-gated modulators (Hayashi, 2015). In particular, their activation upregulates NMDA receptors and carries them to the plasma membrane (Pabba et al., 2014).

Although PREG is described as $\sigma 1$ receptor agonist in some studies (Maurice, Phan, et al., 2001), its affinity for this receptor is higher than 100 μM (Su, London, & Jaffe, 1988). Hence, PREG effects in physiological conditions are probably $\sigma 1$ receptor independent (Table 1).

When $\sigma 1$ receptors are antagonized by NE-100, DHEA potentiation of neuronal NMDA response is blocked, showing that DHEA may enhance NMDA receptor activity, but only via $\sigma 1$ receptor activation (Bergeron et al., 1996). Hence, it has been proposed that modulation of NMDA receptors by PREG-S, DHEA and DHEA-S may result from $\sigma 1$ receptor activation (Fig. 2A) (Monnet & Maurice, 2006; Yoon et al., 2010). These sulfated neurosteroids bind to $\sigma 1$ receptors at micromolar concentrations (Table 1), and probably at the same binding site (Monnet & Maurice, 2006), whereas the affinity of DHEA for $\sigma 1$

receptors is unknown. PREG-S and DHEA-S may be in competition to bind to σ_1 receptors, but this mechanism remains unclear. PREG-S could not only potentiate NMDA receptors directly, but also indirectly via σ_1 receptor activation. In addition, it seems that PREG-S activation of σ_1 receptors may be the predominant mechanism under physiological conditions (Monnet & Maurice, 2006). However, this is an open question since this hypothesis has not been clearly tested yet. The combined action on σ_1 receptors and NMDA receptors could explain why PREG-S is very often described as a potent excitatory neurosteroid. In addition, the norepinephrine release evoked by NMDA is not anymore inhibited by PREG-S and potentiated by DHEA-S when σ_1 receptors are blocked by a selective antagonist, further demonstrating that σ_1 receptors constitute an important intermediate in neurosteroid modulation of NMDA receptors (Monnet, Mahé, Robel, & Baulieu, 1995).

2.3.2. Microtubule-associated proteins

Microtubules are one of the components of cytoskeleton. They serve as tracks for axonal transport, providing dynamic and mechanical functions, and control local signaling events, leading to stable neuronal morphology. They are formed by the polymerization of α - and β -tubulin heterodimers, and disassembled by depolymerization, mostly at their ends (Penazzi, Bakota, & Brandt, 2016). This dynamic is partly regulated by a variety of associated proteins (MAPs) that bind to microtubules and promote their assembly and stability in axons and dendrites (Galjart, 2005; Sánchez, Díaz-Nido, & Avila, 2000). Among them, MAP2 and CLIP-170 have been characterized as neurosteroid receptors (Fig. 3) (Murakami et al., 2000; Weng et al., 2013).

MAP2 is a somatodendritic protein that is 1827 amino acid long and has an average molecular weight of 200 kDa, the exact molecular weight depending on each isoform. The term MAP2 generically refers to three proteins (MAP2a, MAP2b and MAP2c) that are produced from a single gene, and are differentially expressed. MAP2a is expressed from 10 days after birth, MAP2b during development and in adulthood and MAP2c until 10 days after birth in rats (Chung, Kindler, Seidenbecher, & Garner, 1996; Kindler, Schulz, Goerdert, & Garner, 1990). MAP2 contains an amino-terminal projection domain negatively charged and microtubule-binding repeats near the carboxyl terminus composed of conserved KXGS motif which can be phosphorylated. MAP2a and

MAP2b have a longer projection domain than MAP2c. MAP2 displays microtubule-binding domains which contain a F-actin binding domain (Dehmelt et al., 2004) and an amino-terminal domain that can interact with protein kinase A (Vallee, DiBartolomeis, & Theurkauf, 1981). MAP2 is strongly involved in the regulation of microtubule dynamics by enhancing tubulin assembly and promoting microtubule stability (Sánchez et al., 2000). Hence, the deletion of MAP2 in knock-out mice leads to a reduction in both microtubule density in dendrite and dendritic length (Harada, Teng, Takei, Oguchi, & Hirokawa, 2002). MAP2 is a neurosteroid receptor since PREG or DHEA are able to directly interact with the amino-terminal region of this protein, with affinity estimated in the nanomolar range (Fig. 3A and Table 1) (Laurine et al., 2003; Mizota & Ueda, 2008; Murakami et al., 2000). Similarly to PREG, 3 β -methoxypregnenolone, a synthetic PREG derivative, is able to bind to MAP2 at nanomolar concentrations (Table 1) (Fontaine-Lenoir et al., 2006). By binding to MAP2, PREG and derivatives promote tubulin polymerization and microtubule growing (Fig. 3), suggesting that, even not clearly established yet, MAP2 may have a key role in structural plasticity.

CLIP-170 (cytoplasmic linker protein 170) is also a MAP, weighing 170 kDa and located at the elongation plus end of microtubules. It is composed of an amino-terminal microtubule-binding domain, a central coiled-coil domain that allows homodimerization, and a carboxy-terminal metal-binding domain (Pierre, Pepperkok, & Kreis, 1994; Scheel et al., 1999). The amino-terminal domain of CLIP-170 is sufficient for microtubule plus-end tracking (Diamantopoulos et al., 1999; Folker, Baker, & Goodson, 2005). PREG is able to bind to CLIP-170 with nanomolar concentration (Table 1), potentiating the ability of CLIP-170 to promote microtubule assembly (Fig. 3) (Weng et al., 2013). Actually, PREG changes the conformation of CLIP-170 from a folded inactive protein into an opened active form able to interact with microtubules. A recent study demonstrates that cyclin-dependent kinase-like 5 gene deficiency precludes the induction of CLIP-170 active form, leading to microtubule instability. PREG, by maintaining CLIP-170 in the active form, prevents the microtubule from the observed derangement (Barbiero et al., 2017).

PREG and DHEA, probably by diffusing into neurons, seem to act mostly on the microtubular system with affinity in the nanomolar range. PREG-S and DHEA-S hardly cross the plasma membrane, and mostly act via amino-acid ionotropic receptors with affinity in the micromolar range. The sulfated neurosteroids also interact with σ_1 receptors, showing that they are either imported in the cells or produced in them from PREG and DHEA sulfation. Finally, these data are consistent with neurosteroid levels under physiological conditions, since sulfated conjugates are more present than non-sulfated neurosteroids.

3. Neurosteroids: effects on neuroplasticity

3.1. Modulation under physiological conditions

3.1.1. Structural and functional plasticity, and neurogenesis

The pioneer work in neuroplasticity was conducted by Hebb, who postulated that synaptic plasticity involves both structural and functional changes that may occur in excitatory (glutamatergic) and inhibitory (GABAergic) networks (Hebb, 1949).

Structural plasticity refers to the size of the neuronal arbor, including dendritic length and number of ramifications, and to dendritic spine shape and density. Dendritic spines are small protrusions that serve as postsynaptic biochemical compartments and receive excitatory inputs (Sala & Segal, 2014). They are classified into five classes based on their shape: filopodium, thin, stubby, mushroom and cup-shaped (Maiti, Manna, & McDonald, 2015). They are formed and eliminated in an activity-dependent manner (Nägerl, Eberhorn, Cambridge, & Bonhoeffer, 2004). These protrusions are found mostly in glutamatergic neurons such as pyramidal neurons of neocortex, medium spiny neurons in the striatum, CA3 neurons of the hippocampus and Purkinje cells in the

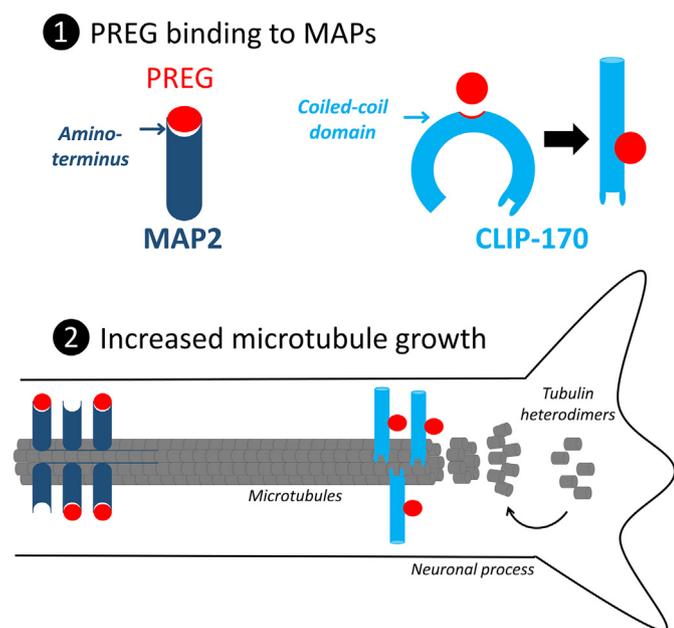


Fig. 3. PREG binds to MAPs and promotes microtubule assembly. ① PREG (red dot) binds to MAP2 (dark blue) in the amino-terminal domain. PREG binds to the coiled-coil domain of CLIP-170 and changes the conformation of the protein. ② The interaction of PREG on MAPs increases tubulin polymerization and microtubule growth in neuronal processes.

cerebellum (Hering & Sheng, 2001), and also in a small population of GABAergic interneurons, mostly located in the hippocampus (Scheuss & Bonhoeffer, 2014). Dendritic spines become mature with the help of glutamate-induced activation of NMDA and AMPA receptors (Mattison, Popovkina, Kao, & Thompson, 2014). In addition, GABA controls the competitive selection of dendritic spines via local Ca^{2+} signaling inhibition (Hayama et al., 2013). Structural plasticity is under control of actin and microtubule dynamics. They are both major dendritic spine regulators since they are necessary to determine spine shape (Gordon-Weeks & Fournier, 2014; Penazzi et al., 2016).

Functional plasticity can be observed when repetitive electrical activity induces a long-lasting increase or decrease of synaptic efficacy in brain regions, called long-term potentiation (LTP) and long-term depression (LTD) respectively (Bliss & Lomo, 1973; Linden & Connor, 1995). These phenomena occur at both excitatory and inhibitory synapses (Castillo, 2012). LTP and LTD are partly sustained by NMDA and AMPA receptors, and can be modulated by GABAergic neurotransmission (Malenka & Bear, 2004; Wu, Guo, Gearing, & Chen, 2014).

Structural and functional plasticity are closely associated: changes in Ca^{2+} levels following glutamate receptor activation lead to actin-dependent effects on dendritic spine motility and morphology (Brüning, Kaech, Brinkhaus, Oertner, & Matus, 2004). LTP induces formation and growth of dendritic spines, and LTD is implicated in spine retraction followed by elimination in the adult mammalian brain (Alvarez & Sabatini, 2007). AMPA receptor expression is positively correlated with the spine head size (Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010). LTD seems to induce morphological changes in presynaptic elements and to reduce their contact with spines (Becker, Wierenga, Fonseca, Bonhoeffer, & Nägerl, 2008). LTP alters astrocyte number and extension volume, and ultimately increases the coverage of excitatory synapses in the hippocampus (Haber, Zhou, & Murai, 2006; Lushnikova, Skibo, Muller, & Nikonenko, 2009).

Adult neurogenesis occurs in the subgranular zone of the hippocampal dentate gyrus and in the subventricular zone. It consists in formation of new mature neurons, using both structural and functional clues. This highly regulated process involves proliferation of neural stem cells and neural progenitor cells, neuronal migration, differentiation and integration into a network of functional synapses within the brain (Lledo et al., 2006). In mice, GABA released by hippocampal interneurons depolarizes progenitor cells and inhibits proliferation via GABA_A receptor activation (Song et al., 2012). NMDA receptor activation leads to decreased neurogenesis in hippocampal dentate gyrus, where NR1 subunit is highly expressed (Cameron, McEwen, & Gould, 1995; Kitayama, Yoneyama, & Yoneda, 2003). By contrast, when NMDA-mediated neurotransmission is blocked, hippocampal neurogenesis is increased, and many of newly born cells become viable neurons that are added to the total population of granule cells (Cameron et al., 1995). The NMDA-receptor inhibitory effect on neurogenesis could involve $\sigma 1$ receptor

activation. Indeed, in $\sigma 1$ receptor knock-out mice, cell proliferation is increased and the lack of $\sigma 1$ receptors induces a hypofunctioning of NMDA receptors, probably due to a decrease in NR2B phosphorylation. Importantly, these alterations are reversed by NMDA receptor agonists, highlighting the role of $\sigma 1$ receptor as intermediate in NMDA-receptor modulation of neurogenesis (Sha et al., 2013).

3.1.2. Neuroplasticity regulation by neurosteroids

Interestingly, the main actors regulating neuroplasticity (amino acid ionotropic receptors and cytoskeleton proteins) are also the targets of neurosteroids, as reported above and illustrated in Table 1. Hence, neurosteroids have been characterized as neuroplasticity modulators (Table 2) (Flood, Morley, & Roberts, 1992; Flood, Smith, & Roberts, 1988). Structural plasticity is enhanced when neurons are treated with PREG or its derivative 3 β -methoxyprogesterone. Indeed, PREG (1–10 μ M) increases MAP2-positive neurite length and 3 β -methoxyprogesterone (μ M) promotes microtubule polymerization in vitro, enhances neurite outgrowth in PC12 cells and extends dendritic trees of motoneurons after spinal cord injury (Table 2) (Duchossoy, David, Baulieu, & Robel, 2011; Fontaine-Lenoir et al., 2006; Matsunaga, Mizota, Uchida, Uchida, & Ueda, 2010; Murakami et al., 2000). These effects are attenuated by a reduced MAP2 expression induced by small interfering RNA specific to MAP2 transcripts, further suggesting that enhanced structural plasticity may rely on the interaction between 3 β -methoxyprogesterone and MAP2 (Fontaine-Lenoir et al., 2006). In addition, since PREG activates CLIP-170 to promote microtubule polymerization, it can be hypothesized that this mechanism also positively regulates structural plasticity, although no direct evidence has been observed. As mentioned above, PREG seems to act mostly inside the cells through microtubule cytoskeleton. It can be hypothesized that PREG acts directly on structural plasticity, leading to modulation of functional plasticity, but via an unknown indirect way.

PREG-S promotes functional plasticity via an increase in trafficking of functional NMDA receptors to the cell surface and enhances synaptic activity depending on Ca^{2+} signaling and c-AMP response element-binding protein activation (Sliwinski, Monnet, Schumacher, & Morin-Surun, 2004; Kostakis et al., 2013; Smith, Gibbs, & Farb, 2014; Smith et al., 2014). This effect obtained with micromolar concentrations of PREG-S, is thought to be mediated by $\sigma 1$ receptors since, as mentioned above, their activation upregulates NMDA receptors and carries them to the plasma membrane (Pabba et al., 2014). PREG-S also promotes neurogenesis in the hippocampal dentate gyrus by increasing glutamate release which leads to presynaptic potentiation and survival of adult-generated hippocampal granule cells (Yang et al., 2011). NMDA receptor potentiation, directly or through $\sigma 1$ receptor stimulation, causes decreased neurogenesis whereas GABA_A receptor blockade has the opposite effect (see Section 3.1.1.). Considering the high affinity of PREG-S for GABA_A receptors (Table 1), it is probable that PREG-S acts

Table 2
Neuroplasticity enhancement by neurosteroids. \uparrow means “increase”.

Neurosteroids	Effects on neuroplasticity	References
PREG	\uparrow Structural plasticity: via MAP2-binding and microtubule cytoskeleton modulation	(Duchossoy et al., 2011; Fontaine-Lenoir et al., 2006; Matsunaga et al., 2010; Murakami et al., 2000)
PREG-S	\uparrow Functional plasticity: through NMDA receptor trafficking \uparrow Neurogenesis: stimulates NMDA and $\sigma 1$ receptors in newborn cells	(Kostakis et al., 2013; Smith, Gibbs, & Farb, 2014) (Mayo et al., 2005; Smith, Gibbs, & Farb, 2014)
DHEA	\uparrow Structural plasticity in vitro and in vivo through an increase in spine density, possibly via $\sigma 1$ receptor \uparrow Functional plasticity: enhances synaptic efficacy \uparrow Neurogenesis: stimulates $\sigma 1$ receptors in newborn cells	(Compagnone & Mellon, 1998; Hajszan et al., 2004; MacLusky et al., 2004; Monnet & Maurice, 2006) (Karishma & Herbert, 2002; Moriguchi et al., 2013)
DHEA-S	\uparrow Functional plasticity: increases LTP via NMDA receptor signaling; increases short-term potentiation via mGluR5 activation	(Chen et al., 2006; Meyer et al., 2002; Xu et al., 2012)
ALLO	\uparrow Structural plasticity: increases mature excitatory synapses in vitro, possibly via presynaptic GABA _A receptors modulation \uparrow Neurogenesis: through GABA _A receptor activation in neuroprogenitor cells	(Iwata et al., 2013; Shimizu et al., 2015) (Irwin & Brinton, 2014; Wang et al., 2008)

via GABA_A receptors inhibition to promote neurogenesis in hippocampal dentate gyrus (Mayo et al., 2005), but this needs to be more studied.

DHEA also acts as a neurotrophic signal and can stimulate axonal growth in vitro (Ulmann et al., 2009). It induces morphological changes in embryonic cortical neurons primary cultures, including axonal length increase, varicosities and basket-like formation around other cell bodies (Compagnone & Mellon, 1998). Since DHEA binds to MAP2 with high affinity (Table 1) (Laurine et al., 2003), it is possible that this interaction could underly the positive effects of DHEA on structural plasticity. In addition, gonadectomized male and female rats treated with DHEA show an increased density of hippocampal CA1 spines compared to control animals (Hajszan, Macluskus, & Leranth, 2004; MacLuskus, Hajszan, & Leranth, 2004). This effect could be mediated by σ 1 receptors, since DHEA interaction with this receptor leads to the formation of membrane lipid microdomains (Monnet & Maurice, 2006), which is critical for dendritic spine shape modulation. Moreover, σ 1 receptor stimulation by DHEA enhances synaptic efficacy and neurogenesis in the hippocampal dentate gyrus, and this effect can be prevented by the σ 1 receptor antagonist NE-100 (Karishma & Herbert, 2002; Moriguchi et al., 2013).

Chronic treatment of rats with DHEA-S (20 mg/kg/day subcutaneous for 7 days) facilitates LTP via an amplification of Src-dependent NMDA receptor signaling in the hippocampal CA1 area (Chen et al., 2006). This study shows that peripheral administration of DHEA-S has an effect in the brain. As mentioned in the Section 2.1., sulfated neurosteroids can cross the blood-brain barrier, but it seems that when in the brain, DHEA-S undergoes desulfation and is much transformed into metabolites (Qaiser et al., 2017). Hence, the effect reported here could be attributed to metabolites rather than DHEA-S itself. In vitro studies show that DHEA-S induces short-term potentiation with concentrations between 0.1 nM and 1 μ M (Xu, Tanaka, Chen, & Sokabe, 2012) and promotes LTP via σ 1 receptor activation in both cultured hippocampal neurons (Meyer, Carta, Partridge, Covey, & Valenzuela, 2002) and rat hippocampal slices (Chen et al., 2006), suggesting a direct effect of DHEA-S in functional plasticity.

ALLO is also able to potentiate GABA_A receptors, particularly those containing δ subunit, located at extra- or peri-synaptic areas (Meera, Wallner, & Otis, 2011). This kind of receptors mediates tonic GABAergic inhibition, which partly regulates neuronal excitability and plasticity in hippocampus, suggesting a role for ALLO in neuroplasticity modulation (Groen et al., 2014). ALLO (10–30 nM) modulates glutamate release via presynaptic GABA_A receptors and directly increases excitatory synapse density along dendrites of hippocampal cell in vitro (Iwata, Wakita, Shin, Fukuda, & Akaike, 2013). This action seems to be mediated by protein kinase A which is critical for neuronal shape modulation, further illustrating the involvement of ALLO in structural plasticity modulation (Shimizu, Ishizuka, Yamazaki, & Shirao, 2015). In addition, ALLO increases the number of dendritic spines in vitro, probably by causing an accumulation of drebrin protein along neurites and an increase in vesicular glutamate transporter 1 clusters, two indicators of synapse maturation (Shimizu et al., 2015). In developmental neurons, ALLO has opposite effect than in mature neurons and inhibits GABA_A receptor activity, leading to an increase in proliferation of neural progenitors in rat hippocampus (Wang, Liu, Irwin, Chen, & Brinton, 2008).

Taken together, these data show that all neurosteroids promote neuroplasticity in physiological conditions. In addition, sulfated neurosteroids seem to act rather on functional plasticity while PREG, DHEA and ALLO mostly regulate structural plasticity. This is consistent with their preferential target described above, even if further studies need to be conducted.

3.1.3. The example of memory

The involvement of neurosteroids can be clearly exemplified in memory mechanisms. LTP and LTD in the hippocampus are thought to be the functional substrates of memory (Bliss & Collingridge, 1993). LTP promotes formation of multiple-spine synapses between a single axon terminal and a dendrite (Toni, Buchs, Nikonenko, Bron, & Muller, 1999). Memory formation is associated with time-dependent development of dendritic spines in the hippocampus (Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009).

NMDA and AMPA receptors play critical roles in long-term memory consolidation. Indeed, activation of NMDA receptors, requiring combined action of both Ca²⁺ influx mediated by AMPA receptors and glutamate binding, is essential for memory formation (Lynch, Larson, Kelso, Barrionuevo, & Schottler, 1983; Morris, Anderson, Lynch, & Baudry, 1986). After memory formation, the rapid trafficking of AMPA receptors to the postsynaptic element is a critical step that eventually leads to the strengthening of synaptic transmission, especially for GluA1-containing AMPA receptors (Henley & Wilkinson, 2013). Within a few hours, they are gradually substituted by GluA2-containing AMPA receptors at the synapse, which form more stable AMPA receptors, resistant to disruption (Shi, Hayashi, Esteban, & Malinow, 2001). GABA_A receptors are also implicated in memory processes, since their pharmacological blockade by picrotoxin leads to learning enhancement (McGaugh & Roozendaal, 2009). In addition, a genetic mutation on GABA_A receptors leading to an impossible modulation triggers memory impairments (Tretter et al., 2009), highlighting the fact that GABA_A receptor is critically involved in memory processes.

Regarding the positive actions of neurosteroids on neuroplasticity, it is established that such compounds could enhance memory processes. Early studies characterized PREG, PREG-S, DHEA and DHEA-S as retention memory enhancers (Table 3) (Flood et al., 1988; Flood et al., 1992).

When administered through the intranasal pathway or in the third ventricle, PREG enhances recognition and spatial memory of rats (Abdel-Hafiz et al., 2016) and memory in the footshock active avoidance test (Flood et al., 1992). Given the fact that PREG seems to act exclusively on microtubule dynamics, those effects may rely on its capacity to promote structural plasticity (Barbiero et al., 2017; Murakami et al., 2000; Weng et al., 2013).

Regarding PREG-S, its effects on memory are less consistent (Table 3). PREG-S infusion (μ M) into the dorsal CA1 hippocampus restores memory of cirrhotic rats, and this effect is probably mediated by NMDA receptors (Dastgheib, Dehpour, Heidari, & Moezi, 2015). Indeed, at micromolar concentrations, PREG-S is able to activate glutamatergic neurotransmission through NMDA and σ 1 receptors, which is consistent with stimulation of functional plasticity, leading to memory enhancement reported in this study. In addition, PREG-S administered at nanomolar concentrations directly in the cerebral ventricles displays a promnesiant activity by increasing recognition memory, in both rats

Table 3
Memory enhancement by neurosteroids.

Neurosteroids	Administration site	Model
PREG	- Third ventricle - Intranasal infusion	- Mice in the active avoidance task in the T-maze (Flood et al., 1992) - Rats in novel object recognition test, Morris water maze and object location task (Abdel-Hafiz et al., 2016)
PREG-S	- Dorsal CA1 - Lateral ventricles	- Cirrhotic rats (Dastgheib et al., 2015) - Spatial recognition memory in mice and rats (Akwa et al., 2001)
DHEA and DHEA-S	- Third ventricle	- Mice in the active avoidance task in the T-maze and mice in the step down passive avoidance tasks (Flood et al., 1988)
ALLO	- Dorsal CA1	- Ovariectomized rats treated with Estrogens and/or Progesterone and tested in the step down inhibitory avoidance task (Escudero et al., 2012)

and mice. In this range of concentrations, PREG-S seems to preferentially act through GABA neurotransmission, by inhibiting GABA_A receptors so it is possible that this action is at least in part responsible for PREG-S effects in this work. Interestingly, this effect is ten times higher when rats are administered with the synthetic (-) enantiomer of PREG-S (Akwa, Ladurelle, Covey, & Baulieu, 2001). Distinct mechanisms are thought to be involved in the pro-mnesic properties of PREG-S versus (-) PREG-S, since the presence of NMDA antagonist prevents the action of PREG-S but not of its (-) enantiomer. In contrast, when infused at micromolar concentration into the lateral septum of rats before the training phase of the novel object recognition task, PREG-S produces an amnesic effect (Nanfaro, Cabrera, Bazzocchini, Laconi, & Yunes, 2010). Regarding these discrepancies, it is difficult to conclude towards a favored involvement of unique target to explain the effects of PREG-S on memory. PREG-S actions likely depend on various parameters, such as the moment and the site it is administered or its concentration.

DHEA and DHEA-S, at millimolar concentrations, are both able to potentiate memory in mice tested in T-maze footshock active avoidance and step-down passive avoidance tasks (Table 3) (Flood et al., 1988). Steroid sulfatase inhibition, which increases DHEA-S and possibly PREG-S levels in brain, demonstrates that the sulfated form of DHEA could be the active agent responsible for such effects (Johnson, Wu, Li, & Maher, 2000). However, in this last study, the levels of PREG-S before and after steroid sulfatase inhibition have not been measured; it is thus unclear whether PREG-S or PREG is the active compound in memory regulation. The memory-enhancing effects of DHEA-S might be attributed to the indirect modulation of NMDA receptors through activation of σ 1 receptors since they have been demonstrated to be implicated in both short-term and long-term memory (Maurice, Urani, Phan, & Romieu, 2001).

Finally, ALLO administration is able to reverse the amnesic effects produced by Estradiol and/or Progesterone administration in ovariectomized rats (Table 2) (Escudero et al., 2012), indicating a positive effect of ALLO on memory. Because GABA_A receptors are the exclusive target of ALLO identified so far (see Section 3.1.2.), the potentiating role of ALLO on memory might rely on their activation. ALLO would act through presynaptic GABA_A autoreceptors located in hippocampal GABAergic neurons, and consequently would inhibit GABA release to stimulate in fine the glutamate transmission (Herd, Belelli, & Lambert, 2007).

The data discussed here show that neurosteroids promote memory processes by acting on multiple targets which enhance neuroplasticity. Further work needs to be conducted to elucidate their exact mechanism of action. In addition, it is not clear whether it is the neurosteroid administered itself or metabolites that cause memory enhancement.

3.2. Modulation under pathological conditions

In several neurological pathologies, neuroplasticity impairments are often observed (Boulle et al., 2012). Interestingly, in some of them, neurosteroid levels are also altered, and many studies have been conducted to attempt to elucidate both their role in pathogenesis and their potential therapeutic use for treatment of these diseases.

3.2.1. Neurosteroids and Alzheimer's disease

In Alzheimer's disease (AD), the grey matter volume is decreased, especially in the hippocampus, which relies on synaptic degeneration that correlates with cognitive decline (Guo et al., 2014; Terry et al., 1991). In the temporal cortex of AD patients, GABA and glutamate levels are lower than in controls, indicating deficient synaptic function and neurotransmission (Gueli & Taibi, 2013). In addition, theta burst stimulations, known to lead to LTD- and LTP-like effects in healthy subjects, fail to produce LTP-like effects in those patients, further demonstrating impaired neuroplasticity in AD (Koch et al., 2012). Most of the altered synapses in the hippocampus are glutamatergic and this loss may be provoked by dendritic spine dysfunctions or abnormal elimination, and reduced neurogenesis (Hamilton et al., 2010; Maiti et al., 2015).

Hippocampal GABAergic neuron loss is relatively modest in AD, but only few of them remain functional (Limon, Reyes-Ruiz, & Mileti, 2012). Alterations in neuroplasticity could be explained by the presence in AD patient brains of amyloid plaques (mostly composed of amyloid β), and aggregation of the MAP Tau in tangles. Indeed, amyloid β dimers extracted from brains of patients with AD and injected in the hippocampus of normal rats inhibit LTP and potentiate LTD (Shankar et al., 2008). This effect is probably the result of NMDA receptor signaling and/or protein kinase A/c-AMP response element-binding protein pathway inhibition (Shankar et al., 2007; Snyder et al., 2005; Vitolo et al., 2002). On the other hand, Tau is necessary for basal synaptic transmission at the mossy fiber-CA3 synapses, but when hyperphosphorylated and aggregated like in AD patient brains, it induces pre- and postsynaptic morphological changes and impairment of LTD (Decker et al., 2015).

Regarding neurosteroids, a heterogeneity of their distribution in brain regions is observed in non-demented patients but not in AD patients (Weill-Engerer et al., 2002), with a general trend toward lower neurosteroid levels in AD patients compared to non-demented patients. The authors also report that amounts of phosphorylated Tau are negatively correlated with DHEA-S levels in the hypothalamus, and those of β -amyloid peptides negatively correlate with PREG-S contents in the striatum and the cerebellum (Weill-Engerer et al., 2002). This involvement of neurosteroids in age-related cognitive deficits is further suggested by unpredicted observations showing higher DHEA content in the hippocampus of AD patients than in healthy subjects (Brown, Han, Cascio, & Papadopoulos, 2003; Kim et al., 2003; Naylor et al., 2008). This high level of DHEA could be explained by a decrease in sulfotransferase activity evidenced in AD brains leading to a decrease in sulfated neurosteroid levels (Kim et al., 2003). In addition, low PREG-S levels in the hippocampus of aged rats are associated with deficient cognitive performances (Vallée et al., 1997). It can be proposed that the lack of neuroactive sulfated steroids DHEA-S and PREG-S in aging may lead to down-stimulation of σ 1 receptors together with up-regulation of GABA_A receptors, and in fine to memory impairments.

As discussed above, some neurosteroids are memory enhancers, making their study in animal models of AD relevant to elucidate their potential therapeutic effects. In a mouse model of AD, PREG-S administration exerts a potent anti-amnesic effect, partly via σ 1 receptor stimulation, and ameliorates the level of hippocampal LTP (Table 4) (Yang et al., 2012). In addition, direct σ 1 receptor stimulation by a synthetic agonist enhances spatial learning in aged rats (Maurice, 2001).

When systemically injected, DHEA-S, which potentiates neuronal excitability through σ 1 and GABA_A receptors, reverses the dendritic shrinkage observed in the cerebral cortex of aging male rats (Table 4) (Chen, Tseng, Wang, & Wang, 2014). However, it is still not clear whether DHEA-S acts by itself, implying an active transport of the sulfated neurosteroid through the blood-brain barrier, or whether its conversion to DHEA and transformation in other metabolites are responsible for their positive effects (see Section 2.1). When steroid sulfatase is inhibited – meaning that the formation of PREG or DHEA from their sulfated forms is inhibited – spatial memory and synaptic plasticity are preserved in a rat model of AD (Yue et al., 2016), evidencing that the imbalance between sulfated and non-sulfated neurosteroids amounts may play an important role in AD pathological processes (Luchetti et al., 2011).

Interestingly, mRNA expression of the enzyme responsible for ALLO biosynthesis increases in the early stages of the disease. Since ALLO potentiates GABA_A receptor activity, this could be a compensatory mechanism in order to counteract the GABAergic inhibition loss (Luchetti, Huitinga, & Swaab, 2011). Furthermore, in a mouse model of AD, ALLO restores hippocampal-dependent learning and memory and neural progenitor survival, suggesting that it could be used therapeutically in Alzheimer's disease (Irwin & Brinton, 2014; Singh et al., 2012). To date, ALLO was tested in clinical studies on healthy subjects to investigate safety, pharmacokinetics and pharmacodynamics (Irwin & Brinton, 2014), waiting for future clinical studies in AD patients.

Table 4
Potential therapeutic effects of neurosteroids in animal models and in humans. AD: Alzheimer's disease.

	PREG	PREG-S	DHEA	DHEA-S	ALLO
AD: Anti-amnesic	Unknown	In animals Yang et al., 2012	Unknown	In animals Chen et al., 2014	In animals Singh et al., 2012
Schizophrenia: Reduced symptoms	In animals and patients Wong et al., 2012; Ritsner et al., 2014; Kreinin et al., 2014; Marx et al., 2014	In animals Wong et al., 2015	Possible marker in patients Harris et al., 2001; Strous et al., 2007	Unknown	Unknown
Anxiety and depression: Anxiolytic and/or antidepressant	In animals and patients Bianchi & Baulieu, 2012; Brown et al., 2014; Parésys et al., 2016	In animals Melchior & Ritzmann, 1994b; Reddy et al., 1998	In animals and in patients Melchior & Ritzmann, 1994a; Sripada et al., 2013; Genuet et al., 2009; Wolkowitz et al., 1995; Wolkowitz et al., 1997; Schmidt et al., 2005	In animals Fleshner et al., 1997; Genuet et al., 2009	In animals Engin & Treit, 2007; Khisti et al., 2000; Shirayama et al., 2011
Epilepsy: anticonvulsant	Unknown	Unknown	Unknown	Unknown	In patients Reddy, 2013; Laxer et al., 2000; Pieribone et al., 2007

3.2.2. Neurosteroids and schizophrenia

Schizophrenia is associated to a decrease in brain volume whereas the ventricles are larger than those of healthy subjects (Antonova, Sharma, Morris, & Kumari, 2004). Besides, abnormalities in regions located in thalamo-cortical networks and disrupted connectivity between dorso-lateral PFC and hippocampus are believed to play a major role in the pathogenesis of this disease (Meyer-Lindenberg & Tost, 2014). Reduced volume and neuronal density in the thalamus have been proposed to be positively correlated with positive symptoms and negatively correlated with negative symptoms (Pakkenberg, 1990; Rao, Kalmady, Arasappa, & Venkatasubramanian, 2010). In addition, a negative correlation between the strength of positive symptoms and CA3 and CA1 volumes has been evidenced, suggesting that hippocampus subfield dysfunctions lead to abnormal integration of sensory representations (Kühn et al., 2012). Negative symptoms, such as cognitive deficits, seem to rely on PFC abnormalities, that may be supported by altered LTP, since dysfunction in glutamatergic, dopaminergic and GABAergic neurotransmissions are also reported in schizophrenia (Salavati et al., 2015). In this pathology, neurosteroids play an important role, both in pathogenic processes and therapy (Cai, Cao, Zhou, & Yao, 2018).

In order to identify markers for diagnostic of schizophrenia, blood levels of neurosteroids have been measured in patients. Some studies report elevated circulating levels of DHEA and DHEA-S in medicated (Di Michele, Caltagirone, Bonaviri, Romeo, & Spalletta, 2005; Oades & Schepker, 1994) and non-medicated first-episode patients compared to controls (Strous et al., 2004). Other studies describe normal DHEA-S circulating levels in medicated patients (Shirayama, Hashimoto, Suzuki, & Higuchi, 2002). Hence, it is possible that antipsychotic drugs normalize DHEA-S blood levels of schizophrenic subjects. It has also been suggested that increased plasmatic ALLO may be associated with aggression and hostility in individuals with schizophrenia (Spalletta et al., 2005). However, in human post-mortem studies, ALLO levels tend to decrease in parietal cortex of patients with schizophrenia compared to healthy subjects (Marx et al., 2006), while PREG and DHEA are both in higher amounts in the posterior cingulate and parietal cortex (Marx et al., 2006). In this case, it can be proposed that abnormalities in central neurosteroidogenesis lead preferentially to the production of DHEA rather than ALLO. As mentioned in Section 2.2.2., DHEA inhibits GABA_A receptors whereas ALLO potentiates them. Hence, changes in their brain levels could explain the final lack in GABAergic functioning reported in schizophrenia (MacKenzie, Odontiadis, Le Mellédo, Prior, & Baker, 2007).

Since a raise in DHEA amounts decreases negative symptoms, anxiety, and depressive symptoms in patients with schizophrenia (Strous et al., 2003), it is possible that high levels of neurosteroids PREG and DHEA found in post-mortem studies are due to medication of patients,

or to compensatory mechanisms in an attempt to decrease negative symptoms. Neurosteroid levels are probably regulated by neuroleptic medication, since both clozapine and olanzapine dose-dependently elevate the neuroactive steroid ALLO in rodent brain (Barbaccia et al., 2001; Marx, Duncan, Gilmore, Lieberman, & Morrow, 2000; Marx, VanDoren, Duncan, Lieberman, & Leslie Morrow, 2003). This could constitute a re-balancing mechanism toward physiological neurosteroid levels. Furthermore, it is noteworthy that schizophrenic patients can develop several comorbidities including anxiety, which could be sustained by neurosteroid levels. Indeed, since DHEA is able to activate NMDA receptors and to inhibit GABA_A receptors, higher DHEA levels in comorbid individuals likely render them more susceptible to anxiety than individuals with schizophrenia alone (Shulman & Tibbo, 2005).

Preclinical findings show that chronic treatments with PREG or PREG-S rescues schizophrenia-like behavior in a mouse model of the disease (Table 4) (Wong et al., 2012; Wong, Sze, Chang, Lee, & Zhang, 2015). To exert its actions, PREG-S acts through the Akt/Glycogen Synthase Kinase 3 β pathway, which is deeply involved in neuroplasticity, whereas PREG modulates it only slightly (Wong et al., 2015).

Clinical studies reported that DHEA has therapeutic potential in schizophrenia: higher DHEA levels were significantly correlated with lower symptoms and enhanced memory (Harris, Wolkowitz, & Reus, 2001; Strous et al., 2007), lower extrapyramidal symptoms in antipsychotic treated schizophrenia patients (Nachshoni et al., 2005), and improvement in negative, depressive and anxiety symptoms (Strous et al., 2003). Several mechanisms can be proposed regarding such effects, even if it remains an unsolved question. DHEA may exert its actions via the cytoskeleton, since it binds to MAP2. It is also possible that DHEA is converted into metabolites that eventually mediate its therapeutic effects. More recently, clinical studies in schizophrenic patients show that it could reduce negative symptoms (Ritsner, Bawakny, & Kreinin, 2014), ameliorate cognitive deficits in recent-onset schizophrenia (Kreinin, Bawakny, & Ritsner, 2014) and improve functional capacities (Marx et al., 2014) (Table 4). In the latter study, increase in both PREG and ALLO serum levels were reported after PREG administration, suggesting that PREG therapeutic effect observed in human could be mediated by its metabolites, at least partly. Hence, it cannot be excluded that combined mechanisms in the brain involving modulation of the microtubular system by PREG associated with activation of GABAergic neurotransmission by ALLO could explain the influence of these neurosteroids in schizophrenia.

3.2.3. Neurosteroids and stress-related disorders

Many of depressive and anxiety disorders are partly due to chronic stress exposure, leading to changes in brain structure and function. In patients suffering from depressive disorders, hippocampal volume is reduced, due to neuronal atrophy including shrinkage of dendritic

arborization and reduction of dendritic spine integrity (Dias et al., 2014; Duman & Duman, 2015; Leuner & Shors, 2013). In addition, a decrease in hippocampal neurogenesis has also been suspected in depressive patients, leading to mood dysregulation and cognitive impairments, including memory deficits (Drew & Hen, 2007). In rodents, chronic stress triggering a depressive-like phenotype produces dendritic atrophy in CA3 region and frontal cortex, two brain regions deeply involved in cognitive functions (Cook & Wellman, 2004; Watanabe, Gould, & McEwen, 1992). It also prolongs the elevation of glucocorticosteroid amounts that can result in cell death (Sapolsky, Krey, & McEwen, 1985). In addition, in chronically stressed rodents, an increase in LTD in the hippocampus, and a decrease in LTP in both the hippocampus and the frontal cortex are reported (Kim & Diamond, 2002).

Anxiety disorders are characterized by excessive fear, typically in response to specific objects or situations that do not pose or predict any danger (Shin & Liberzon, 2010). This excessive fear is partly the consequence of disrupted neuroplasticity in amygdala. Neurons in this region exhibit a large number of glucocorticosteroid receptors, meaning that they are highly sensitive to raising hormone levels occurring in stress response (Mitra & Sapolsky, 2008). In addition, patients with anxiety disorders display abnormal activation of the PFC and amygdala, and decreased functional connectivity between these areas (Hilbert, Lueken, & Beesdo-Baum, 2014). Evidences suggest that glutamatergic neurotransmission plays a critical role in the pathogenesis of anxiety disorders. Indeed, glutamate receptor activation affects both hippocampal-dependent associative learning and amygdala-dependent emotional processing during and after a stressful event (Bermudo-Soriano, Perez-Rodriguez, Vaquero-Lorenzo, & Baca-Garcia, 2012). In addition, it has been evidenced in humans with anxiety disorders that GABA_A receptor functions decline in the amygdala and the hippocampus (Möhler, 2012). In rodents, chronic stress provokes enhanced dendritic arborization together with an increased volume in the amygdala, and a dendritic atrophy and debranching in CA3 pyramidal neurons of the hippocampus (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002).

Neurosteroids levels in plasma or CSF of patients affected by anxiety disorders are found either lower or higher when compared to healthy subjects, while all studies available in depressed patients exhibit decreased neurosteroid levels (Longone et al., 2008). Indeed, PREG levels decrease in plasma or CSF of patients suffering from unipolar depression or during the depressive phase of bipolar depression (George et al., 1994; Rupprecht et al., 1998). However, in post mortem analyses, PREG amounts seem to be slightly increased in the cerebral cortex of depressed patients (Marx et al., 2006). It is possible that this observation reflects a compensatory upregulation as a consequence to the pathology, although some confounding factors including uncontrolled medication status at the time of the death might limit the interpretations of these data. DHEA and DHEA-S are also in lower amounts in the plasma of depressed patients suffering from chronic fatigue syndrome (Pluchino et al., 2015). It is noteworthy that circulating levels of these neurosteroids in major depression remitter patients are found in higher concentrations both before and after antidepressant pharmacotherapy compared to non-remitters patients (Hough et al., 2017). DHEA and DHEA-S contents in the blood may constitute a marker for prediction of major depression remission. Nevertheless, it is unclear whether or not these blood levels could reflect brain levels of neurosteroids in pathological conditions. Similarly, whether the lack of these neurosteroids measured in plasma or CSF of patients is only due to a decrease in their precursor PREG, or to a general reduction in steroidogenic enzymes activity is not established yet.

Circulating PREG-S, DHEA-S and ALLO are also lower in patients with anxiety disorders compared with control subjects (Heydari & Le Mellédo, 2002; Semeniuk, Jhangri, & Le Mellédo, 2001). However, no data is available regarding their brain levels in this disease. Because of the correlation between ALLO brain levels and stress-stimulated hypothalamo-pituitary-adrenocortical axis activity (Patchev, Hassan, Holsboer, & Almeida, 1996), it was proposed that ALLO could modulate

this axis function (Biggio, Pisu, Biggio, & Serra, 2014). Acute stress results in both 5 α -R up-regulation and rapid increase of ALLO, reaching levels known to modulate GABA_A receptor activity (Purdy, Morrow, Moore, & Paul, 1991). However, 5 α -R and ALLO levels are shown to be decreased under chronic stress in rodent, such as social isolation (Bali & Jaggi, 2014). This could be reconciled with the low ALLO concentration found in CSF of patients affected by unipolar depression (Zorumski, Paul, Izumi, Covey, & Mennerick, 2013). Consistently, fluoxetine, a selective serotonin reuptake inhibitor, increases ALLO amounts in female rat brain (Fry et al., 2014; Pinna, Costa, & Guidotti, 2004; Pinna, Dong, Matsumoto, Costa, & Guidotti, 2003; Uzunov, Cooper, Costa, & Guidotti, 1996), and the anxiolytic compound etifoxine also enhances PREG and ALLO concentrations in the rat brain (Do Rego et al., 2015; Verleye et al., 2005), further suggesting that antidepressant and anxiolytic compounds could modulate neurosteroid levels to exert their therapeutic action. Interestingly, in preclinical studies, neurosteroids PREG, PREG-S, DHEA, DHEA-S and ALLO display antidepressant- and/or anxiolytic-like properties (Engin & Treit, 2007; Fleshner, Pugh, Tremblay, & Rudy, 1997; Genud et al., 2009; Khisti, Chopde, & Jain, 2000; Melchior & Ritzmann, 1994a; Melchior & Ritzmann, 1994b; Reddy, Kaur, & Kulkarni, 1998) (Table 4). 3 β -methoxyprogesterone, a PREG derivative, seems to exert fast-acting and prolonged antidepressant-like property in the rat subjected to social isolation after weaning (Bianchi & Baulieu, 2012). In another model of depression, 3 β -methoxyprogesterone seems to prevent stress-triggered avoidance behavior, and also to normalize transversal parameters related to depression, including hormone hypersecretion, hypothermia and sleep disturbances (Parésys et al., 2016).

PREG-S, on the other hand, has dual effects in animal models of anxiety disorders: it is anxiolytic-like at low doses (0.1 μ g/kg) and anxiogenic-like at higher doses (10 μ g/kg) (Melchior & Ritzmann, 1994b) (Table 4). At low concentrations, PREG-S preferentially inhibits GABA_A receptors, which is not consistent with classical anxiolytic-like compounds that usually enhance GABA_A receptor activity. At higher concentrations, PREG-S activates NMDA receptors, either indirectly through σ 1 receptor stimulation or by a direct binding, to produce anxiogenic effects. Other mechanisms of action may be involved to explain PREG-S anxiolytic-like effects at low concentrations.

Regarding DHEA antidepressant-like activity, several hypotheses have been proposed. DHEA efficacy could be mediated by androsterone, a DHEA metabolite which may potentiate GABAergic neurotransmission (Ben Dor, Marx, Shampine, Rubinow, & Schmidt, 2015; Gartside, Griffith, Kaura, & Ingram, 2010; Genud et al., 2009). On the other hand, DHEA, similarly to PREG, modulates microtubule cytoskeleton by binding to MAP2. Since PREG actions on microtubule cytoskeleton may be responsible for its antidepressant-like activity, it is probable that a similar mechanism could occur for DHEA. DHEA anxiolytic-like effects could also be sustained by its capacity to modulate functional plasticity. Indeed, it reduces the amygdala activity, the connectivity between the amygdala and the peri-amygdala and between the amygdala and the insula, and increases the activity in the medial PFC (Sripada et al., 2013; Sripada, Welsh, Marx, & Liberzon, 2014) (Table 4).

ALLO infused into the hippocampus or the amygdala produces antidepressant-like effects (Shirayama et al., 2011) (Table 4). ALLO displays also anxiolytic-like effects when infused into amygdala, where it may increase tonic GABAergic inhibition (Engin & Treit, 2007; Romo-Parra, Blaesse, Sosulina, & Pape, 2015). In humans, ALLO elevation is associated with an enhanced activation of neurocircuits regulating emotions, themselves largely implicated in stress-related disorders (Sripada et al., 2013).

Finally, in clinical studies, PREG and DHEA display antidepressant activity in depressed patients with major depression (Wolkowitz et al., 1995; Wolkowitz et al., 1997), in depressive phase of bipolar patients (Brown et al., 2014), and in patient affected with midlife-onset major and minor depression (Schmidt, Daly, Bloch, Smith, et al., 2005) (Table 4).

3.2.4. Neurosteroids and epilepsy

Epilepsy is characterized by recurrent, unprovoked epileptic seizures, caused by excessive synchronous discharge of neurons in the brain, mostly due to an imbalance between glutamatergic and GABAergic neurotransmissions. In temporal lobe epilepsy, during a process called reactive plasticity, the structure of the hippocampus changes in response to excessive excitation and not enough inhibition of networks, particularly in dentate granule cells (Kobayashi & Buckmaster, 2003). Whereas some neurons die, including mossy cells and GABAergic interneurons, other modify their connectivity leading to aberrant connections (Artinian, Peret, Mircheva, Marti, & Crépel, 2015). This phenomenon could reflect a compensatory mechanism which is not sufficient to rescue memory impairments in temporal lobe epilepsy (Zenonos & Richardson, 2014). In addition, alterations in extra-synaptic δ subunit-containing GABA_A receptors in principal cells and interneurons could contribute to increased seizure susceptibility in hippocampus of a temporal lobe epilepsy model (Peng, Huang, Stell, Mody, & Houser, 2004).

To date, no clearly established data are available regarding measurements of neurosteroid levels in epileptic brain, under medication or not. However, anti-epileptic treatments can modify neurosteroid levels in blood, since women with frequent seizures and treated with anti-epileptic drugs display a decrease in serum DHEA-S levels (Galimberti et al., 2005; Löfgren, Tapanainen, Koivunen, Pakarinen, & Isojärvi, 2006). Nevertheless, since DHEA-S cannot easily cross the blood-brain barrier, it is possible that these circulating levels do not reflect those in the brain.

PREG-S and DHEA-S have been characterized as pro-convulsant drugs, whereas ALLO displays anti-convulsant properties (Table 4) (Kokate et al., 1999; Reddy, 2013). As mentioned above, sulfated neurosteroids are modulators of GABA_A and ionotropic glutamate receptors. Hence, their pro-epileptic effects could be the consequence of an inhibition of the GABAergic neurotransmission together with a potentiation of the glutamatergic neurotransmission (Williamson et al., 2004). DHEA displays anti-epileptic activity as it suppresses iron-induced experimental seizures, but the mechanism of this action is not established yet (Mishra, Singh, & Sharma, 2010). In theory, σ 1 receptor activation and GABA_A receptor inhibition by DHEA would lead to pro-convulsant effects, suggesting that its anti-epileptic actions could be explained by another mechanism of action, possibly implying MAP2 binding and cytoskeleton modulation.

The anti-convulsant action of ALLO seems to be mediated by enhancing inhibitory transmission through synaptic and extrasynaptic GABA_A receptor modulation (Kokate, Cohen, Karp, & Rogawski, 1996). In epileptic animal models, GABA_A receptors mediating tonic and synaptic inhibition become less sensitive to neurosteroid modulation than in controls (Sun, Mtchedlishvili, Erisir, & Kapur, 2007) (Table 4). Interestingly, Ganaxolone, a synthetic ALLO derivative, is a positive allosteric modulator of the GABA_A receptor which provides protection in a wide variety of induced seizure animal models (Beekman et al., 1998; Carter et al., 1997; Gasior, Carter, Goldberg, & Witkin, 1997), and in patients (Laxer et al., 2000; Pieribone et al., 2007) (Table 4).

Together, the data discussed here show that in AD, stress-related disorders, schizophrenia and epilepsy, both neuroplasticity and neurosteroid levels are altered. In turn, neurosteroid administration in those pathological conditions may have beneficial effects.

4. Conclusions

This review attempted to better characterize non-genomic effects of neurosteroids on neuroplasticity and their involvement in pathophysiology of neurological diseases. PREG, PREG-S, DHEA, DHEA-S and ALLO exert their actions through modulation of amino acid receptors and/or microtubule-associated proteins, which undoubtedly regulate neuroplasticity. It seems that PREG and DHEA preferentially act on the microtubular system to regulate structural plasticity, whereas PREG-S,

DHEA-S and ALLO preferentially act directly or not via ionotropic glutamate receptors to modulate functional plasticity.

Although these non-genomic actions are quite well described in the literature, several questions remain unanswered so far and need further investigations. Among them, brain levels of steroids are not established and require techniques that are difficult to set up. In addition, the stability of neurosteroids versus their respective metabolites under both physiological and pathological conditions are also unclear. Then, the intracellular pathways elicited by these neurosteroids are poorly described to date. Cytoskeleton modulation by PREG, DHEA, and perhaps other neurosteroids, could indeed constitute an innovative mode of action that could be involved in pathophysiological conditions. Investigations regarding other MAPs as receptor for neurosteroids need also to be conducted.

As highlighted in this review, whether cytoskeleton modulation could account for understanding the link between neuroplasticity alterations and neurosteroid level impairments in neurological diseases, should also be addressed. Because of their strong impact on neuroplasticity, this could explain why neurosteroids specifically studied in this review have potential therapeutic effects and could act as new candidates for treatment of some neurological diseases for which to date, no treatment is fully satisfactory

Conflict of interest statement

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Research article 1

Antidepressant compounds currently prescribed in clinic display significant limitations since about a third of patients do not achieve remission. In addition, cognition is still impaired in patients responding to pharmacotherapy, meaning that these molecules do not completely cure depressed subjects.

MAP4343 is a pregnenolone derivative that was previously shown to exert antidepressant-like activity in animal models of depression. The starting point of the present thesis was to investigate the behavioral outcomes of chronic administration of MAP4343. Using two animal models, its antidepressant-like activity was confirmed and further investigated through the cognitive performances assessment. We first selected the transgenic mice GRi as a model of depression, since it displays alterations in the stress response. The depressive-like behavior of these animals was measured using the Splash test, as previously published by our lab. Unfortunately, in our experiments, GRi mice did not exhibit any cognitive deficits. Hence, to evaluate the effect of MAP4343 on cognition, we used another mouse model of depression, the chronic social defeat stress that leads to memory impairments. The depressive-like behavior is classically assessed in this model using the social avoidance test. The novel object recognition test was used in this study to evaluate the cognitive performances because it was shown to test a type of memory that is hippocampus-dependent. We also used the Y-maze to test the working memory of mice, but the results are not presented here because all animals failed to exhibit any deficit.

Since cognition is sustained by neuroplasticity processes, the resultant of repeated administration of MAP4343 on several plasticity-related features were studied *in vivo*. We aimed to evaluate the effect of the chronic treatment on neuronal proliferation and on plasticity-related gene expression in the hippocampus of GRi mice. These mice are quite difficult to breed, and there were only few animals that were available for our study. Hence, because of this constraint, we had to test each animal for both kind of experiments. Mice were sacrificed and each hemisphere were used for either Ki-67 immunostaining, or PCR analyses.

This research article will be soon submitted to peer-reviewing, for publication.

MAP4343 antidepressant-like effects in mice: insights from genetic and environmental models of depression

Marina Schverer, Kaamula Smith, Isabelle Villey, Etienne-Emile Baulieu, Nicolas Froger, and Laurence Lanfumey

Abstract

Although current antidepressants can restore mood and normalize hypothalamic-pituitary-adrenocortical axis activity in about 40% of patients, none of them could efficiently alleviate cognitive deficits associated to depression, which are still present in remitted patients. MAP4343 is a new pharmacological compound that displayed a therapeutic-like activity in stress-related behaviors in two animal models. The present study aimed to further characterize the antidepressant-like effects of MAP4343, particularly on cognition and hypothalamic-pituitary-adrenocortical axis. To this end, we used two mouse models in which we investigated the effect of MAP4343 on stress-related behavior, memory, neuroplasticity and HPA axis activity.

We confirmed the therapeutic-like activity of the molecule, showing that it could be specific to depressive-like states. In addition, chronic MAP4343 treatment reversed stress-induced memory deficits, and promoted performances of unstressed mice. Cell proliferation and plasticity-related genes expression, including *MAP2* and *Bdnf*, were measured in the hippocampus, and found potentiated by repeated administration of MAP4343. Finally, these results could be associated with HPA axis modulation.

To conclude, we showed for the first time that MAP4343 exhibited procognitive activity based on neuroplasticity enhancement. Hence, it could be considered as a serious candidate for depression pharmacological treatment.

Introduction

Depression is a mental illness characterized by various symptoms, including dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis and alterations in cognitive functions. The HPA axis, which involves both neuroendocrine structures such as the hypothalamus, the pituitary and adrenal glands, and brain areas including the hippocampus and the prefrontal cortex, is a major actor for regulating energy homeostasis (see (Herman et al., 2016) for a review) via the release of glucocorticoids, corticosterone in rodents or cortisol in human. In addition, it is also mainly involved in the stress response (Lupien et al., 2009), and can be altered in disease. For example, in major depression, the negative feedback governed by the glucocorticoids on the glucocorticoid receptor (GR) is impaired, leading to hyperactivation of the HPA axis (Burke et al., 2005; Merali et al., 2004; Raadsheer et al., 1994) that results in a maladaptive response to stress.

Stress has also been shown to decrease the brain-derived neurotrophic factor (BDNF) levels in brain in several stress related models (Levy et al., 2018), and the cellular mechanisms by which GR activity could repress BDNF expression has been recently demonstrated in neuron-like cells (Chen et al., 2017). BDNF is a protein that mediates its actions mainly through the tropomyosin-related kinase receptor B (TrkB). BDNF and TrkB levels are correlated with the expression of synaptic proteins and glutamate receptors (Duric et al., 2013; Law & Deakin, 2001), which determines the strength of the synapse, and therefore the functional integrity of neuronal networks. Hence, BDNF/TrkB signaling was proposed to have a critical role in neuroplasticity processes (Levy et al., 2018), especially in response to environment challenges, that triggers both structural and functional adaptive changes in several brain regions. In depressed patients, low BDNF and TrkB expression has been found in the hippocampus (Dwivedi et al., 2003; Pandey et al., 2008). This was proposed to be related to neuronal network alterations, leading to cell death and dendritic shrinkage, which could explain, at least in part, the reduction of the volume of the hippocampus reported in this disease (Rajkowska et al., 1999; Sheline et al., 1996). On the other hand, adult hippocampal neurogenesis alteration has been consistently reported in rodent model of depression (Drew & Hen, 2007). Neurogenesis is the formation of new neurons that can occur in

adulthood in restricted brain areas including the dentate *gyrus* of the hippocampus (Drew & Hen, 2007), which requires neuroplasticity integrity. This process, among others, is suspected to significantly participate to the correct execution of cognitive functions (Zunszain et al., 2011). Therefore, disrupted neuroplasticity could be the cause of cognitive deficits, including memory loss and altered attentional processes, described in patients suffering from depression (Roca, Vives, Lopez-Navarro, Garcia-Campayo, & Gili, 2015). Although current antidepressants are able to restore mood and normalize HPA axis activity in about 40% of depressive patients, none of them could efficiently cure the cognitive deficits related to this pathology, which are still present in remitted patients (Soria et al., 2018). Hence, new approaches are needed to treat these symptoms. Recently, the pregnenolone derivative MAP4343 has demonstrated antidepressant-like properties in several animal models (Bianchi & Baulieu, 2012; Parésys et al., 2016). Interestingly, this molecule could potentiate neuroplasticity. MAP4343 was shown to bind to microtubule-associated protein 2 (MAP2), and to stimulate MAP2 ability to interact and stabilize microtubules (Fontaine-Lenoir et al., 2006). This could be involved in its effect on the locomotor recovery after spinal cord injury in rats, possibly by promoting the expression of MAP2 and the total dendritic arbor of lumbar spinal cord motoneurons in injured rats (Duchossoy et al., 2011).

In order to further characterize MAP4343 therapeutic-like effects in relation with the cognitive deficits induced by depressive disorders, we hypothesized that its antidepressant-like activity could be related to its ability to modulate neuroplasticity through microtubule cytoskeleton. We therefore investigated the effects of chronic administration of MAP4343 on cognition, neuroplasticity and stress axis in two animal models of depressive-like disorders. The first one was the chronic stress induced by social defeat (CSDS) in mice, leading to anxiety- and depressive-like behavior, together with memory impairments (Martin et al., 2017). We also studied MAP4343 actions in a transgenic mouse model of reduced GR-induced negative feedback regulation of the HPA axis, the glucocorticoid receptor-impaired (GRi) mice (Pepin et al., 1992). We further demonstrated the antidepressant-like activity of MAP4343 in these mice models, and showed that it also reversed cognitive deficits in the CSDS model, probably via its action on neuroplasticity.

Material and methods

Animals. Adult male C57BL/6J mice (7 week-old; Janvier Labs, France) were housed 4 per cage for one week before starting experiments. CD-1 retired breeder male mice (12 month-old; Janvier Labs, France) were isolated before screening. Transgenic mice (strain B6C3F1; line 1.3) bearing a transgene for the glucocorticoid receptor (GR α) and paired wild-type (WT) mice were bred at Inserm UMR894 animal facility. Mice issued from the same gestation were housed in the same cage. During all experiments, mice were kept under standard laboratory conditions ($22 \pm 1^\circ\text{C}$, 60% humidity, 12-h light-dark cycle with lights on at 07:00, food and water ad libitum). All procedures concerning animal care and treatment were performed by observers who were blind to experimental conditions and were carried out in accordance with the protocols approved by the Paris Descartes ethical committee for the use of experimental animals, licensed by the Directorate General for Research and Innovation (French Ministère de l'Enseignement Supérieur et de la Recherche), under protocol authorization #15357. Animal studies are reported in compliance with the ARRIVE guidelines (Drummond, Paterson, & McGrath, 2010; McGrath & Lilley, 2015).

Chronic social defeat stress. Chronic social defeat stress (CSDS) was performed as previously described (Martin et al., 2017). Male CD-1 mice were screened for aggressive behavioral response, evaluated by measuring the latency to the first aggression that should be less than 60 seconds in at least two consecutive 180 second-screening sessions. Once selected, CD-1 mice were singly housed in one side of a standard cage separated by a clear perforated Plexiglas divider, which allowed only sensory contacts. The day after, a C57BL/6J intruder mouse was exposed to a CD-1 aggressor for 10 min, during which the intruder mouse was attacked and displayed subordinate posturing. To avoid physical injury, mice were briefly isolated in case of over-aggressive behavior from the CD-1. At the end of the session, the Plexiglas divider was placed back, separating the C57BL/6J and the CD-1 mouse. This procedure was repeated for 10 consecutive days (Figure 1) between 10:00 and 11:00 am, using a different CD-1 mouse aggressor each day. Control mice were housed by pair in a similar cage, separated by a similar clear Plexiglas divider, in another housing

room. Immediately after the last defeat session, stressed and control mice were singly housed in a new standard cage.

Dosage of plasmatic MAP4343. To validate the *per os* administration route of MAP4343 as described by Corbett et al. based on voluntary oral administration (Corbett, MCGowin, Sieber, Flannery, & Sibbitt, 2012), the dosage of plasmatic levels of MAP4343 was performed after either gavage or cookie intake. Briefly, WT mice received a fixed dose of MAP4343 (150 mg/kg) either under classical gavage protocol, in 100 μ L, or in a small cookie weighing around 150 mg (Biscuits à la cuillère, Carrefour, France), filled by 100 μ L MAP4343 and 2, 4 or 24 hours after, they were sacrificed by cervical dislocation and trunk blood was collected into tubes containing heparin, centrifuged at 5000 rpm for 10 min at 4°C to obtain plasma. All tissue and plasma samples were stored at -80 °C until use. The dosage was done using a liquid chromatography/mass spectrometry assay.

Treatments. To assess the effect of chronic MAP4343 administration (22 days) on stressed or control mice, each group of the CSDS model was divided into two: mice treated with MAP4343 (150 mg/kg/day, *per os*), and those treated with the vehicle (hydroxyethylcellulose, HEC 0.5%). GRi mice were either treated with vehicle (HEC 0.5%) or MAP4343 (150 mg/kg/day, *per os*), whereas WT mice received HEC administration (15 days). In both models, treatment was administrated at 6 pm, via a cookie weighing around 150 mg (Biscuits à la cuillère, Carrefour, France), filled by 100 μ L HEC or MAP4343 (37,5 mg/mL). The intake was directly monitored by the experimenter. The latency to the cookie intake was 5-15 seconds.

At the end of the experiments, animals were sacrificed by cervical dislocation, and the brain was quickly dissected and frozen in liquid nitrogen. For GRi mice and their paired WT, half of the brain was rapidly frozen into isopentane at -30°C, and the other half was dissected and frozen in liquid nitrogen. Plasma was also sampled from mice of the CSDS model. Trunk blood was collected as described above.

Behavioral testing. The behavioral tests were performed under low light conditions (10 lux). Mice were placed in the behavior room 1 hour before the beginning of each test for acclimatization.

Open-field test. In both CSDS and GRi models, anxiety-like behavior was assessed using the open-field test (OFT). In CSDS mice, the apparatus consisted in a white open area (50 x 50 cm). The test lasted 1 hour and mice were tested at D11 and D27 (Figure 1A). WT and GRi mice were tested in a black open field (30 x 30 cm) for 15 minutes at D10. At the beginning of the test, mice were placed at one angle of the open field, and were free to explore the entire area. The time spent and the number of entries in a virtual region designated as the central zone were measured, using Viewpoint software (Civrioux, France).

Social avoidance. To evaluate their depressive-like phenotype, the social skills of CSDS mice were assessed using the social avoidance test, as previously described (Martin et al., 2017). Mice were tested in two successive sessions lasting 150 seconds, in the white open field (50 x 50 cm) used for the OFT. During the first session, mice were in presence of an empty circular wire cage (18 × 9 cm), located against a wall of the area. Then, the empty circular wire cage was replaced by another containing an unknown CD-1 aggressive mouse (called as “target”). The time spent in a virtual interaction zone around the circular cage was scored manually using XNote StopWatch software, allowing the calculation of the interaction index as follow: $(\text{time spent in the interaction zone with the target}) / (\text{time spent in the interaction zone without the target}) \times 100$. As previously described (Krishnan et al., 2007), stressed mice were classified as resilient or susceptible when their interaction ratio was higher or lower than 100, respectively. Only susceptible animals were kept for behavioral and molecular analyses. The social avoidance test was performed both before and after MAP4343 chronic treatment (D12 and D28; Figure 1A).

Novel object recognition. The recognition memory of mice from the CSDS model was assessed using the novel object recognition (NOR) test, performed as previously described (Martin et al., 2017). For this test, a black open field (30 × 30 cm) and three different objects (red plastic cylinders, transparent plastic flasks and transparent glass

bottles) were used. In the morning (9:00 am) of D29 (Figure 1A), mice were free to explore the open field for 15 minutes. In the afternoon (2:00 pm), mice were placed in the presence of two identical objects, for the acquisition phase. 1 hour later (intertrial interval (ITI) 1h), one of the two objects was randomly replaced by another one unknown from the animals. The procedure was replicated at D30 (Figure 1A), 24 hours after the acquisition phase (ITI 24h), with a third different object. The time mice spent exploring the objects was manually scored from recorded video using XNote StopWatch software, allowing the calculation of the recognition index as follow: (exploration time of the novel object – exploration time of the familiar object)/(total exploration time). Mice that explored both objects less than 5 seconds were excluded.

Splash test. The depressive-like behavior of GRi mice was assessed using the splash test, consisting in squirting a 10% sucrose solution on the dorsal region of mice in their home cage, and inducing a grooming behavior. The total time spent grooming was manually scored from recorded video using XNote StopWatch software.

Immunohistochemistry. The effect of MAP4343 on hippocampal cell proliferation was assessed using the Ki67 marker in GRi mice. Hippocampal coronal sections of 20 μm (between bregma levels -1.340 and -2.300) were collected on double dipped gelatin coated glass slides, using a cryostat (Leica CM 3050, Leica Microsystems, Wetzlar, Germany), and stored at -20°C until use. Then, sections were fixed with 4% paraformaldehyde at 4°C for 10 minutes, permeabilized with Tris-buffered saline-triton 0.05%, and incubated with Ki67 antibody (1:1000, Vector Laboratories, Burlingame, Calif., USA) overnight at 4°C . Sections were then incubated with biotinylated secondary antibodies (2 hours at room temperature), and the reaction was amplified using the ABC staining system (1 hour at room temperature; Vector Laboratories, Burlingame, Calif., USA). Sections were treated with 3,30-diamino-benzidine for a few minutes, dehydrated and mounted with cover slip. The positive cells in the entire dentate *gyrus* were counted under a microscope (Nikon Eclipse E400, Nikon Instruments Inc., Shanghai, China) using Mercator software (Mercator, Explora Nova, France).

Quantitative reverse transcriptase PCR (RT-qPCR). Total mRNA extraction in the whole hippocampus, frontal cortex and hypothalamus was performed using TRI Reagent Solution (Life Technologies, Saint Aubin, France) following manufacturer's instructions. Then, cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtaboeuf, France). The following cycling protocol was applied: 25°C for 10 minutes, 37°C for 2 hours and 85°C for 5 seconds. The amplification was made with KAPA SYBR FAST qPCR Master Mix (Clinisciences, Nanterre, France), using the 7300 Real Time PCR System (Applied Biosystems, Courtaboeuf, France), with the cycling protocol: 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. *Gapdh*, a gene encoding for glyceraldehyde-3-phosphate dehydrogenase, was used as housekeeping gene for gene expression normalization. The $2^{\Delta\Delta CT}$ (Delta–Delta Comparative Threshold) method was used to normalize the fold change in mRNA expressions. The primer sequences used in this study are reported in the Table 1.

Corticosterone measurement. Plasma from CSDS mice was collected in the morning of D32 (Figure 1A) between 7:30 and 10:00 am (about 30 hours after the last treatment). Plasmatic corticosterone levels were measured using a specific enzyme-linked immunosorbent assay (ab108821; Abcam, Cambridge, UK), following the manufacturer's instructions. The standard curve (0-100 ng/ml) and samples were run in duplicate.

Statistical analyses. For all experiments, statistical analyses were performed using Prism 7 software (GraphPad, San Diego, USA). The normal distribution of data (displayed as mean \pm S.E.M.) was tested by means of Shapiro-Wilk normality test and KS normality test. To compare two groups, an unpaired Student t-test with equal standard deviation was performed. Data from behavioral studies in CSDS mice were analyzed using a one-way and 2 way ANOVA respectively, both followed by Fisher's LSD multiple comparison test was used to compare all groups.

Results

Characterization of anxiety- and depressive-like behavior of defeated and GRi mice

Chronic stressed C57BL/6J mice were tested in the OFT 24 h after the last CSDS session, at D11. Stressed mice displayed significant decreased time (95.02 ± 4.29 versus 122.70 ± 6.25 seconds; $p=0.0004$) and entries (119.50 ± 7.45 versus 179.10 ± 10.76 ; $p<0.0001$) in the central zone, compared to control mice (Figure 1A), evidencing an anxiety-like phenotype in defeated animals. The social avoidance performed at day 12 revealed that 21 stressed mice, called “susceptible”, had a significant lower interaction ratio than controls ($p=0.0003$). That of other 14 stressed mice (called “resilient”) was significant higher than the interaction ratio of susceptible mice ($p<0.0001$) (Figure 1A).

Transgenic GRi mice displayed spontaneous anxiety- and depressive-like phenotype. GRi mice spent less time (20.25 ± 1.87 versus 33.93 ± 2.63 seconds; $p=0.0004$) in the OFT and entered less often (37.09 ± 3.02 versus 61.17 ± 4.63 ; $p=0.0003$) in the central zone than WT animals (Figure 1B). In the splash test, these animals had a grooming duration higher than WT mice (119.80 ± 18.17 versus 80.80 ± 5.90 seconds; $p=0.0649$) (Figure 1B).

Validation of the administration mode

MAP4343 plasmatic concentration following oral administration was assessed to compare it to classical gavage route drug administration. We found no difference in concentrations between animals treated via gavage or via cookie (Figure 1C). 2 hours after treatment, these levels were around 100 ng/mL. They declined under the limit of detection (fixed at 2.50 ng/mL) after 24 hours (Figure 1C), and 2 way ANOVA revealed a significant effect of the time ($p<0.0001$).

Effects of chronic MAP4343 administration in anxiety- and depressive-like behavior of defeated and GRi mice

The anxiety-like behavior of mice from both models was evaluated in the OFT. When tested at D27, the susceptible CSDS mice treated with HEC displayed no difference

with the control HEC mice regarding the time spent in the central zone ($111,40 \pm 13.55$ versus $116,40 \pm 11.29$ seconds) and the number of entries (137.90 ± 16.89 versus 150.80 ± 10.41 ; Figure 2A), suggesting that the anxiety-like phenotype induced by CSDS was not anymore observed 16 days after the end of the CSDS protocol.

In the transgenic GRi mice, and as observed before the beginning of the treatment, all the parameters measured in the OFT were reduced in HEC treated GRi compared to HEC treated WT mice ($p < 0.0001$, one-way ANOVA,) (Figure 2B).

In both models, MAP4343 chronic administration had no effect on these variables (Figure 2A and B).

The effect of chronic MAP4343 treatment on depressive-like behavior was tested in CSDS mice using the social avoidance test. A 2 way ANOVA revealed a significant interaction between stress and treatment ($p = 0.0328$), although none of those variables had a significant effect alone. After a 17-day chronic treatment *per os*, defeated mice still displayed a depressive-like behavior and their interaction ratio were about 50% lower than that of control mice (40.01 ± 7.76 versus 89.80 ± 14.89 ; $p = 0.0550$). Chronic MAP4343 administration in susceptible mice reversed this phenotype (103.20 ± 19.57 versus 40.01 ± 7.76 ; $p = 0.0256$), whereas no change was observed in control non stressed mice (77.29 ± 13.72 ; Figure 2C).

In GRi mice, the depressive-like phenotype was assessed in the splash test, where one-way ANOVA revealed significant differences between groups ($p = 0.0346$). GRi mice spent more time to groom than WT (119.80 ± 18.17 versus 80.80 ± 5.90 seconds; $p = 0.0266$), and this was reversed by chronic MAP4343 administration (80.55 ± 5.13 ; $p = 0.0225$) (Figure 2D).

Effects of chronic MAP4343 on neuroplasticity in the hippocampus of GRi mice

The effect of chronic MAP4343 administration on neuroplasticity was assessed on hippocampal cell proliferation and BDNF expression in GRi mice, since previous studies demonstrated a deficit for both plasticity index in these mice compared to their WT paired control (Boulle et al., 2016; Paizanis et al., 2010). For neural cell proliferation, in both the ventral and dorsal regions of the dentate *gyrus*, the number of Ki67-positive cells (Figure 3A) was increased with MAP4343 treatment (ventral: 125.40

± 11.33 versus 93.67 ± 4.36 ; $p=0.0322$; dorsal: 142.60 ± 9.05 versus 113.60 ± 5.89 ; $p=0.0151$) (Figure 3B).

Neuroplasticity was also studied through measures of total *Bdnf* and *Bdnf* exon IV mRNA expressions in GRi mice. Total *Bdnf* amounts slightly increased in GRi mice treated chronically with MAP4343 but this was not statistically significant (Figure 3C).

Effects of CSDS and chronic MAP4343 administration in recognition memory of defeated mice

The short-term recognition memory was evaluated in the ITI 1h phase. A 2 way ANOVA revealed a significant effect of both stress ($p=0.0493$) and treatment ($p=0.0276$). Defeated HEC mice had a recognition index significantly lower than controls HEC (-0.13 ± 0.08 versus 0.07 ± 0.06 ; $p=0.0290$, Figure 4A). This effect was reversed by chronic administration of MAP4343 (0.09 ± 0.05 ; $p=0.0365$; Figure 4A). Control mice receiving HEC were not able to discriminate the novel object since the recognition index was not different from 0. When these mice received chronic MAP4343 administration, the recognition index was significantly higher than 0 (one sample t-test compared to 0; $p=0.0041$; Figure 4A).

The long-term recognition memory was assessed in the ITI 24h phase, where all mice were able to discriminate the novel object (one sample t-test compared to 0; $p<0.01$ for all groups). A 2 way ANOVA revealed a significant effect of the treatment ($p=0.0223$). Defeated HEC mice had lower memory performances than control HEC mice (0.17 ± 0.05 versus 0.35 ± 0.03 ; $p=0.0137$; Figure 4A). Chronic MAP4343 administration had no effect on unstressed animals, but significantly increased the recognition index of susceptible mice (0.39 ± 0.05 versus 0.17 ± 0.05 ; $p=0.0088$; Figure 4A).

Effects of chronic MAP4343 on neuroplasticity in the hippocampus of CSDS mice

In this model, neuroplasticity was assessed by measuring mRNA levels of total *Bdnf*, *Bdnf* exon IV and MAP2 in the hippocampus. These analyses were conducted in control mice, to evidence a possible proplastic effect of MAP4343 under physiological

conditions (Figure 4B); and in stressed animals, to possibly explain the therapeutic-like effect of MAP4343 on memory (Figure 4C). In control mice, MAP4343 significantly surged *Bdnf* exon IV mRNA expression (1.28 ± 0.09 versus 1.02 ± 0.05 ; $p=0.0290$) (Figure 4B), but no change was observed regarding total *Bdnf* and MAP2 expressions. In the stressed group, MAP4343 induced a surge in both *Bdnf* exon IV (1.42 ± 0.13 versus 1.04 ± 0.09 ; $p=0.0299$) and MAP2 (1.11 ± 0.03 versus 1.00 ± 0.03 ; $p=0.0408$) mRNA levels (Figure 4C).

Effects of chronic MAP4343 on stress axis of defeated mice

GR and CRH mRNA levels were measured in the hypothalamus of defeated mice. GR was unchanged with the stress or treatment. One-way ANOVA revealed differences between groups regarding CRH mRNA expression ($p=0.0362$), it was significantly increased with MAP4343 compared to HEC ($p=0.0122$) (Table 2).

The levels of basal corticosterone in plasma of defeated mice were measured to further elucidate the impact of MAP4343 chronic treatment on the stress axis (Table 3). A 2 way ANOVA showed no effect of stress or treatment on basal corticosterone levels.

Discussion

This study was aimed to better characterize the pro-cognitive and proplastic outcomes of the antidepressant-like effect of MAP4343 using two different mouse models of depression. We showed that the antidepressant-like activity of this molecule was accompanied by the reversal of stress-induced cognitive deficits. In addition, we provide evidences demonstrating that these therapeutic-like effects could be relied on neuroplasticity enhancement.

Antidepressant-like activity of repeated MAP4343 treatment

Both defeated and GRi mice displayed anxiety- and depressive-like behavior, as assessed by the behavioral tests carried out just after the CSDS sessions at D11 for stressed mice or in basal conditions for GRi mice. However, the anxiety-like phenotype of mice submitted to CSDS was no longer observed after a 16-day chronic treatment

with HEC, while this was the case in previous studies (Martin et al., 2017). Basal corticosterone levels were unchanged in our experiments, which is consistent with previous findings (Macedo et al., 2018). Indeed, social defeat was described as inducing a robust increase in corticosterone levels after the first session, and decreasing to reach the levels of unstressed animals 10 days after the last day of defeat (Macedo et al., 2018). Similarly, in our study, CSDS did not induce any change in the expression of GR in the hypothalamus, which was in accordance with a study where GR level was measured by *in situ* hybridization in the paraventricular nucleus of the hypothalamus, after a similar stress procedure (Wagner et al., 2011). Overall, our data on hypothalamic GR expression, corticosterone levels and probably anxiety-like behavior suggested an adaptation of the HPA axis after the treatment, which could be partially due to the method we used to administrate MAP4343 or HEC (cookies) to the mice. Indeed, in most of the studies that used this CSDS paradigm followed by chronic antidepressant treatment, drug administration was performed through intraperitoneal injections (Krishnan & Nestler, 2011; Martin et al., 2017), that may represent an additional chronic stress and constitute a negative reinforcement for the mice (Appleyard, Taylor, & Little, 1990). In contrast, we chose a technique based on voluntary intake by animals (Corbett et al., 2012). This treatment protocol probably tended to normalize the stress state of animals and to suppress the negative reinforcement of anxiety after CSDS. We then could not conclude on the potential anxiolytic properties of MAP4343 on this test. However, in the second model we used, the GRi mice that have not been stressed, MAP4343 chronic treatment was not able to reverse the anxious state of animals, as it was previously shown using other antidepressant, e.g., agomelatine (Boulle et al., 2016), allowing us to conclude toward no effect of this compound on anxious behaviors. In contrast, when assessed in the social avoidance test in CSDS mice, depressive-like behavior, which was still observed in mice after a HEC treatment through cookies, was reversed by chronic administration of MAP4343. The interaction ratio of MAP4343-treated defeat mice was not anymore different than that of control non stressed HEC-treated mice, supporting the antidepressant action of this molecule previously demonstrated in other rodent models, such as rats and tree shrews (Bianchi & Baulieu, 2012; Parésys et al., 2016). In GRi mice also, the abnormal behavior in the splash test observed in HEC treated mice was also reversed by chronic MAP4343 treatment, the grooming behavior of MAP4343-treated GRi mice being no longer different to that of HEC-treated WT mice. However,

it can be underline that in the present study, the phenotype of GRi mice in this test was opposite to that previously published (Boulle et al., 2016), where the authors found significant lower grooming duration in GRi compared to WT mice. Although the environmental conditions were slightly different in both cases, this led us to questioning the meaning of an evoked grooming in depressive-like mice, since data regarding this parameter are rather divergent in the literature. Indeed, chronic mild stress was shown to surge induced grooming in mice (Isingrini et al., 2010; Kompagne et al., 2008), which could be explained by an attempt to reduce stress (Smolinsky et al., 2009). Conversely, depressive-like behavior was often related to reduced grooming time (Shiota et al., 2016), that may reflect a despair behavior (Smolinsky et al., 2009). Although it is hence difficult to speculate regarding such complex behavior in regard to depressive like behavior in rodents, the important data we obtained here was that chronic MAP4343 could reverse the phenotype of GRi mice, and allowed them to behave as controls. Therefore, our data strongly suggested that MAP4343 could reverse a depressive-like phenotype in two different mouse models, an environmental and a genetic one, without acting on anxiety-related behaviors. Depression and anxiety are often associated because they are both stress-related disorders. In addition, it is well known that antidepressants belonging to the selective serotonin reuptake inhibitors family can also be used to treat anxiety disorders when administrated chronically (Kolcsar, Gáll, & Dogaru, 2014; Popovic, Vieta, Fornaro, & Perugi, 2015; Strawn, Welge, Wehry, Keeshin, & Rynn, 2015). Regarding the low rate of success of current antidepressants, MAP4343 could be an innovative strategy because of its potential selectivity for depressive states. It is noteworthy that we did not use any comparative drug, because Bianchi and Baulieu showed that MAP4343 therapeutic-like actions were more rapid and more persistent than that of fluoxetine, a common antidepressant molecule targeting the serotonergic system (Bianchi & Baulieu, 2012). Given the fact that MAP4343 do not act on this system (unpublished data from Mapreg), its mechanisms of action cannot be compared to any other current antidepressant.

Effects of repeated MAP4343 administration on hippocampal plasticity and cognition

Previous work in our group demonstrated that cell proliferation was reduced in the hippocampal dentate *gyrus* of GRi mice compared to WT (Boulle et al., 2016; Païzanis

et al., 2010). Here, we showed that chronic MAP4343 administration in GRi mice could increase this parameter in both the ventral and dorsal dentate *gyrus*. The hippocampus has been described as a functionally heterogeneous structure, with the dorsal part involved in learning and memory processes, and the ventral region regulating mood-related behaviors (Fanselow & Dong, 2010). In addition, it has been demonstrated that cell proliferation was necessary for antidepressant activity of fluoxetine, imipramine, desipramine and haloperidol, which are molecules that may enhance mood by acting on the monoaminergic system (Santarelli et al., 2003). Hence, the positive action of MAP4343 on cell proliferation in the ventral part of the hippocampal dentate *gyrus* could, at least partially, sustain its antidepressant-like action.

Because cognitive deficits are among the major symptoms reported in depressed patients (Roca et al., 2015), the antidepressant efficacy of a compound should encompass enhancement of cognition. Because of MAP4343 proplastic effects in the dorsal hippocampus of GRi mice, we hypothesized that this molecule could impact cognitive processes. Recognition memory of GRi was not different from that of WT mice (unpublished data from our lab), so we conducted investigations only in the CSDS model in which repeated stress was shown to induce memory deficits (Martin et al., 2017). First, we found that, when short-term memory was tested, only control mice receiving MAP4343 were able to discriminate the novel object. This came along with a PSD-95 in hippocampal *Bdnf* exon IV mRNA levels. Since this exon has been described as a key regulator of memory processes (Chhatwal, Stanek-Rattiner, Davis, & Ressler, 2006; Cunha et al., 2010), its upregulation could be the origin of MAP4343 promnesic action observed in unstressed animals. In stressed mice, repeated MAP4343 administration could reverse both short- and long-term memory deficits induced by CSDS. This was consistent with a previous study that found enhanced object recognition memory in social isolated rats treated acutely or sub-chronically with MAP4343 (Bianchi & Baulieu, 2012). In our work, this phenomenon also occurred with a surge in hippocampal *Bdnf* exon IV, MAP2 and hypothalamic *Crh* mRNA levels. *Bdnf* exon IV can be subjected to epigenetic regulation and increased by antidepressant pharmacotherapy (Boulle et al., 2012). In addition, both CRH knockdown in the paraventricular nucleus and the blockage of its type 1 receptor led to memory deficits in mice, illustrating its potential role in cognitive processes (Deak et al., 1999; Lucas, Chen, & Richter-Levin, 2013). The increase of its levels in the hypothalamus after

treatment could hence participate to MAP4343 reversal of cognitive impairments reported in our study. Interestingly, MAP2 mRNA expression was also upregulated by chronic MAP4343 in stressed animals. Because MAP2 is the receptor for MAP4343, these data strongly suggested that the therapeutic-like effect of this molecule was directly linked to MAP2 activity. Indeed, MAP4343 has been evidenced to be able to promote microtubule polymerization, through its binding to MAP2, and this has been showed to enhance locomotor recovery after spinal cord injury in rats (Duchossoy et al., 2011; Fontaine-Lenoir et al., 2006), hence highlighting its role of neuroplasticity enhancer. In addition, a recent report mentioned a possible positive correlation between blood MAP2 amounts and improved depressive symptoms when patients were under pregnenolone therapy (Daftary et al., 2017), further underlying the relation between MAP2 and neuroplasticity in the antidepressant-like effects of MAP4343.

Overall, our data suggested that MAP4343 could promote plasticity leading to enhanced cognitive performances in both healthy and stressed animals, through upregulation of Bdnf exon IV mRNA. When animals exhibited cognitive impairments related to their depressive-like state, MAP4343 also recruited MAP2 and CRH, hence causing a potential therapeutic-like action. Therefore, a new hypothesis emerged from the present work, postulating that under physiological conditions, MAP4343 effects on cognition may involve BDNF regulation, whereas under pathologic conditions, it could require the additional intervention of other partners such as MAP2 and CRH. Further investigations are needed to characterize the exact mechanism of action triggered by MAP4343 and eliciting both procognitive and therapeutic-like effects.

The investigations conducted in this study with the use of two animal models of depression showed that chronic MAP4343 could promote neuroplasticity through modulating both BDNF and MAP2 activities. The consequence of such regulation induced antidepressant-like effects, including correction of cognitive deficits induced by chronic stress. For these reasons, MAP4343 can be considered as an innovative candidate for treating depression.

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Table 1. Primer sequences used for mRNA expression analyses by RT-qPCR.

Gene	3' primers	5' primers
<i>Gapdh</i>	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
<i>Total Bdnf</i>	AAAACCATAAGGACGCGGAC	TAGACATGTTTGCGGCATCC
<i>BDNF IV</i>	CTCTGCCTAGATCAAATGGAGCT	GAAGTGACAAGTCCGCGTCCTT
<i>MAP2</i>	TCAGGAGACAGGGAGGAGAA	GTGTGGAGGTGCCACTTTTT
<i>GR</i>	GACTCCAAAGAATCCTTAGCTCC	CTCCACCCCTCAGGGTTTTAT
<i>CRH</i>	CCTTGAATTTCTTGCAGCCG	GGAATTCTGTTGAGATTCCCC

Table 2. Effects of chronic MAP4343 on stress-related gene expression of CSDS mice.

GR and CRH mRNA expression levels were analyzed in the hypothalamus of CSDS and non-stressed control mice. No difference between groups were evidenced for GR, while a significant increase in hypothalamic CRH mRNA amounts was found in stressed mice treated with MAP4343 compared to HEC ($p=0.0122$; ANOVA $p=0.0362$). All data passed normality tests and are expressed as mean \pm S.E.M. Statistical analyses were performed using one-way ANOVA followed by uncorrected Fisher's LSD post-hoc test.

Gene mRNA levels in the hypothalamus of CSDS mice				
Gene	Control-HEC	Stressed-HEC	Stressed-MAP4343	ANOVA result
GR	1.004 \pm 0.02819	1.016 \pm 0.03467	1.006 \pm 0.01232	n. s.
CRH	1.012 \pm 0.05164	0.9382 \pm 0.06515	1.238 \pm 0.1165	$p=0.0362$

Table 3. Effects of chronic MAP4343 on plasmatic corticosterone levels in CSDS mice.

Corticosterone levels (ng/mL) were measured under basal conditions, 30 hours after the last MAP4343 administration. Data passed normality tests and are expressed as mean \pm S.E.M. 2way ANOVA failed to find any difference between groups.

Control HEC	Control MAP4343	Stressed HEC	Stressed MAP4343	ANOVA result
37.64 \pm 2.75	41.19 \pm 4.42	33.55 \pm 2.06	38.57 \pm 1.44	n. s.

Figure 1. Study design and behavioral characterization of CSDS and GRi models.

A: Mice were submitted to chronic social defeat stress (CSDS) for 10 days and treated with MAP4343 (150 mg/kg) or its vehicle hydroxyethylcellulose (HEC; *per os*) for 21 days. Anxiety-like phenotype was assessed at D11 and D27 in the open-field test (OFT), and depressive-like phenotype was evaluated using the social avoidance test (SA) at D12 and D28. 24h after the last CSDS session, defeated mice exhibited both anxiety- and depressive-like behaviors. The interaction ratio measured in the social avoidance allowed the discrimination of susceptible and resilient mice among stressed animals. Susceptible mice had a significant lower interaction ratio than controls ($p=0.0003$) and resilient mice ($p<0.0001$). Only susceptible mice were kept for the rest of the study. Memory was tested in the novel object recognition task (NOR) at D29 and D30.

B: WT and GRi mice were treated with MAP4343 (150 mg/kg) or HEC for 14 days. They were tested in the OFT (at D0 and D10) and splash test (D0 and D14). GRi mice had a spontaneous anxiety- and depressive-like behavior at basal level (D0), before the treatment.

C: Dosage of plasmatic MAP4343 in WT mice treated 2, 4 and 24 hours with a fixed dose (150 mg/kg) of MAP4343, through either gavage or cookie. There was no difference in MAP4343 plasmatic concentration between these two administration modes, and there was a significant effect of time ($p<0.0001$), with a mostly complete disappearance of the compound 24h after administration by both routes.

All data passed normality tests, and are expressed as mean \pm S.E.M. Statistical analyses were performed using either unpaired t-test, one-way or 2 way ANOVA, both followed by uncorrected Fisher's LSD post-hoc test.

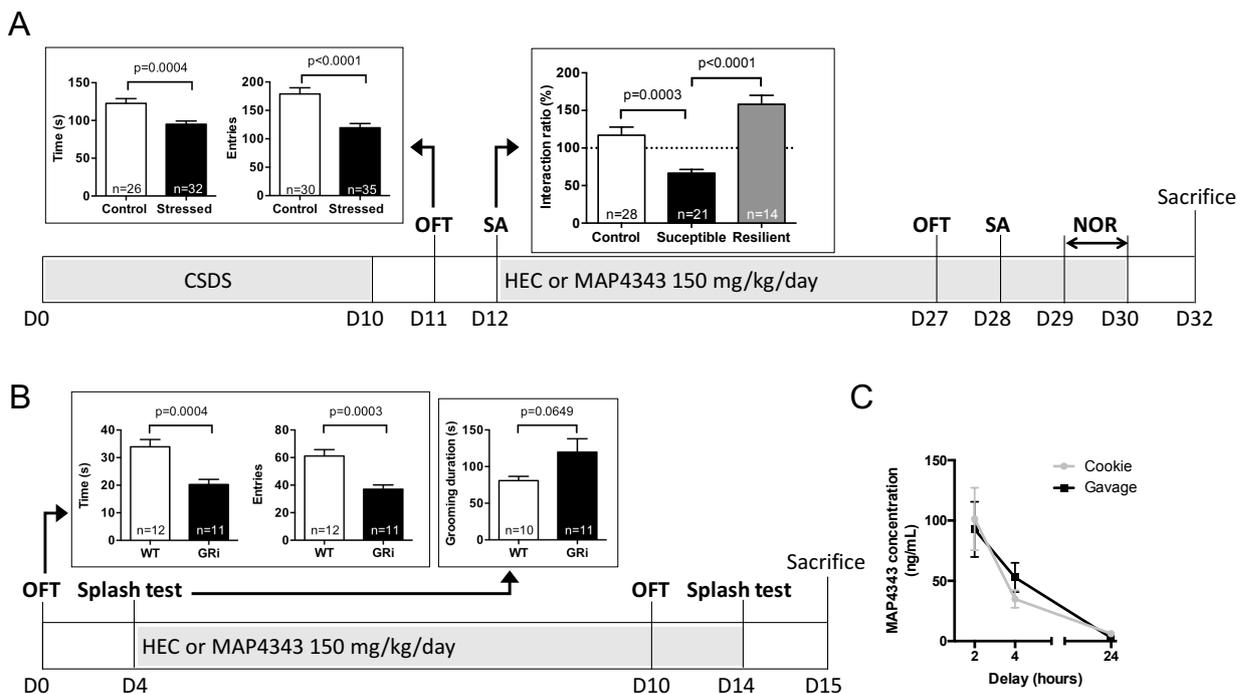


Figure 2. Effects of chronic MAP4343 administration on stress-related behaviors in CSDS and GRI mice.

Anxiety-like behavior of defeated or GRI mice was assessed in the OFT after a 21-day chronic administration of MAP4343 or HEC. In the CSDS procedure, neither stress nor MAP4343 modify the behavior of the mice (A). In mutated mice, the anxiety observed in GRI mice was not corrected by MAP4343 (B).

The effect of chronic MAP4343 on depressive-like phenotype was evaluated in CSDS mice tested in the social avoidance (C) and in GRI mice tested in the splash test (D). MAP4343 was able to reverse the depressive-like phenotype in both models.

All data passed normality tests, and are expressed as mean \pm S.E.M. Statistical analyses were performed using either one-way ANOVA or 2-way ANOVA, both followed by uncorrected Fisher's LSD post-hoc test.

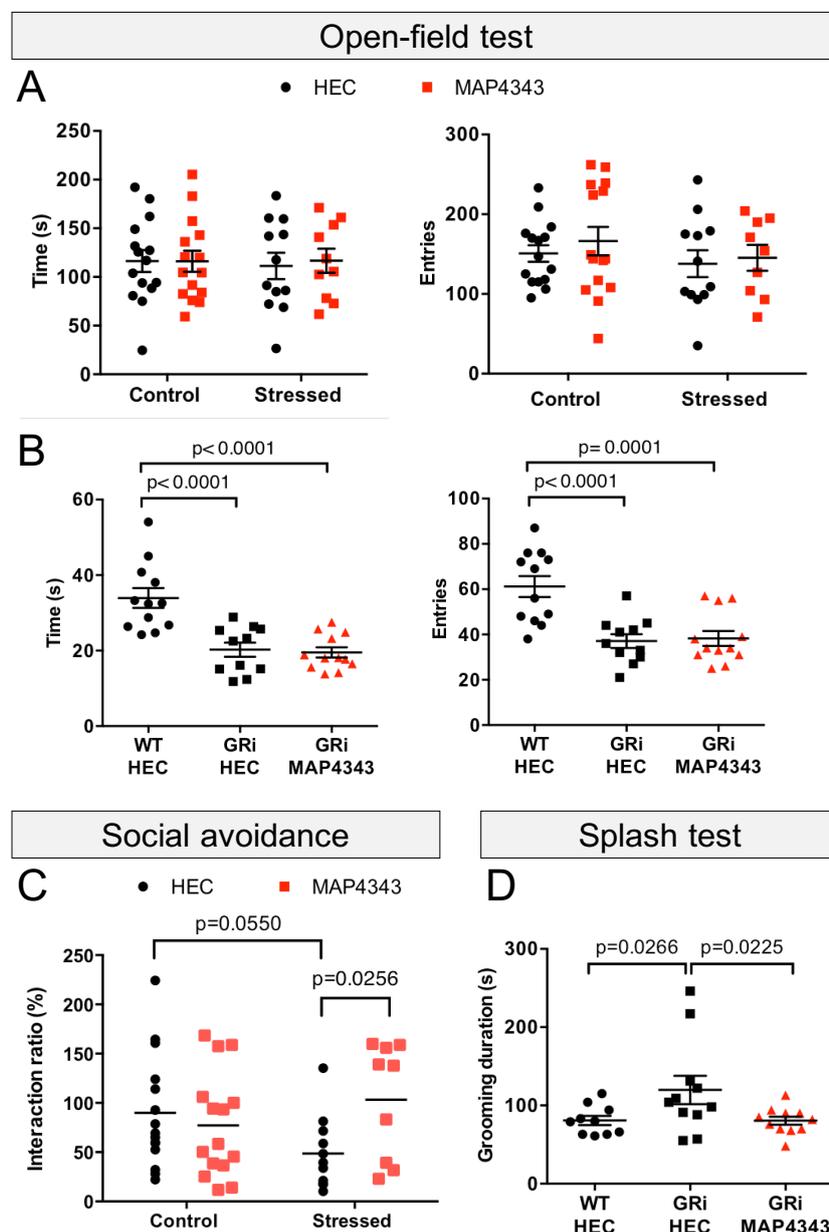


Figure 3. Effects of MAP4343 chronic administration on hippocampal plasticity in GRi mice.

A: Illustration of the distribution of Ki67-immunoreactive cells in the dorsal (upper panel) and ventral (lower panel) parts of the hippocampal dentate *gyrus* of GRi mice treated with HEC (left panel) or MAP4343 (right panel).

B: Quantification of Ki67-immunoreactive cells expressed in the hippocampal dentate *gyrus*. MAP4343 significantly increased the number of Ki67-positive cells in both the ventral and dorsal parts of the hippocampus.

C: Total *Bdnf* and *Bdnf* exon IV mRNA expressions were measured in the hippocampus of GRi mice. No significant difference between groups was observed.

All data passed normality tests, and are expressed as mean \pm S.E.M. Statistical analyses were performed using unpaired t-test.

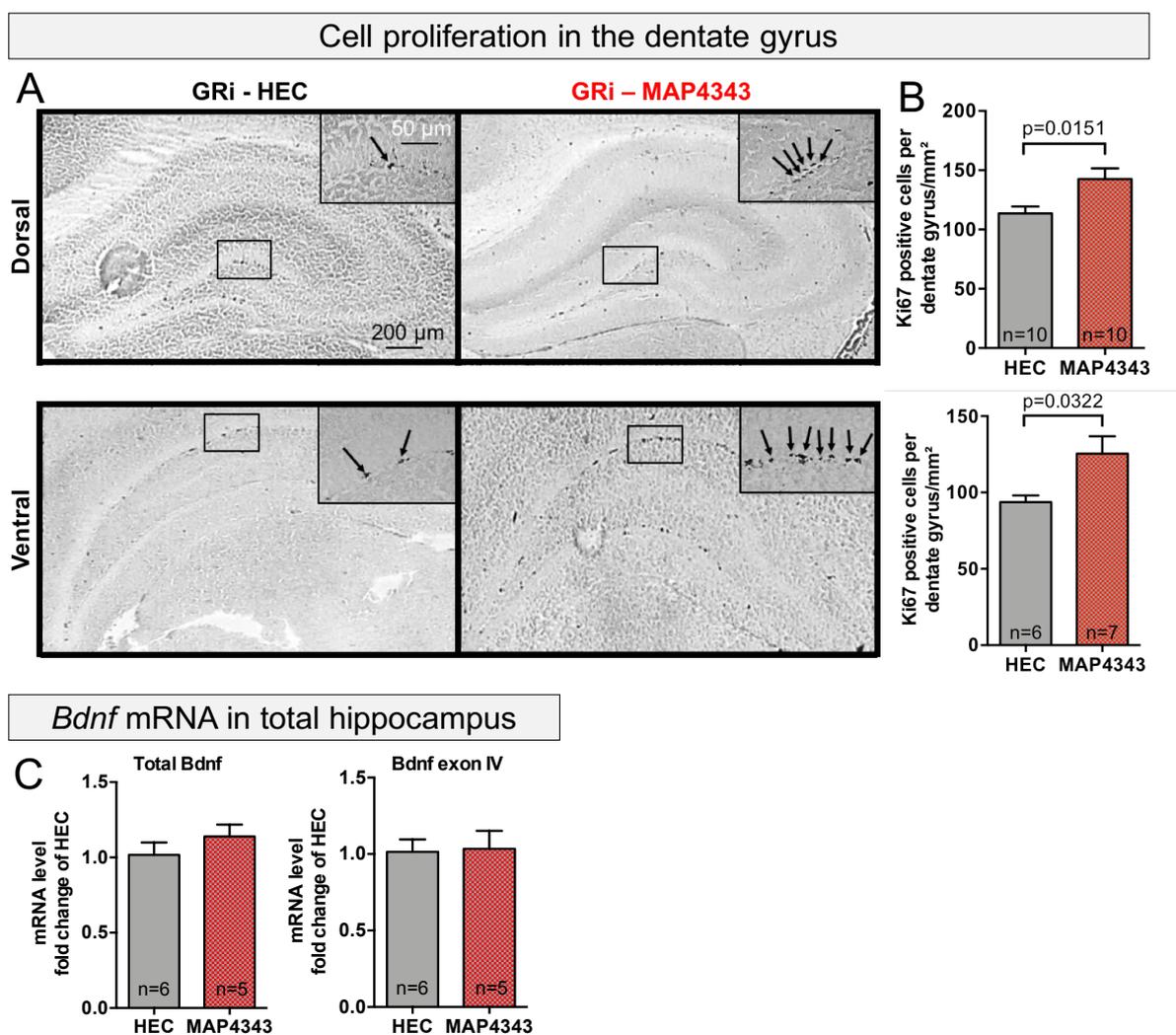
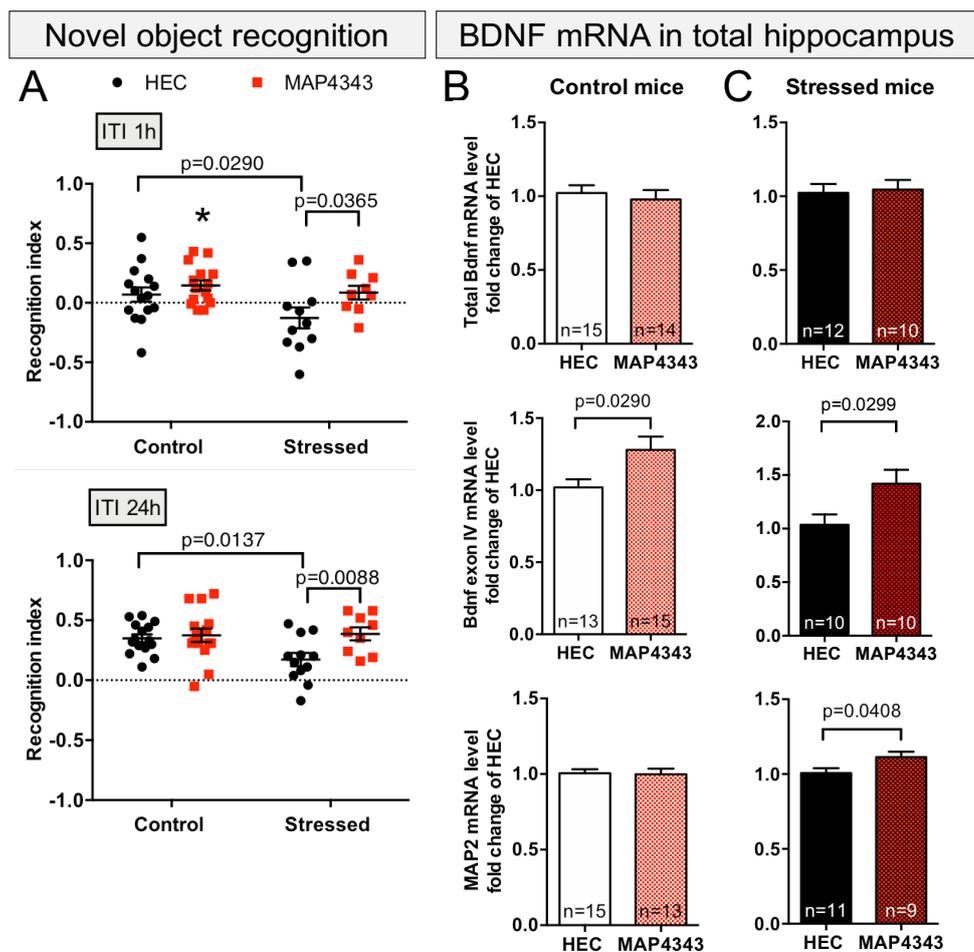


Figure 4. Effects of MAP4343 chronic administration on cognition and hippocampal plasticity in CSDS mice.

A: CSDS mouse memory was assessed in the NOR, using two ITIs. For ITI 1h, only control mice receiving MAP4343 treatment were able to discriminate the novel object (one sample t-test compared to 0; * $p=0.0041$). Stressed mice were less performant than controls ($p=0.0290$), and chronic MAP4343 could significantly reverse this phenotype ($p=0.0365$). For ITI 24h, all mice were able to discriminate the novel object (one sample t-test compared to 0; $p<0.01$ for all groups). Stressed mice still showed memory deficits compared to controls ($p=0.0137$), and this was reversed by MAP4343 ($p=0.0088$). Statistical analyses were performed using 2-way ANOVA followed by uncorrected Fisher's LSD post-hoc test.

Total *Bdnf*, *Bdnf* exon IV and *MAP2* mRNA expressions were measured in the hippocampus of control (B) and stressed (C) mice. In both groups, MAP4343 significantly increased *Bdnf* exon IV expression, but that of total *Bdnf* remained unchanged. MAP4343 also significantly increased the expression of *MAP2* in stressed mice ($p=0.0408$). Statistical analyses were performed using unpaired t-test. All data passed normality tests, and are expressed as mean \pm S.E.M.



Research article 2

The previous experiments showed that repeated MAP4343 treatment could enhance the memory performances of unstressed or stressed mice. This was associated with an increase in cell proliferation and an upregulation of BDNF and MAP2 expression at the mRNA level in the hippocampus, suggesting that MAP4343 could exert its antidepressant-like activity, and more precisely its procognitive action, through enhancement of neuroplasticity.

In the following study, we used a cellular model to elucidate the effects of MAP4343 on neuronal morphology and determine whether it could actually promote neuroplasticity. This question was assessed through measures of the neuronal shape (including the neurite length and width, the number of branching points), which can allow us to characterize the maturity of the cells. Since MAP4343 is known to target the microtubule cytoskeleton, we also measured the expression of cytoskeleton-related proteins. In parallel, we also examined the effect of MAP4343 on the expression of synaptic proteins. Indeed, they could be an additional indicator of neuronal maturation. Then, we investigated the mechanism of action of MAP4343, focusing on its action on the kinases involved in BDNF intracellular pathways elicited by its binding to TrkB, because of its role in both neuroplasticity and depression. It is noteworthy that in protein studies, the level of each protein was measured after 3, 5, 7, 10, 13 days of treatments. For clarity, only the significant changes were kept in the following article.

The results presented in this article will be completed by further findings obtained following experiments that are currently conducted in the lab, before its submission to peer-reviewing.

The new antidepressant compound MAP4343 promotes neuronal plasticity through cytoskeleton and kinase-dependent signaling pathway

Schverer Marina, Jennifer Leandri, Julie Cottin, Larbi Amazit, Etienne-Emile Baulieu, Isabelle Villey, Laurence Lanfumey, Nicolas Froger

Abstract

Neuroplasticity refers to the ability of the nervous system to adapt its structure and function in response to internal and external stimuli. It can be altered in psychiatric diseases, such as major depression. MAP4343 is a pregnenolone derivative that displayed antidepressant-like activity in several animal models, and is suspected to enhance plasticity by binding to the microtubule-associated protein 2 (MAP2). The aim of the present work was to characterize the effect of this molecule on neuroplasticity, and the potential role of the signaling pathways triggered by brain-derived neurotrophic factor (BDNF) and its high affinity receptor tropomyosin receptor kinase B (TrkB) in the mechanism of action of MAP4343. Using primary neuronal culture, we investigated MAP4343 action on morphological features, and evidenced that it induced a faster maturation of neurons, enhancing neurite length and bundling of growth cones. The treatment also increased the expression of cytoskeleton-related proteins cortactin and MAP2, as well as that of synaptic markers PSD-95 and synapsin I. Given the critical role of BDNF/TrkB signaling in neuroplasticity and depression, we investigated the effects of MAP4343 on several kinases activated by BDNF binding to TrkB. We found that the outcomes following MAP4343 treatment were time-dependent, since it first promoted the phosphorylation of both extracellular signal-regulated kinase (ERK) and cyclic adenosine monophosphate-responsive element binding (CREB), and then increased the levels of BDNF, TrkB, Akt, ERK and CREB in neurons. Overall, we evidenced that MAP4343 had a positive activity on neuroplasticity, and this action may recruit intracellular kinases such as ERK.

Introduction

Cognitive deficits, considered among the major symptoms in depressed patients, are described to be related to neuroplasticity alterations (Marsden, 2013; Soria et al., 2018). Although some antidepressant molecules have been characterized as neuroplasticity enhancers, they all failed to correctly alleviate cognitive deficits in patients with major depression (Levy et al., 2018; Soria et al., 2018). MAP4343 is a compound that displayed persistent antidepressant-like activity in animals, triggering faster efficacy than fluoxetine (Bianchi & Baulieu, 2012). Indeed, MAP4343 rescued the depressive-like phenotype induced by the isolation-rearing model of depression in rats (Bianchi & Baulieu, 2012), and restored locomotor and exploratory activity, additionally to physiological features increased after chronic psychosocial stress in tree shrews (Parésys et al., 2016). Moreover, MAP4343 was shown to bind to microtubule-associated protein 2 (MAP2) (Fontaine-Lenoir et al., 2006), leading to an outgrowth of PC12 cell processes and an increased expression of MAP2 *in vitro* (Fontaine-Lenoir et al., 2006). Hence, MAP4343 may modulate structural plasticity, at least partly, through its interaction with MAP2.

In neurons, plasticity is highly regulated by several actors, including neurotrophins such as the brain-derived neurotrophic factor (BDNF) that interacts with its high-affinity receptor tropomyosin receptor kinase B (TrkB) (Boulle et al., 2012; Levy et al., 2018). The binding of BDNF to TrkB conducts to the transphosphorylation of the receptor, and this leads to the phosphorylation and activation of several targets involved in different intracellular pathways (Numakawa et al., 2010). For example, BDNF/TrkB association triggers the phosphatidylinositol pathway that may activate Akt and extracellular signal-regulated kinase (ERK), both eventually leading to phosphorylation of the transcription factor cyclic adenosine monophosphate-responsive element binding (CREB) (Du & Montminy, 1998). CREB activation by BDNF binding to TrkB is described to promote the production of proteins involved in several events such as survival, growth and plasticity (Cunha, Brambilla, & Thomas, 2010; Leal, Comprido, & Duarte, 2014). In the hippocampus, the activation of Akt, and probably ERK, by BDNF/TrkB interaction is suspected to be involved in cognitive processes, in particular in spatial memory formation (Mizuno et al., 2003). BDNF and TrkB levels are reported to be reduced in the hippocampus and prefrontal cortex of patients that committed suicide (Dwivedi et

al., 2003; Pandey et al., 2008), possibly leading to the neuroplasticity alteration observed in depression.

We hypothesized that MAP4343 antidepressant-like activity may rely on its capacity to regulate plasticity. The present study aimed to better characterize the effect of MAP4343 on neuronal plasticity, and to investigate the potential implication of BDNF/TrkB signaling in MAP4343 mechanism of action. We showed that MAP4343 promoted both structural and synaptic plasticity in cultured neurons, and this effect involved a potentiation of BDNF/TrkB signaling.

Materials and methods

Animals. Pregnant female SWISS mice were used as a source of embryonic tissue for primary neuronal cultures. Animals were housed in the same room under controlled conditions ($21 \pm 1^\circ\text{C}$, 12h/12h light/dark cycles, light on at 8 a.m., and food and water available *ad libitum*). All experiments were carried out in accordance with the Directive 2010/63/UE.

Primary cultures of cortical or hippocampal neurons. Pregnant females were euthanized by cervical dislocation and cortices or hippocampi from E16 embryos were dissected and placed in cold phosphate-buffered saline (PBS) containing glucose (1 g/L). Brain tissue was mechanically dissociated. All plates used for cell seeding, were previously coated with poly-D-lysine ($2 \mu\text{g}/\text{cm}^2$), during at least 2 hours at 37°C . Cell density was different depending on the experiment (morphometric studies: 6,000 cells/ cm^2 , protein studies: 50,000 cells/ cm^2 , protrusion experiments: 25,000 cells/ cm^2). Seeding was done in Neurobasal medium (Thermofisher Scientific, Les Ulis, France) containing B-27 supplement (50 X; Thermofisher Scientific) and L-glutamine (2 mM, Sigma-Aldrich, St-Quentin Fallavier, France) in 6-well Greiner plates, previously coated with poly-D-lysine (Sigma-Aldrich) at $2 \mu\text{g}/\text{cm}^2$. Cells were maintained at 37°C and 5% CO_2 . Cortical neurons were treated with vehicle (1% ethanol) or three doses of MAP4343 (0.1, 1 and 5 μM) just after seeding, and kept 3 days *in vitro* (DIV) for morphometric studies and immunocytochemistry experiments, in which neurons were fixed using 4% paraformaldehyde for one hour at 4°C . For the measures of protein expression, cells were collected in a lysis buffer (NaCl 150 mM, Tris-HCl 50 mM,

Glycerol 20%, NP40 1%) after 5, 7, 10 or 13 days. Hippocampal neurons were used for the investigation of spine-like protrusions because they are enriched in protrusions in basal condition. They grew on coverslips for 14 DIV (with a medium change after 10 days), and were treated for 3 days with vehicle or MAP4343 1 μ M. Finally, cells were fixed with 2% paraformaldehyde at DIV 17 for 15 minutes at RT, and kept in PBS at 4°C until use.

Morphometric measurements. After 3 DIV, neurons were incubated at 37°C and 5% CO₂ for 1 hour 15 minutes with calcein AM (0.33 mg/ml final, ThermoFisher Scientific) to label living cells, and DRAQ5 at 500 nM for 3 minutes to stain nuclei. High content screening was performed using the Cellomics ArrayScan VTI (ThermoFisher Scientific) for image acquisition, and Studio software for image analyses. Each well was scanned with a 10x magnification using an epifluorescent inverted microscope (Zeiss Observer Z1) with a high resolution camera integrated to the platform. The fluorescent signal from calcein was detected at 485 nm and the one from Draq5 at 549 nm. A neuron profiling was obtained, encompassing several of automatic measures of morphologic features, such as the number of branching points, neurite length, total area or width.

Immunocytochemistry. Fixed cells were permeabilized in PBS-0.1% of triton (Sigma-Aldrich) for 10 minutes and unspecific sites were saturated using SeaBlock blocking buffer (Thermoscientific) for 1 hour, at room temperature. Cells were then incubated overnight at 4°C with mouse anti-tyrosinated α -tubulin primary antibodies (mouse, clone TUB-1A2, 1:500; Sigma-Aldrich), diluted in the blocking buffer. After rinses in PBS, cells were incubated for 1 hour at room temperature with goat anti-mouse secondary antibodies (IgG Alexa Fluor, 1:1000; ThermoFisher Scientific), diluted in the blocking buffer. Coverslips were finally mounted on Superfrost slides with a fluorescent mounting medium (Dako, Agilent, Les Ulis, France). Fluorescent images of primary neurons were imaged using a confocal laser-scanning microscope (TCS SP8, Leica Microsystems, Wetzlar, Germany).

Growth cone analyses. As previously described (Tanaka & Kirschner, 1991; Teng et al., 2001), growth cones can be classified as spread or bundled. Spread cones are paused and contain spayed microtubule believed to be stable, while bundled cones are rapidly extending and contain straight microtubules supposed to be dynamic (Dent & Gertler, 2003; Tanaka, Ho, & Kirschner, 1995). Growth cone analyses were performed from neurons immunostained for tyrosinated tubulin. Experimenters blindly evaluated the proportion of spread versus bundled cones, on an average of 50 neurons per culture, from 3 independent cultures.

Western blot. Protein concentration of each sample was determined using Pierce^TBCA Protein Assay Kit according to the manufacturer's instructions (ThermoFisher Scientific). Loading samples were diluted in lysis buffer, denatured in DL-dithiothreitol (Sigma-Aldrich) and boiled for 5 minutes at 95°C. Proteins were separated on 10% bisacrylamide/trisacrylamide gel electrophoresis (ThermoFisher Scientific). A pre-stained molecular size marker (260–3.5 kDa range, ThermoFisher Scientific) was used to locate proteins on the basis of their position on the gel. After electrophoresis (150 V, 1 hour), proteins were transferred onto a nitrocellulose membrane (ThermoFisher Scientific) using a dry blotting system (7 minutes, 20 V, iBlot 2, ThermoFisher Scientific). After blocking in Odyssey blocking buffer (Li-cor, Lincoln, NE, USA) for 1 hour at room temperature, membranes were incubated with primary antibodies (Table 1) overnight at 4°C, and with secondary antibodies 1 hour at room temperature (Table 1). All antibodies were diluted in Odyssey blocking buffer containing 0.2% of Tween 20 (Sigma-Aldrich). Infrared fluorescence emission was analyzed at 800 nm and 700 nm using an Odyssey imager (Li-Cor). The relevant immunoreactive fluorescent bands were quantified using ImageJ software (National Institutes of Health, USA). Phosphorylated/total protein ratio was calculated, and all data were normalized with β -actin. For each blot, results were expressed as ratio of vehicle.

Enzyme-linked immunosorbent assay (ELISA). The BDNF Emax ImmunoAssay System kit (Promega, Madison, WI, USA) was used to detect the BDNF contents in

cell lysates, according to the manufacturer instructions. Samples were either used as collected to measure free mature BDNF, or acid treated to measure total BDNF.

Diolistic labeling was performed on hippocampal neurons as previously described (Heck, Betuing, Vanhoutte, & Caboche, 2012). Tungsten beads (Bio-rad, Marne-la-Coquette, France) were mixed with solid Dil (1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate; Molecular Probes, D282) dissolved in methylene chloride. Dil-coated beads were suspended in water, sonicated, and coated on the inner surface of a PolyVinylPyrrolidone (Sigma-Aldrich) pre-treated Teflon tube from which cartridges were cut. Beads were delivered through a 3- μ m pore-size filter (Isopore polycarbonate, Millipore, Molsheim, France) on coverslips using helium gas pressure (150 psi) using the genegun device (Bio-Rad). After labeling, coverslips were kept in buffer at room temperature for at least 2 hours and mounted in Mowiol 4-88 (Sigma-Aldrich).

Neuritic protrusion analyses. Image stacks of neurites from hippocampal neurons were acquired using a confocal laser-scanning microscope (TCS SP8, Leica, Wetzlar, Germany). The head and width of protrusions were semi-automatically quantified by means of Reconstruct software (<http://synapses.bu.edu>), with a fixed width head threshold (Risher, Ustunkaya, Alvarado, & Eroglu, 2014). Protrusions having larger or smaller width head than 0.6 μ m were classified as mature or immature shapes respectively.

Statistical analyses. For all experiments, statistical analyses were performed using Prism 7 software (GraphPad, San Diego, USA). The normal distribution of data was tested by means of Shapiro-Wilk normality test or KS normality test. The contingency of data from growth cone analysis was evaluated using a Chi-square test. In morphometric experiments, data having a Gaussian distribution were compared using a One-way ANOVA followed by Fisher's LSD multiple comparison *post-hoc* test, and experimental groups that did not followed a Gaussian distribution were compared with a Kruskal-Wallis test. For protrusion counting and Western blot analyses, data were normalized for each culture, and a one-sample t-test compared to the theoretical value

“1” corresponding to vehicle conditions was used for each treatment condition. Differences between groups were considered significant at the 95% confidence level ($p \leq 0.05$).

Results

MAP4343 effects on structural plasticity

After 3 DIV, MAP4343 produced changes in neuron morphology. Neurons were more polarized, with longer neurite extensions (Figure 1A). The structure of growth cones was studied by ranking the proportion of spread versus bundled cones. When neurons were treated with MAP4343 (1 and 5 μM) for 3 days, the proportion of spread growth cones was significantly reduced, and the bundled ones were increased compared to vehicle-treated neurons (Chi-square test: $p=0.018$ and $p=0.0002$ respectively) (Figure 1A-B).

3 days of treatment with MAP4343 also induced a dose-dependent increase in neurite length (One-way ANOVA, $F_{(3, 20)}=6.484$; $p=0.0030$, Figure 1C), with significant effects obtained with the concentrations 1 μM (One-sample t-test compared to “1” that refers to vehicle, +33%; $p=0.0067$) and 5 μM (+50%, $p=0.0004$). MAP4343 induced a dose-dependent decrease of the total neurite width (Kruskal-Wallis test; $p=0.0046$), that was significant for MAP4343 5 μM (-10%, $p<0.01$; Figure 1C). Concomitantly, the neurite total area was also dose-dependently raised in the presence of MAP4343 ($F_{(3, 20)}=4.133$, $p=0.0197$; +18% at 1 μM , $p=0.0085$ and +20% at 5 μM , $p=0.0064$; Figure 1C). The number of branching points tended toward increase with the 3 doses of MAP4343, but this was not statistically significant (Figure 1C).

MAP4343 effects on expression of cytoskeleton-related proteins

After 5 DIV, MAP4343 had no significant effect on α -tubulin expression (Figure 1D-E). The three doses of MAP4343 increased the expression of cortactin compared to vehicle (0.1 μM : +10%, $p=0.0264$; 1 μM : +14%, $p=0.0202$), even if the effect of MAP4343 5 μM was only a tendency (+14%, $p=0.0702$). MAP2 expression was increased after treatments with MAP4343 1 μM (+34%, $p=0.0234$) and 5 μM (+24%, $p=0.0056$) (Figure 1D-E).

MAP4343 effects on expression of synaptic proteins and spine-like protrusions

When 5 μM of MAP4343 were applied to neurons for 3 days, western blot analyses revealed a significant decrease in the expression of synapsin I (-30%, $p=0.0184$) and PSD-95 (-24%, $p=0.0160$), when compared to the baseline referring to vehicle-treated condition (Figure 2A-B). The lower concentration of MAP4343 (0.1 μM) was also able to decrease the neuronal expression of synapsin I (-18%, $p=0.0379$) (Figure 2A-B).

In contrast, after 5 DIV, the expression of synapsin I was higher in presence of MAP4343 than in vehicle-treated neurons (0.1 μM : +15%, $p=0.0065$; 1 μM : +22%, $p=0.0159$; 5 μM : +9%, $p=0.0294$). Similarly, PSD-95 protein level was also enhanced with MAP4343 5 μM (+22%, $p=0.0458$) (Figure 2A-B).

In hippocampal neurons, MAP4343 1 μM did not modify the density of precursor-, immature- and mature-like protrusions (Figure 2C-D). Regarding precursors, the filopodia shape tended toward decrease, whereas the stubby shapes seemed to be increased under MAP4343 treatment, but failed to reach statistical significance, probably because of the high variability in values.

MAP4343 effects on BDNF content and TrkB protein expression

Cortical neurons were treated with 3 doses of MAP4343 (0.1, 1, 5 μM) and cultured during 5, 7, 10 or 13 days. BDNF levels and TrkB protein expression were measured by ELISA and western blot respectively. After 5 DIV, no significant difference was observed in neuronal BDNF content between vehicle-treated and MAP4343-treated conditions. At 7, 10 and 13 DIV, the MAP4343 induced a dose-dependent increase in neuronal BDNF content, that was significant when the molecule was used at 5 μM (DIV 7: +69%, $p=0.0425$; DIV 10: +66%, $p=0.0170$; DIV 13: +86%, $p=0.0504$) (Figure 3A).

Regarding TrkB protein expression, it was unchanged at DIV 5, and an increase was found after 7 days of treatment with the 3 doses of MAP4343 (0.1 μM : +18%, $p=0.0349$; 1 μM : +18%, $p=0.0605$; 5 μM : +20%, $p=0.0077$). At DIV 10, the surge in TrkB expression was persistent, since MAP4343 1 μM enhanced it (+22%, $p=0.0042$), and a tendency was observed with MAP4343 5 μM (+25%, $p=0.0522$) (Figure 3B-C). Finally, after 13 days of treatment, the expression of TrkB seemed to be still higher than in control condition, although these changes were not statistically significant.

MAP4343 effects on targets of the BDNF/TrkB pathway

The expression of phosphorylated/total form of proteins was measured to assess their activation levels. While Akt expression was unchanged (Figure 4A-B), MAP4343 increased the activation of both ERK and CREB when used at 1 μ M (ERK: +86%, $p=0.0367$; CREB: +23%, $p=0.0173$) and 5 μ M (ERK: +60%, $p=0.0498$; CREB: +60%, $p=0.0664$) compared to vehicle (Figure 4A-B).

After 13 DIV, the amount of activated proteins was unchanged (data not shown), and all doses of MAP4343 induced a surge in total levels of Akt (0.1 μ M: +18%, $p=0.0258$; 1 μ M: +30%, $p=0.0092$; 5 μ M: +43%, $p=0.0197$), ERK (0.1 μ M: +24%, $p=0.0033$; 1 μ M: +41%, $p=0.0001$; 5 μ M: +66%, $p=0.0002$), and CREB (0.1 μ M: +15%, $p=0.0041$; 1 μ M: +29%, $p=0.0005$; 5 μ M: +38%, $p=0.0012$), compared to vehicle (Figure 4C-D).

Discussion

The present study was designed to investigate the effects of MAP4343 on neuronal plasticity. Indeed, this molecule previously showed pro-plasticity effects *in vitro*, promoting microtubule polymerization via its interaction with MAP2 (Fontaine-Lenoir et al., 2006). Here, we characterized these pro-plasticity effects in neurons, and demonstrated for the first time that they may be due to MAP4343 ability to modulate kinase activity, leading to a potentiation of BDNF/TrkB signaling pathway.

We found that MAP4343 was able to shorten changes in neuronal shape toward a potential mature form. Indeed, it increased neurite length, decreased their width, an enhanced growth cone bundling. Since neurites mostly consisted of microtubule cytoskeleton, the changes in neurite shape may be related to MAP4343 ability to interact with MAP2 (Fontaine-Lenoir et al., 2006), or with other microtubule-related proteins, such as CLIP-170, a protein that tracks the growing end of microtubules. Indeed, pregnenolone, the parent molecule, was shown to bind to CLIP-170 (Weng et al., 2013), additionally to MAP2 (Mizota & Ueda, 2008; Murakami, Fellous, Baulieu, & Robel, 2000). Hence, it is possible that MAP4343 activity engages MAP2 and CLIP-170, that both promote microtubule elongation (Goodson & Jonasson, 2018; Swiech et al., 2011). MAP4343 increased the neuronal expression of MAP2, without changing

the total amounts of α -tubulin. As mentioned above, MAP2 promotes microtubule stabilization and is a receptor for MAP4343 (Johnson & Jope, 1992). Our results suggest that neurite elongation might be due to microtubule cytoskeleton rearrangement induced by the direct binding of MAP4343 to MAP2 and the upregulation of this protein.

Neuronal plasticity also occurs at specific sites, such as growth cone and dendritic protrusions, which are actin-enriched structures. More precisely, the distal part of growth cones mostly contains actin filaments, whereas the proximal part is enriched in microtubules (Erik W Dent, Gupton, & Gertler, 2011). Protrusions are largely composed by actin filaments, while microtubules are often restricted to dendritic shafts, although it was demonstrated that they could transiently enter in the neck of the mature spines, mostly for synaptic protein delivery (Jaworski et al., 2009; McVicker et al., 2016). MAP4343 had no effects on β -actin (data not shown), but we found that it increased cortactin expression in neurons. Cortactin was described as an actin-related protein strongly expressed in dendritic spines of mature neurons and in axonal and dendritic growth cones of hippocampal neurons (Konietzny, Bär, & Mikhaylova, 2017; Spence, Kanak, Carlson, & Soderling, 2016). This protein was characterized as a plasticity regulator (He et al., 2015), and its upregulation by MAP4343 could explain the effect of this molecule on growth cone shape. Therefore, we evidenced here for the first time the ability of MAP4343 to modulate the actin cytoskeleton, at least indirectly. Further investigations are needed to know if the action of MAP4343 on cortactin eventually leads to actin filament reorganization, in a same way that MAP4343-induced rearrangement of the microtubule cytoskeleton.

The analysis of the density of spine-like protrusions revealed no effect of MAP4343 treatment, while a decline in the levels of the synaptic markers synapsin I and PSD-95 was observed (Thiel, 1993; Tu et al., 1999). Previous work revealed that variations in synapsin I amounts were associated with changes in clustering of PSD-95 in spines, suggesting that both proteins could act in concert to regulate synaptic plasticity (Morita et al., 2006). Besides its role of anchoring protein for synaptic elements, PSD-95 was shown to have a nonsynaptic function during neuron development. Indeed, it can interact with the microtubule end-binding protein EB3 (Sweet et al., 2011), leading to disrupted microtubule organization and reduced proximal dendrite branching (Charych et al., 2006). We measured the expression of both proteins after 2 additional days of

treatment, and found that they were upregulated by MAP4343. It is possible that at this time, synapse maturation was initiated since the expression of synaptic markers was reported to be a clue of dendritic spine maturation (Sweet, Tseng, & Firestein, 2011). It would be interesting to measure the density of protrusions after longer MAP4343 treatment, and see whether it might correlate with the surge in synaptic marker expression.

Since intracellular pathways triggered by BDNF/TrkB interaction are critically involved in both structural and synaptic plasticity (Leal et al., 2014), but also in the therapeutic response of several antidepressants (Levy et al., 2018), we hypothesized that MAP4343 action on neuronal plasticity could rely on the recruitment of these signaling pathways. We first showed that when applied at least 7 days, MAP4343 could increase both BDNF and TrkB levels in neurons. These effects could be persistent in time since these levels seemed to be still high after 13 days of treatment. BDNF binding to TrkB is known to induce the activation of several targets, including ERK and Akt, whose activity was reported to increase the phosphorylation of synapsin I (Jovanovic, Czernik, Fienberg, Greengard, & Sihra, 2000). Here, we found that MAP4343 applied for 5 days was able to potentiate the phosphorylation of both ERK and CREB. In addition, long-term (13 days) treatment with MAP4343 also induced a surge in protein expression of Akt, ERK and CREB, suggesting an upregulation of mRNA expression that has to be investigated. Because CREB is a target of ERK, it was unclear if the increase in CREB activation was directly due to MAP4343 activity, or if it was only the consequence of the enhanced ERK phosphorylation.

In the present report, we found that MAP4343 effect on the different variables measured was time-dependent. Indeed, it was likely that this molecule induced early structural changes including neurite outgrowth and growth cone bundling (at DIV 3), then plasticity-related proteins and synaptic markers were upregulated, concomitantly with a surge in the activation of ERK and CREB (at DIV 5). This was followed by BDNF/TrkB levels increase (at DIV 7, 10 and probably 13), and eventually a rise in total amount of Akt, ERK and CREB (at DIV 13). It is probable that the rise in BDNF content was only due to CREB activation. Indeed, CREB is described as responsible for the production of this protein, leading to reinforcement of BDNF/TrkB signaling (Finkbeiner et al., 1997). The increasing amounts of Akt, ERK and CREB may stand for bringing more substrate for MAP4343 effects. Overall, our data suggest that the

starting point of MAP4343 mechanism of action could involve cytoskeleton modulation, through MAP2 and cortactin, as discussed above. A study using mesenchymal stem cells reported that ERK could be activated by the rearrangement of actin cytoskeleton occurring after application of mechanical forces (Müller, Langenbach, Kaminski, & Rychly, 2013). This could be paralleled with MAP4343 effects, although how its activity might conduct to an increase in ERK phosphorylation remains an open question.

MAP4343 has been described as a compound having antidepressant-like properties (Bianchi & Baulieu, 2012; Parésys et al., 2016). In animal models of depression, dysregulations of the cytoskeleton were reported (Bianchi, Heidbreder, & Crespi, 2003; Yang, Wang, Wang, Liu, & Wang, 2009), and this could participate to neuronal plasticity impairments observed in this disease (Marsden, 2013). The present study demonstrated that MAP4343 could be a serious candidate for neuronal plasticity enhancement required in depressive disorders.

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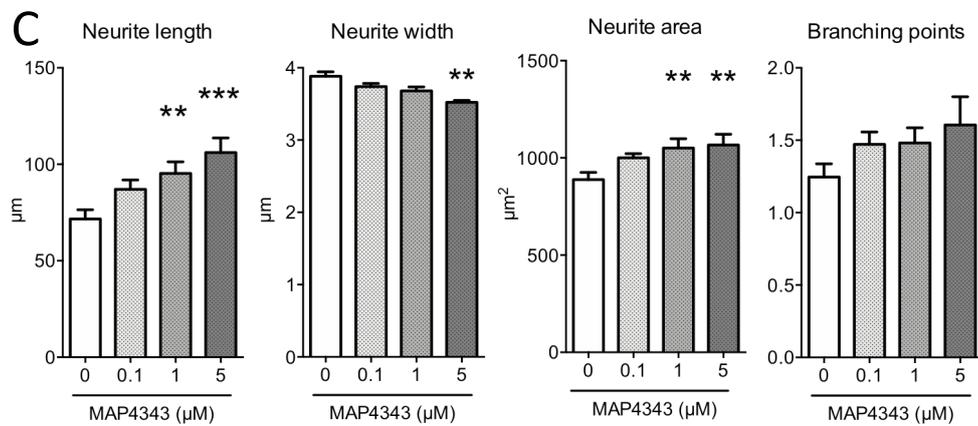
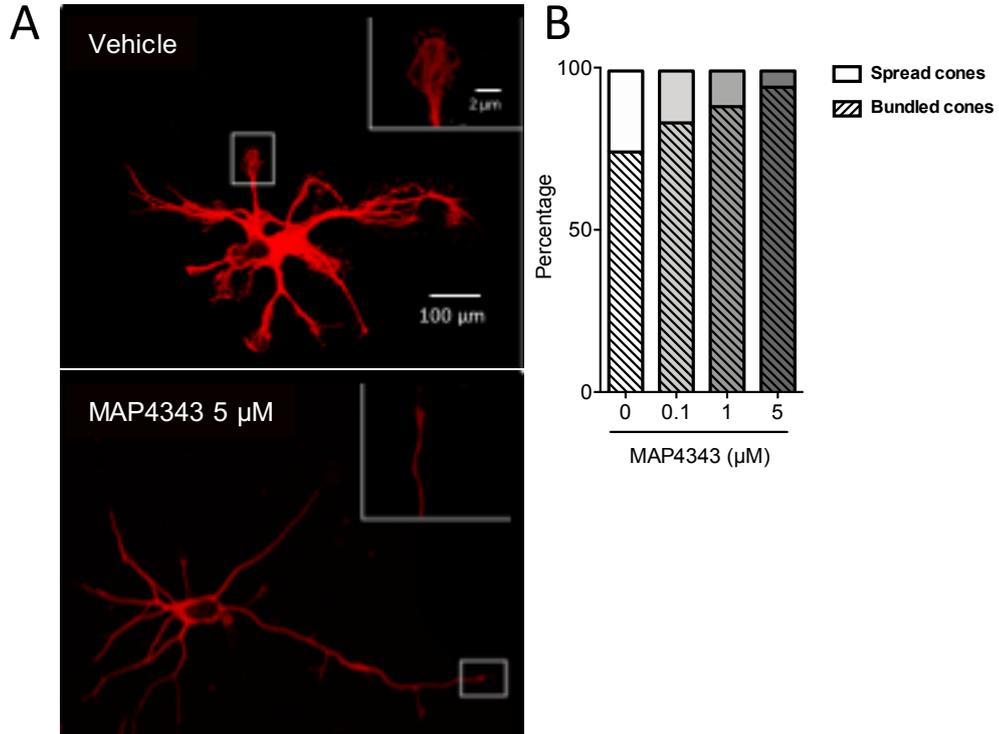
Table 1. Antibodies used for western blot analyses

Target	Produced in	Clone	Dilution	Supplier	Reference
β -Actin	mouse	AC-74	1:100,000	Sigma-Aldrich	A2228
α -Tubulin	mouse	DM1A	1:2,000	Sigma-Aldrich	T6199
MAP2	mouse	HM2	1:1,000	Sigma-Aldrich	M4403
Cortactin	rabbit	E1922Y	1:1,000	Abcam	ab81208
Synapsin I	rabbit	polyclonal	1:1,000	Sigma-Aldrich	S193
PSD-95	mouse	7E3-1B8	1:500	Sigma-Aldrich	P246
TrkB	rabbit	polyclonal	1:1,000	Millipore	07-225
Phospho-TrkB (Tyr816)	rabbit	polyclonal	1:350	Millipore	ABN1381
Akt	rabbit	polyclonal	1:1,000	Cell Signaling	9272
Phospho-Akt (Ser473)	mouse	587F11	1:500	Cell Signaling	4051
ERK1/2	rabbit	polyclonal	1:2,000	Millipore	06-182
Phospho-ERK1/2 (Thr202/Tyr204)	rabbit	polyclonal	1:500	Cell Signaling	9101
CREB	rabbit	D76D11	1:1,000	Cell Signaling	4820
Phospho-CREB (Ser133)	rabbit	87G3	1:500	Cell Signaling	9198
IRDye 680RD anti-mouse	goat	polyclonal	1:10,000	Li-cor	926-68070
IRDye 800CW anti-rabbit	goat	polyclonal	1:10,000	Li-cor	926-32211

Figure 1. Effects of MAP4343 on neuronal plasticity in cortical neurons *in vitro*.

A. Representative fluorescent images showing neurons labelled against tyrosinated tubulin (red) when treated with MAP4343 (5 μ M) as compared to vehicle-treated neurons. Islet pictures focused on the morphology of the growth cone indicated in the white square. B. Measurement of proportions of spread versus bundled growth cones in neurons treated with 3 doses of MAP4343 or with vehicle (Ethanol 1%). C. Automatic morphometric measures including neurites length, neurite width, neurite area and branching point count, showing that MAP4343 potentiated neurite outgrowth. Neurite length, area and branching points data passed normality tests and were compared using One-way ANOVA followed by Fisher's LSD multiple comparison test. Data related to neurite width did not have a Gaussian distribution and groups were compared with a Kruskal-Wallis test; (** $p < 0.01$; *** $p < 0.001$). All results are expressed as mean \pm S.E.M. $n = 5-8$ independent cultures per group. D. Immunodetection by Western blot of tubulin, cortactin, and MAP2 proteins from cortical neurons under MAP4343 treatment (0.1, 1 and 5 μ M). The values in brackets refer to the molecular weight of the protein in kDa. E. Quantification of protein bands, showing that MAP4343 increased cortactin and MAP2 expression, without changing α -tubulin expression. Data passed normality tests and are expressed as mean \pm S.E.M. $n = 5-7$ independent cultures per group. MAP4343 effects were assessed using a one-sample t-test comparing each group to 1, that refers to vehicle condition (* $p < 0.05$; ** $p < 0.001$).

Morphometric measurements



Cytoskeleton-related proteins

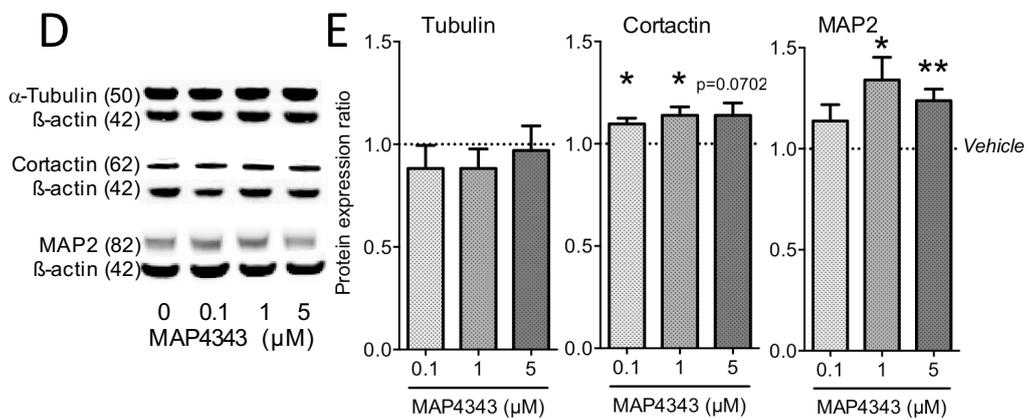
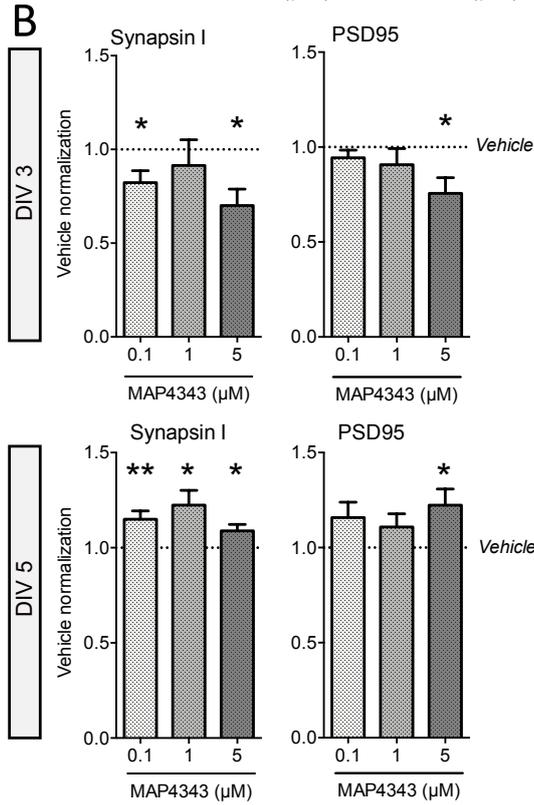
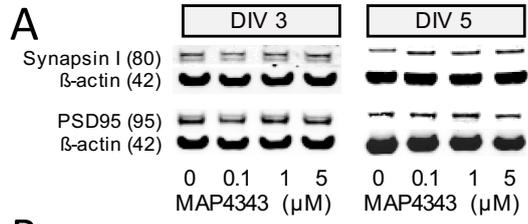


Figure 2. MAP4343 effects on synaptic markers and spine-like protrusions in cortical and hippocampal neurons *in vitro*.

A. Immunodetection by Western blot of synapsin I and PSD-95 proteins from cortical neuron under MAP4343 treatment (0.1, 1 and 5 μ M), after 3 (left panel) or 5 (right panel) DIV. The values in brackets refer to the molecular weight of the protein (kDa). B. Quantification of protein bands, showing that MAP4343 decreased synapsin I and PSD-95 levels in neurons after 3 days of treatment, while it increased them after 5 days. Data passed normality tests and are expressed as mean \pm S.E.M. n=6-11 independent cultures per group. MAP4343 effects were assessed using a one-sample t-test comparing each group to 1, that refers to vehicle condition (* p<0.05; ** p<0.001). C. Density of protrusion in neurons treated with 1 μ M of MAP4343. Data passed normality tests and are expressed as mean \pm S.E.M. n=8 independent cultures per group. MAP4343 effects were assessed using a one-sample t-test comparing each group to 1, that refers to vehicle condition.

Synaptic markers



Protrusions

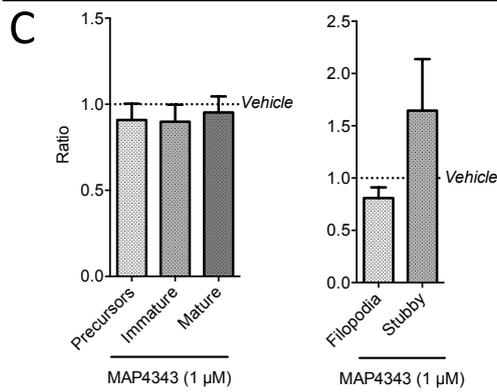


Figure 3. MAP4343 effects on BDNF and TrkB levels in cortical neurons *in vitro*.

A. BDNF ELISA assay in neurons treated for 5, 7, 10 and 13 days with MAP4343 (0.1, 1 and 5 μM), showing an increase in BDNF content in neurons after 7, 10 and 13 days under MAP4343 treatment.

B. Immunodetection by Western blot of TrkB protein in neurons treated for 5, 7, 10 and 13 days with the same doses of MAP4343. The values in brackets refer to the molecular weight (kDa).

C. Quantification of protein bands, showing that after 7 and 10 DIV, MAP4343 potentiated the expression of TrkB. All data passed normality tests and are expressed as means \pm S.E.M. $n=5-8$ independent cultures per group. MAP4343 effects were assessed using a one-sample t-test comparing each group to 1, that refers to vehicle condition (* $p<0.05$).

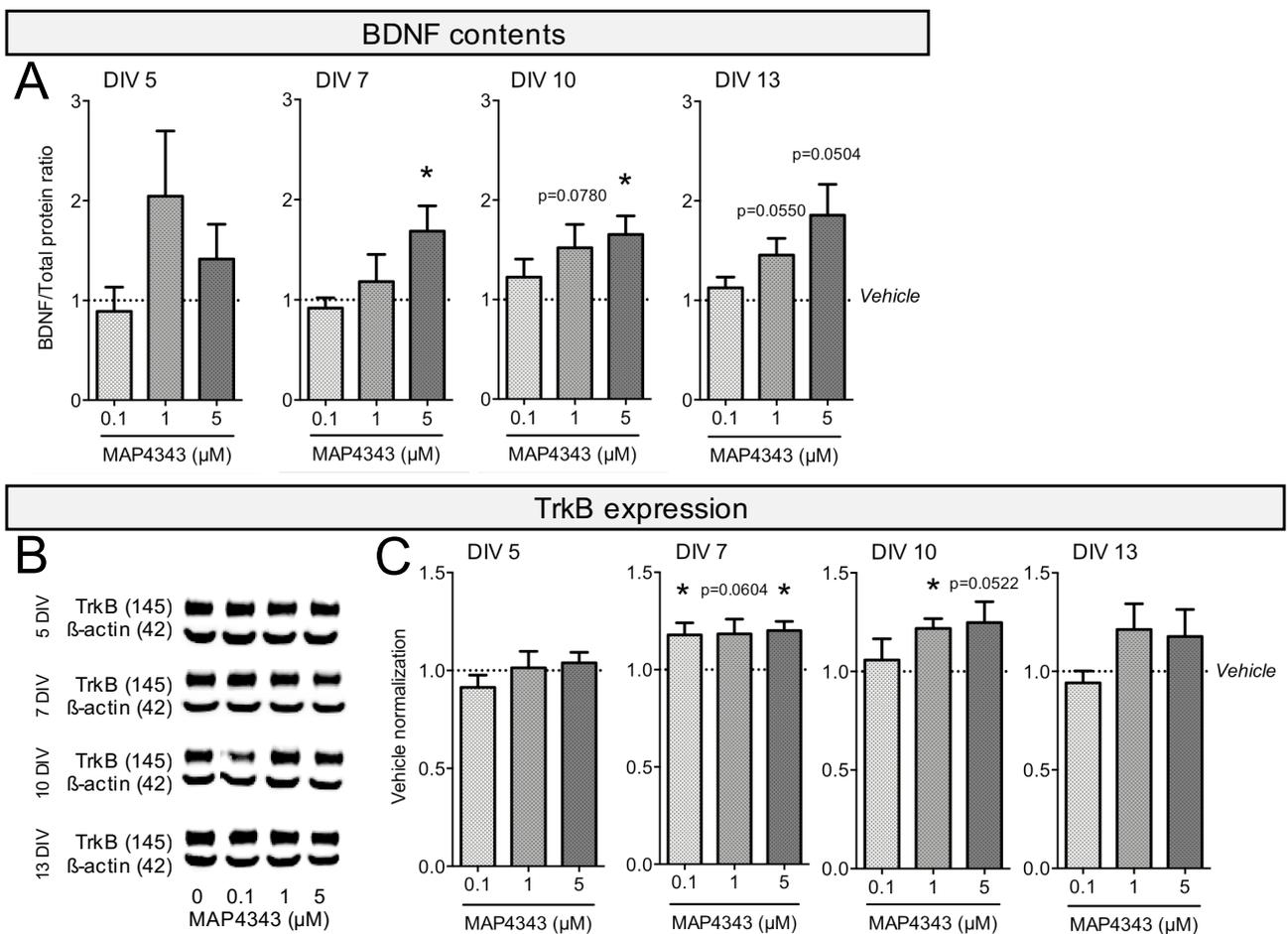


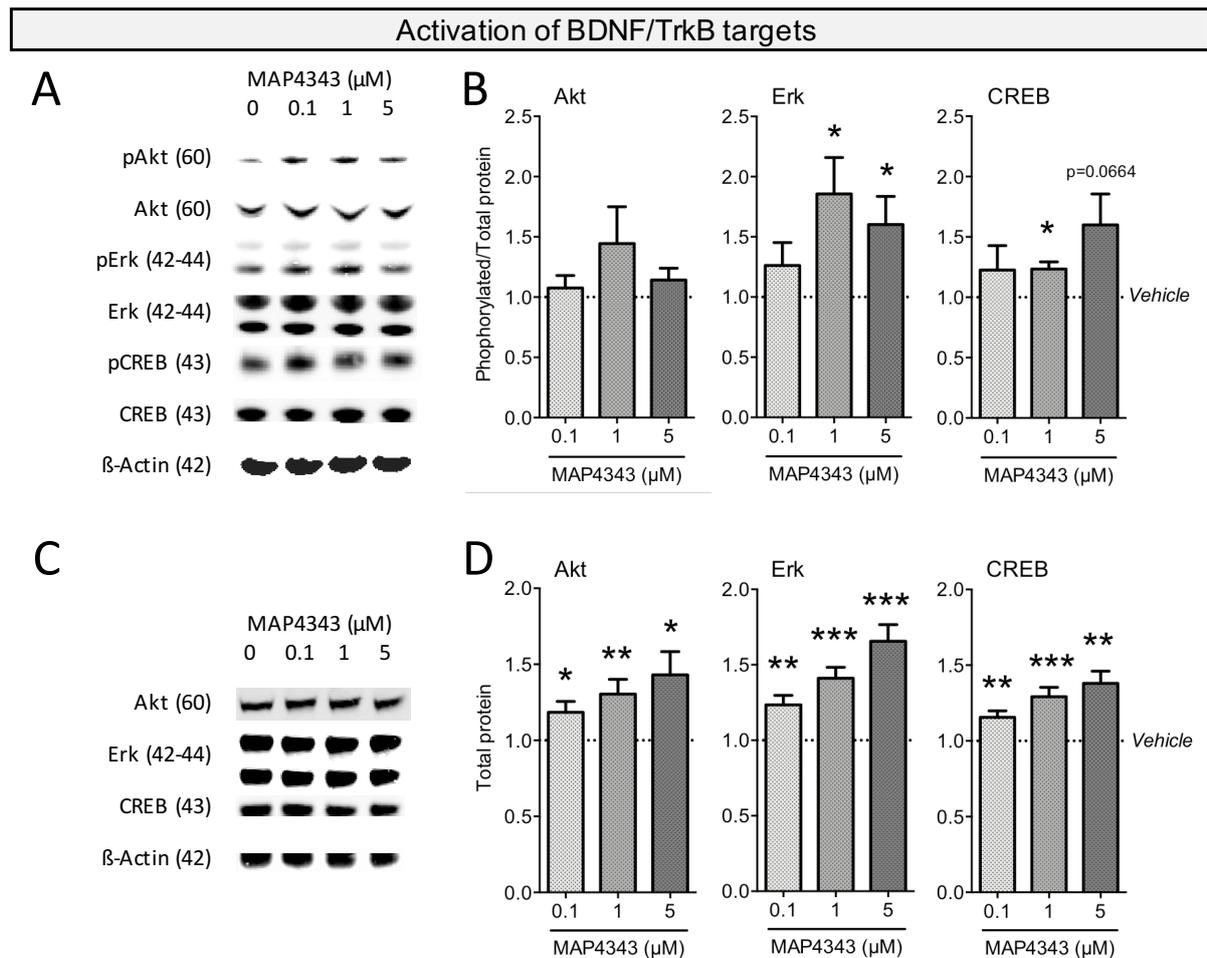
Figure 4. MAP4343 effects on targets of BDNF/TrkB signaling pathway in cortical neurons *in vitro*.

A. Western blot immunodetection of Akt, ERK, CREB proteins, and their respective phosphorylated forms in neurons treated for 5 days with MAP4343 (0.1, 1 and 5 μM).

B. Quantification protein bands, showing that MAP4343 (1 and 5 μM) increased the phosphorylated protein/total protein ratio of ERK and CREB, which reflected larger activation of these proteins when neurons were under MAP4343 treatment. n=5-7 independent cultures per group.

C. Western blot immunodetection of Akt, ERK and CREB in neurons treated for 13 days with MAP4343 (0.1, 1 and 5 μM).

D. Quantification protein bands, showing that MAP4343 (0.1, 1 and 5 μM) increased the total amount of Akt, ERK and CREB. n=10-13 independent cultures per group. The values in brackets refer to the molecular weight of the protein in kDa. All data passed normality tests and are expressed as mean \pm sem. MAP4343 effects were assessed using a one-sample t-test comparing each group to 1, that refers to vehicle condition (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).



General discussion

Overview of the major findings in the present work

MAP4343

Cytoskeleton

- ↗ Cortactin — Actin
- ↗ MAP2 — Microtubules

Intracellular signaling

- ↗ pERK and pCREB
- ↗ BDNF and TrkB
- ↗ Akt, ERK and CREB

Neuroplasticity processes

- ↗ Neurite outgrowth
- ↗ Bundled growth cones
- ↗ Hippocampal cell proliferation

Behavioral outcomes

- Antidepressant-like
- Procognitive

Figure 21. Main findings obtained in the present study. MAP4343 potentiated cytoskeleton- and signaling-related proteins, promoted neuroplasticity, and had antidepressant-like and procognitive activity.

This thesis project was conducted to further characterize the antidepressant-like activity and to identify the mechanisms of action of the pregnenolone derivative MAP4343. Our main data, listed in Figure 21, showed that MAP4343 leads to therapeutic-like and promnesic behavioral outcomes that could be sustained by positive modulation of neuroplasticity through cytoskeleton and BDNF/TrkB signaling.

Therapeutic-like activity of MAP4343

In the present thesis, we first aimed to further characterize the therapeutic-like activity of MAP4343. We showed, using behavioral approaches, that chronic administration of MAP4343 could reverse the depressive-like behavior of mice submitted to CSDS, and of mutated GRi mice. These data confirmed those reported in previous works conducted by Mapreg in which MAP4343 exhibited antidepressant-like activity in the isolation-rearing model in rats, and in the chronic psychosocial stress model in tree shrews (Bianchi & Baulieu, 2012; Parésys et al., 2016). Interestingly, our present results strongly suggested that MAP4343 therapeutic-like action could be specific to depressive states, since it could not reverse the anxiety-like state of GRi mice, in contrast to previous work from Mapreg reported by Bianchi and Baulieu (2012). This discrepancy between these data could be due to the way we assessed anxiety behavior, the open-field test, versus the elevated-plus maze previously used, and the rodent model, GRi mice versus rats. Moreover, the stress response is altered in GRi mice, through impairment in GR function, and it has been shown that when animals are submitted to these tests - elevated plus maze or open field test - anxiety-like behavior was accompanied by changes in the HPA axis function (Ennaceur, 2014). Hence, it is probable that the reported anxiolytic-like activity of MAP4343 may require the proper functioning of the HPA axis, which is not the case in GRi mice, and also probably in a subtype of depressive patients. In the CSDS model, we could not conclude on the potential anxiolytic properties of MAP4343. As deeply discussed in Article 1, the anxiety-like phenotype of mice submitted to CSDS was no longer observed after a 16-day chronic treatment with the vehicle of MAP4343. This was probably linked to the way the treatment was delivered (cookies), which, in contrast to intraperitoneal injections that may represent an additional chronic stress and constitute a negative reinforcement in mice, tended to normalize the stress state of animals and suppress the negative reinforcement of anxiety after CSDS.

However, regarding depressive-like behavior in the CSDS mice, MAP4343 clearly reversed altered interactive behavior in the social avoidance test. The interaction ratio of defeted MAP4343-treated mice was not anymore different than that of control unstressed HEC-treated mice, supporting the effective antidepressant action of this molecule previously demonstrated in other rodent models, such as rats and tree shrews (Bianchi & Baulieu, 2012; Parésys et al., 2016). Those data were further confirmed in GRi mice, where the splash test was used to assess the depressive-like behavior of these mice. Although the grooming phenotype of stress-related mice in this test is still a matter of debate, as discussed in the section 1.2.2 of the Introduction, we nevertheless found that the abnormal behavior of GRi mice was rescued by a chronic MAP4343 treatment. We actually found an increase in grooming duration in GRi mice, in opposite than that previously reported (Boulle et al., 2016). Hence, the increase in induced grooming can be related to an attempt to stress reduction, but a decrease could also reflect a despair behavior (Smolinsky et al., 2009). Moreover, these divergent effects could be due to the mouse model itself. Indeed, it is possible that its phenotype shifted due to changes in the testing environment in our lab. However, in any cases, the behavior in transgenic mice was different than that in control animals, and could be rescued by chronic treatment with different classes of antidepressants (Boulle et al., 2016), including MAP4343 as we showed here.

To assess the therapeutic-like effect of MAP4343 on cognition, we used the CSDS model since it was shown that this social stress induced long-lasting deficits in both short- and long-term memory, which can be assessed in the novel object recognition task (Jin et al., 2015; Zhao et al., 2013). Repeated MAP4343 treatment after the CSDS session reversed these memory alterations, evidencing another dimension of the antidepressant-like effects of MAP4343. Indeed, cognitive deficits are among the major symptoms in MDD, which are hardly treated by current antidepressants. Although some pharmacological compounds showed some positive action on cognition (Rosenblat, Kakar, & McIntyre, 2016), this symptom is insufficiently treated and a recent study reported that the cognitive deficits observed in MDD patients were still present in remitter patients (Soria et al., 2018). In this frame, our data clearly evidenced that MAP4343 rescued the cognitive deficits observed in a stress-related model of depression, and hence suggested that it could enhance cognition in depressed subjects, making this molecule a good candidate for acting on several

features, including mood and cognition. It should be nevertheless important to replicate these effects on tests that investigate other types of memory, or in other rodent models of impaired cognition. Unfortunately, although GRi mice were reported to have impairments in both short-term olfactory and fear memory (Boulle et al., 2016; Montkowski et al., 1995), we found no deficit in both short- and long-term recognition memory (data from our lab), making impossible to explore the cognitive effects of MAP4343 on these mice. Hence cognition could be altered in these animals only in specific memory subfields.

Several brain structures are impacted in MDD, including those that belong to the limbic system and the HPA axis. This is the case for the hippocampus, that is reduced in size, partly because of a loss in cell proliferation and a reduced complexity of the neuronal arbor (Sheline et al., 1996; Woolley, Gould, & McEwen, 1990). The paraventricular nucleus of the hypothalamus is also changed in MDD, since there is an increase in the number and activity of CRH-releasing neurons (Keck & Holsboer, 2001; Raadsheer et al., 1994). These data led us to conduct our molecular investigations in the hippocampus and the hypothalamus. In addition, both mood and cognition require the integrity of neuroplasticity processes as exposed in the Introduction of this manuscript. We selected several neuroplasticity markers to elucidate the effects of MAP4343 *in vivo*, including hippocampal cell proliferation, *Bdnf* exon IV and *MAP2* mRNA levels in the hippocampus, and hypothalamic amounts of *Crh*. Our results showed that MAP4343 therapeutic-like activity on depressive-like state and memory came with a surge in neuroplasticity markers, suggesting that this molecule improved *in vivo* neuroplasticity.

Cell proliferation in the hippocampus participates to neurogenesis. In human, most of the *post-mortem* studies have evidenced that in adult, new neurons continued to be generated with a modest decline during aging (Eriksson et al., 1998; Reif et al., 2006; Spalding et al., 2013). Moreover, although questioned by Sorrells and colleagues (Sorrells et al., 2018) who suggested that human neuroplasticity could differ from other species, it has been recently confirmed that in human dentate *gyrus*, about 700 new neurons were generated per day whatever the age (Boldrini et al., 2018). Although in MDD, *post-mortem* studies revealed disparities regarding neurogenesis (Lucassen, Stumpel, Wang, & Aronica, 2010; Reif et al., 2006), animal studies actually evidenced a reduction of this process after a chronic stress (Drew & Hen, 2007; Warner-Schmidt

& Duman, 2006), probably because of the long-term exposure to glucocorticoids in this context (Mirescu & Gould, 2006; Sapolsky et al., 1985). Adult hippocampal neurogenesis has even been proposed to be necessary for the behavioral efficacy of antidepressants (Santarelli et al., 2003). In our study, we also found that in GR1 mice, where neurogenesis was shown to be altered (Boulle et al., 2016; Paizanis et al., 2010), MAP4343 was able to recruit this process, further linking antidepressant activity and proplastic effects of this molecule.

We also obtained additional data further evidencing that this compound could modulate neuroplasticity *in vivo*. As mentioned in the section 5.2.1 of the Introduction, the main function of MAP2 is to promote microtubule stabilization (Goodson & Jonasson, 2018), suggesting a central role in structural plasticity. *Post-mortem* studies in MDD patients revealed a decrease in the hippocampal expression of MAP2 gene (Duric et al., 2013). Consistently, animal studies showed that hippocampal MAP2 mRNA and protein levels were decreased in mice submitted to chronic stress, and normalized by antidepressant treatments (Martin et al., 2017; Stein-Behrens et al., 1994; Yang et al., 2014). In addition, a correlation between altered expression of MAP2 in the hippocampus and cognitive decline has been proposed (Liu, Zhang, Zheng, & Zhang, 2005). Interestingly, we showed here that MAP2 mRNA levels were decreased in defeated stress mice, and that the behavioral improvements induced by MAP4343 chronic administration in these animals were associated with an increase in the expression of MAP2 mRNA levels in the hippocampus. Although no correlation has been found, these data strongly suggested that the therapeutic-like effect of MAP4343 on both the depressive-like phenotype and memory performances might be related to its action on neuroplasticity. Given the fact that MAP2 is a neurosteroid receptor (Fontaine-Lenoir et al., 2006), it is probable that MAP4343 upregulated the expression of this protein to potentiate its own activity and it can be hypothesized that the rise in MAP2 in this structure could be responsible for MAP4343 behavioral effects, at least in part.

Whether the HPA axis, and more particularly the hypothalamic CRH, plays a role in the effects of MAP4343 was also investigated in this study. Hypothalamic CRH has several actions. It is described as the key hypothalamic factor controlling the HPA axis because it triggers its activation. In *post-mortem* analyses of patients that committed suicide, *Crh* mRNA levels were found increased in the hypothalamus

(Merali et al., 2004). This is believed to be related to the HPA axis hyperactivity. Nevertheless, animal studies revealed that both CRH knockdown in the paraventricular nucleus and the blockage of its type 1 receptor led to memory deficits in mice (Deak et al., 1999; Lucas, Chen, & Richter-Levin, 2013), suggesting its role in cognition. In the present study, we found that MAP4343 induced a surge in the hypothalamic *Crh* mRNA amounts, and this could be related to the memory reversal observed in defeated mice after chronic administration of the molecule. These data questioned the meaning of the surge in hypothalamic CRH under MAP4343 treatment, regarding what was described in findings from *post-mortem* analyses of depressed patients. It is possible that the rise observed in the disease is a compensatory mechanism, which would be nevertheless insufficient to reverse the depressive state. Currently prescribed antidepressants, mainly targeting the monoamine systems, were shown to downregulate the HPA axis activity (Buhl et al., 2010), probably causing reduced hypothalamic CRH amounts. In the light of our data, it could be hypothesized that a lack in CRH levels would explain why these pharmacological compounds do not properly correct the cognitive deficits reported in MDD. In contrast, the beneficial effect of MAP4343 on cognition could be related to the increase in CRH levels under this treatment.

Finally, at the neurotrophic level, it has been consistently shown that *Bdnf* exon IV was strongly involved in mood since, for example, its deletion *in vivo* leads to depressive-like behavior in mice (Sakata, Jin, & Jha, 2010). It is also a key actor of cognition, because it has been clearly demonstrated that consolidation of fear learning was associated with an upregulation of this exon (Lubin, Roth, & Sweatt, 2008). In the present study, defeated mice displayed increased *Bdnf* exon IV levels under MAP4343 chronic treatment, which is consistent with the fact that antidepressants are able to induce a surge in BDNF amounts in limbic brain regions such as the hippocampus (Boulle et al., 2012; Castrén & Kojima, 2017). Hence, MAP4343 therapeutic-like effects probably requires BDNF function. The involvement of BDNF in the mechanism of action of MAP4343 was further investigated and will be developed below.

In this first part, we aimed to further characterize the therapeutic-like activity of MAP4343. We could conclude that when chronically administered, MAP4343 upregulates neuroplasticity-related genes, such as *Bdnf* exon IV and *MAP2* in the

hippocampus, but also hypothalamic *Crh*, that are probably responsible for the antidepressant-like action of the molecule, which includes the reversal in the stress-induced mood and memory deficits.

Proplastic effects of MAP4343

In the previous section, we showed that MAP4343 therapeutic-like effects required neuroplasticity-related processes. Interestingly, when chronically treated with this molecule, unstressed mice had greater memory performances than vehicle-treated mice. These promnesic properties were accompanied by an increase in the expression of *Bdnf* exon IV mRNA in the hippocampus. These findings suggest that, besides its antidepressant-like activity, MAP4343 also has procognitive activity, further evidencing the recruitment of neuroplasticity actors.

In vitro, neuroplasticity can also be assessed by protrusion study, and measurement of synaptic protein expression. We used DiOloistic labeling of cultured hippocampal neurons, considering that it was a good strategy for dendritic spine staining, because of its high reproducibility, and unlike the Colgi-Cox technique, its rapidity to complete and its safety, since it does not require any harmful component (Staffend & Meisel, 2011). In our experiments, a 3-day treatment of hippocampal neurons with MAP4343 was not sufficient to evidence a potential enhancement of both protrusion maturation and pre- and post-synaptic marker expression. Rather, we reported a decline in PSD-95 and synapsin I, that seemed to be related to their non-synaptic functions during development (Sweet et al., 2011). Indeed, PSD-95 could interact with microtubules, causing a disruption in their organization and a reduction in dendritic branching (Charych et al., 2006). When MAP4343 was applied 2 more days, neurons displayed a surge in synaptic marker expression. Interestingly, at this stage, cortactin, a protein associated to the actin cytoskeleton and described as being enriched in dendritic spines (Konietzny, Bär, & Mikhaylova, 2017; Spence, Kanak, Carlson, & Soderling, 2016), was also increased with MAP4343. We did not measure the protrusion profile in this case, making difficult to conclude toward an enhancement in synaptic plasticity, but since the expression of cortactin and synaptic markers was upregulated, it is possible that MAP4343 induced a rise of mature protrusion density after 5 days of treatment.

Further investigations showed that MAP4343 increased the proportion of bundled growth cones. The distal part of growth cones mostly contains actin filaments, whereas the proximal part is enriched in microtubules (Dent, Gupton, & Gertler, 2011). Hence, this effect could be sustained by MAP4343 ability to modulate microtubule cytoskeleton and perhaps actin, through cortactin upregulation. More precisely, it can be hypothesized that the cross-talking between the two kinds of cytoskeleton was responsible for such effect. Indeed, this cross-talk is critical for several cellular processes, such as dendritic arbor morphology (Geraldo, Khanzada, Parsons, Chilton, & Gordon-Weeks, 2008; Swiech et al., 2011). For example, the growing end of microtubules could interact with the actin cytoskeleton through a complex formed by CLIP-170 and the GTPase-activating protein 1 containing a IQ motif (Fukata et al., 2002; Watanabe et al., 2004). Because CLIP-170 is a receptor for pregnenolone and possibly for MAP4343 also (Weng et al., 2013), MAP4343 could exert its proplastic effects on neuronal morphology by acting on microtubule and actin cytoskeletons, through MAP2, cortactin and CLIP-170.

We also evidenced that neurite outgrowth of hippocampal neurons was enhanced under MAP4343 treatment. Because neuronal processes are mostly composed of microtubules, this could be due to the MAP4343 ability to bind to MAP2 and to promote microtubule assembly (Fontaine-Lenoir et al., 2006). These findings came with a surge in MAP2 expression, which was consistent with the data we obtained *in vivo*, where a chronic treatment with MAP4343 also increase the expression of MAP2 in the hippocampus of defeated mice. Interestingly, early studies demonstrated that dendrite branching was correlated with MAP2 phosphorylation state *in vitro* (Audesirk et al., 1997; Diez-Guerra & Avila, 1993). In fact, when dephosphorylated, MAP2 binds to microtubules and promotes their stability and rigidity. It seems that MAP2 action on microtubules does not occur at the growing end. Hence, it may provide stability at the other end, enabling the microtubule to grow through polymerization. When phosphorylated, MAP2 dissociates from microtubules, that are therefore more dynamic, and this results in an increase in neurite branching (Sánchez et al., 2000). Overall, we can propose that when binding to microtubule, which induces stability, MAP2 could be involved in neurite elongation, whereas microtubule dynamics, observed following MAP2 phosphorylation and dissociation, could be critical for branching. It is noteworthy that taxol, a drug that prevents

microtubule depolymerization and promotes their stability, inhibits neurite growth (Letourneau & Ressler, 1984). Hence, it seems that neurite growth requires an optimal state between microtubule dynamic and stability. Because MAP4343 acted only slightly on neurite branching, and significantly on elongation, it is possible that in our experiments, MAP2 was rather dephosphorylated and attached to microtubules. Additional studies regarding the phosphorylation state of MAP2 should be addressed to test this hypothesis, which would suggest that under MAP4343 treatment, the increase in the binding complex MAP2/microtubule, hence favoring microtubule polymerization, led to neurite outgrowth.

MAP2 is a substrate of several kinases, including Akt, PI3K, CaMKII, and ERK (Sánchez et al., 2000), that are necessary for neurite elongation (Figure 22A). For example, Akt has dual role in neurite outgrowth. When acting through the inactivation of GSK3, Akt induces a reduction in MAP2 phosphorylation, leading to microtubule polymerization and neurite elongation (Figure 22B) (Lim & Walikonis, 2008). However, Akt favors neurite branching via mTOR activation, suggesting that its action involves MAP2 phosphorylation (Figure 22B) (Verma et al., 2005). In addition, the transcription factor CREB, which can be activated by Akt, PI3K, CaMKII, and ERK, is strongly involved in neuroplasticity processes including neurite outgrowth (Jessen et al., 2001). Conversely, it has been demonstrated that MAP2 is an anchoring protein of PKA in dendrites, and is necessary for CREB activation induced by this kinase (Figure 22A) (Harada et al., 2002). In addition, this interaction could be independent from MAP2 phosphorylation, hence suggesting the involvement of other actors in this mechanism (Huang et al., 2013).

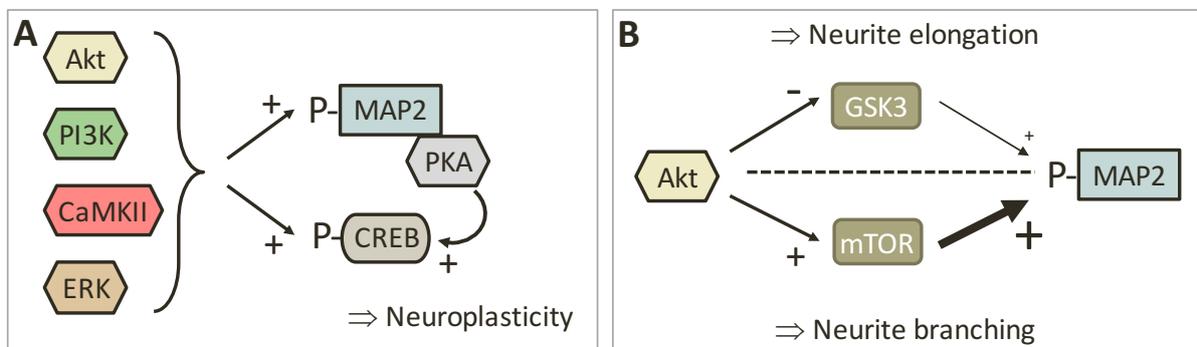


Figure 22. Interaction between MAP2 and intracellular kinases. A. Kinases Akt, PI3K, CaMKII, and ERK can phosphorylate MAP2 and CREB to trigger neuroplasticity. PKA anchors to MAP2, leading to CREB activation. B. Akt differentially modulates the phosphorylation state of MAP2 through either GSK3 or mTOR to promote neurite elongation or branching respectively.

We evidenced here that MAP4343 could enhance neuroplasticity through cytoskeleton modulation, including by the mobilization of its target MAP2, that may promote proper intracellular signaling to enhance neurite outgrowth. All the kinases mentioned above and potentially acting on MAP2 can be activated by BDNF binding to its high-affinity receptor TrkB. Given the role of BDNF in antidepressant treatment and in plasticity, we hypothesized that MAP4343 mechanism of action could involve BDNF/TrkB signaling.

MAP4343 and BDNF/TrkB signaling

In vivo and *in vitro* investigations conducted in the present work showed that MAP4343 could enhance BDNF amounts, both in the hippocampus of unstressed and defeated mice, and in cultured cortical neurons. We decided to focus on kinases CaMKII, Akt, ERK that all activate CREB, a transcription factor. CREB activity leads to the production of neuroplasticity proteins (Wadhwa et al., 2015), but it also induces a surge in BDNF levels in cells, meaning that this protein is able to upregulate its own activity (Cunha et al., 2010).

Regarding CaMKII, we failed to obtain the right tool to study its activation state in presence or absence of MAP4343. Experiments are currently conducted in the lab because this kinase is critical for neuroplasticity processes, and was shown to be involved in learning in rats (Ghosh et al., 2015).

In cultured cortical neurons, MAP4343 treatment stimulated different actors of the signaling pathways triggered by BDNF binding to TrkB. Actually, its effects were time-dependent: first, a surge in the activation of ERK and CREB was observed, followed by an increase in BDNF and TrkB expression, finally resulting in elevated cellular levels of total Akt, ERK and CREB (Figure 23).

The delayed action of MAP4343 on BDNF, TrkB, Akt, ERK and CREB levels could be explained by the fact that a surge in ERK phosphorylation leads to increased CREB activation. As mentioned above, CREB is responsible for the production of the different proteins recruited by BDNF/TrkB binding (Cunha et al., 2010). So it is probable that the changes in protein level observed under treatment could be the consequence of CREB activation. However, the mechanism by which MAP4343 induced this activation remains elusive. Indeed, MAP4343 could directly activate

CREB by increasing its phosphorylation, which would suggest a kinase activity of this molecule, although the most plausible hypothesis is that it is the result of ERK activation by MAP4343.

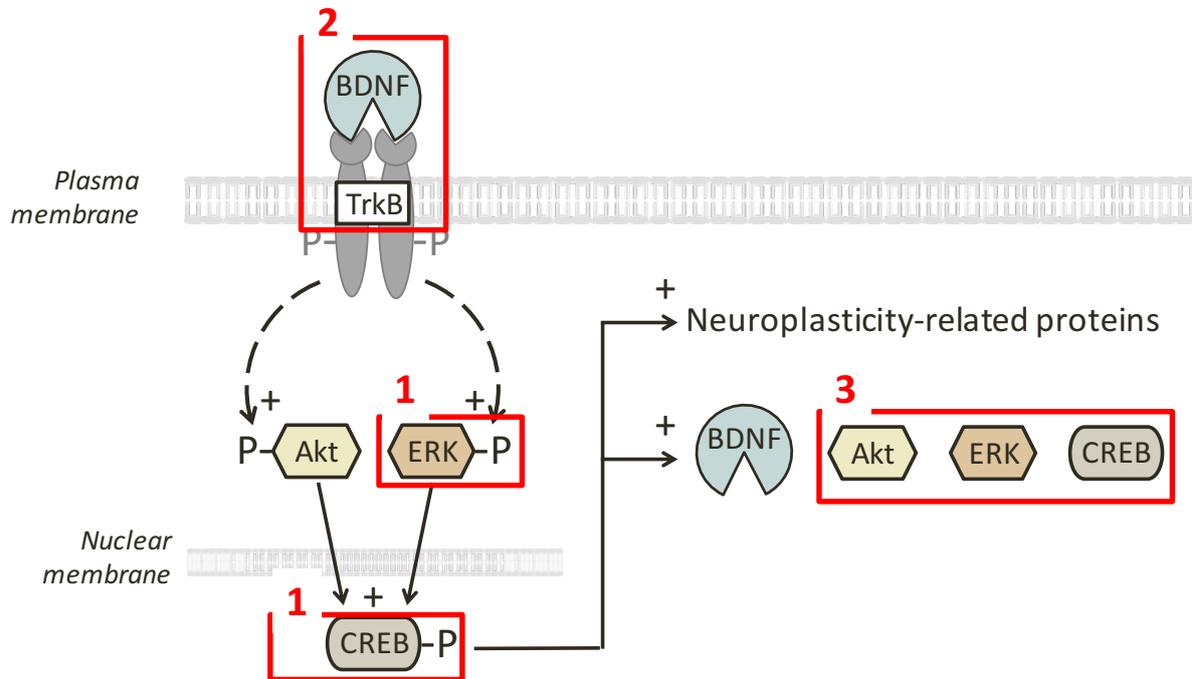


Figure 23. MAP4343 time-dependent action in BDNF/TrkB signaling. 1. A rise in ERK and CREB phosphorylation occurred after 5 days of treatment; 2. BDNF and TrkB expression are increased after 7 and 10 days; 3. Total Akt, ERK and CREB are surged after 13 days.

ERK was shown to be associated with both microtubules and actin (Müller, Langenbach, Kaminski, & Rychly, 2013; Reszka, Seger, Diltz, Krebs, & Fischer, 1995). Also, both ERK and Akt can be directly activated by actin cytoskeleton rearrangement, induced by mechanical pressure in plasma membrane, and this activation highlights the role of actin cytoskeleton in the conversion from mechanical to biochemical signals (Müller et al., 2013). The data we obtained and described in the previous section suggested that MAP4343 could directly modulate the cytoskeleton. Hence, it is possible that it activates ERK by means of both actin filaments and microtubules. ERK can also be activated by intracellular calcium, coming from the extracellular compartment or the endoplasmic reticulum, and acting through a cascade involving Ras-GTP and Raf kinase (Thomas & Huganir, 2004). Calcium influx has been demonstrated to be critical for neuroplasticity processes, including actin-dependent morphological plasticity in dendritic spines (Brünig et al., 2004). Hence, it would be

interesting to measure the effect of MAP4343 treatment in neuronal calcium levels, since the exact mechanism by which this molecule increases the phosphorylation of ERK still needs to be investigated. In addition, MAP4343 effects on ERK, MAP2 and cortactin occurred in the same time-scale (after 5 days of treatment), so it is unclear if all these events are independent or if one is responsible for another.

Overall, a partial intracellular mechanism of action of MAP4343 could be evidenced. We showed that MAP4343 increased the expression of cortactin, demonstrating for the first time a potential regulation of the actin cytoskeleton by this molecule. MAP4343 was also able to increase the phosphorylation of several intracellular kinases that are involved in BDNF/TrkB signaling. Finally, all these actions have been found to result in enhanced neuroplasticity, and this is strongly suspected to be responsible for MAP4343 therapeutic-like activity.

Perspectives

The findings gathered during this thesis project brought answers to several questions, but also gave rise to new hypotheses. The therapeutic-like activity of MAP4343 in models of depression is now well established, as well as its proplastic properties, both *in vivo* and *in vitro*. However, its mechanism of action remains in part unknown.

We evidenced that MAP4343 was able to modulate several actors of the BDNF/TrkB signaling. To date, it is still unestablished whether this compound actually requires the binding of BDNF to TrkB to mediate its actions. A strategy using antagonists for the receptor TrkB could allow to estimate the involvement of this interaction in MAP4343 effects. Moreover, to know whether the proplastic activity of MAP4343 was rather mediated by kinase activations, inhibitors against these enzymes could be applied under MAP4343 treatment, to see if they could prevent the proplastic effects of MAP4343. These experiments would give evidence regarding the direct involvement of BDNF/TrkB and different kinases in MAP4343 mechanism of action.

MAP4343 was able to increase ERK activation, via an unknown mechanism (Figure 24). Since it has been proposed that ERK can be directly activated by both actin filaments and microtubules (Müller et al., 2013; Reszka et al., 1995), the activity of MAP4343 on the cytoskeleton would need to be further characterized. First, MAP4343 can bind to MAP2, leading to enhanced microtubule polymerization (Fontaine-Lenoir et al., 2006), more precisely at the growing end. On the other hand, MAP4343 enhanced cortactin expression. The type of interaction MAP4343 has with this protein and the subsequent consequences on actin filaments remain elusive. As mentioned above, the cross-talk between actin filaments and the growing end of microtubules is involved in neuroplasticity processes (Geraldo et al., 2008; Swiech et al., 2011), and requires a plethora of actors, including CLIP-170 (Fukata et al., 2002; Watanabe et al., 2004). This protein is a receptor for pregnenolone (Weng et al., 2013), similarly than MAP2, and it is hence possible that MAP4343 potential effect on the cross-talk between actin and microtubules could act through CLIP-170. Thus, the emerging hypothesis would be that MAP4343 can activate ERK through modulation of microtubule and/or actin cytoskeleton.

ERK can also be activated by calcium influx (Thomas & Huganir, 2004), leading to promoted neuroplasticity (Brünig et al., 2004) (Figure 24). Hence another hypothesis

regarding MAP4343 mechanism of action could be that it induces a surge in calcium levels in neurons.

Once ERK is activated, it can induce the phosphorylation of MAP2, leading to its dissociation from microtubule. This results in an increase in microtubule dynamics associated to potentiated formation of branching points *in vitro* (Sánchez et al., 2000). We showed that MAP2 expression is upregulated concomitantly with increased ERK activation under MAP4343 treatment. Therefore, the measure of MAP2 phosphorylation and neurite branching points at this stage could further enlighten the MAP4343 mechanisms of action.

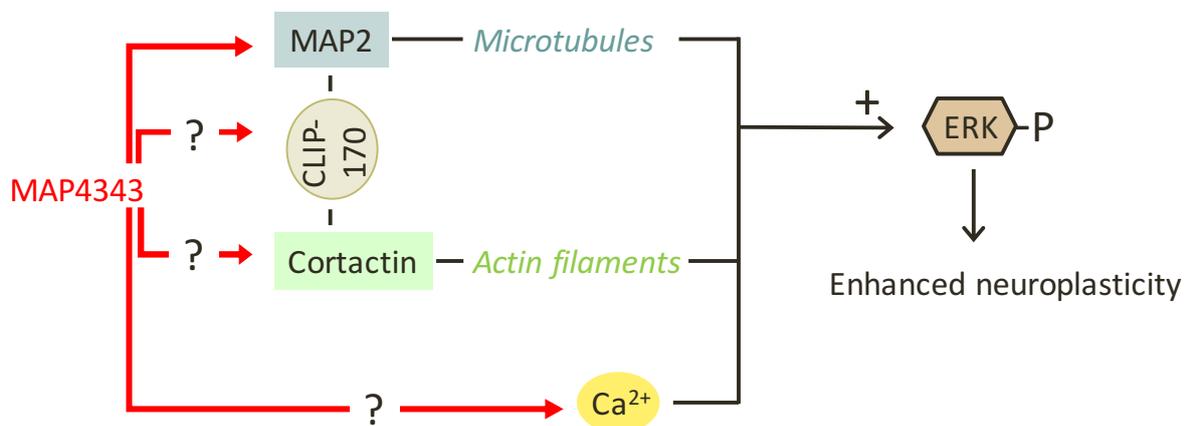


Figure 24. Possible mechanisms of action of MAP4343 leading to ERK activation.

Conclusions

The aim of the present thesis project was to further characterize the antidepressant-like activity of MAP4343, and to study its mechanism of action.

We confirmed the antidepressant-like activity of MAP4343 in stress-related behaviors that was previously reported, and showed that it had additionally procognitive properties. This conducted us to investigate the mechanism of action of this molecule focusing on neuroplasticity-related features. MAP4343 could increase neuroplasticity *in vivo*, by promoting cell proliferation and the expression of plasticity-related genes in the hippocampus. Neuroplasticity was also enhanced *in vitro* under MAP4343 treatment, since it induced morphological changes together with upregulation of cytoskeleton-related proteins and intracellular kinases.

Altogether, the gathered data obtained in this work allowed us to conclude that MAP4343 has an antidepressant-like activity in several animal models, also including the reversal of stress-induced memory deficits. Therefore, MAP4343 could be a new and promising strategy to reverse symptoms in MDD patients more efficiently than currently prescribed antidepressants. This is supported by its original mode of action since, rather than targeting the monoamine system as most of compounds used in clinic, MAP4343 acts through the cytoskeleton to enhance neuroplasticity and exert its therapeutic activity.

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Abstract

Major depressive disorder is a mental illness characterized by several symptoms, including low mood and cognitive deficits, that could rely on neuroplasticity alterations. Current pharmacotherapy displays significant limitations since 30 to 40% of patients fail to achieve remission. In addition, the classical treatments do not properly alleviate the cognitive impairments observed in depressive patients. MAP4343 is a pregnenolone derivative that displayed antidepressant-like activity in animals, triggering faster efficacy than fluoxetine, a selective serotonin reuptake inhibitor currently prescribed in patients. Moreover, MAP4343 promoted cellular process outgrowth, probably through its binding to microtubule-associated protein 2 (MAP2). Given these findings, we hypothesized that MAP4343 antidepressant-like activity may encompass the reversal of cognitive deficits. Hence, the aim of this thesis was to evidence the effects of MAP4343 on stress-induced cognitive impairments, and to elucidate its mechanism of action related to the cellular pathways that trigger plasticity. MAP4343 action on depressive-like behavior was evaluated in mice subjected to chronic stress induced by social defeat, and in transgenic mice having altered stress response. MAP4343 had antidepressant-like activity in both models, and restored cognition in defeated animals. This molecule also had pro-cognitive action in unstressed mice, suggesting that MAP4343 could promote neuroplasticity. Using primary neuron cultures, we found it promoted neurite outgrowth and growth cone bundling, and upregulated the expression of both actin- and microtubule-related proteins. Moreover, MAP4343 increased the activation and/or the expression of various intracellular kinases, including extracellular signal-regulated kinase (ERK), together with the transcription factor cyclic adenosine monophosphate-responsive element binding (CREB), considered as key actors in neuronal plasticity pathways. The emergent hypothesis was that MAP4343 activity on the cytoskeleton could be responsible for ERK and CREB activation, leading to an upregulation of brain-derived neurotrophic factor signaling pathways triggered by its binding to its high affinity receptor tropomyosin receptor kinase B. Overall, the results obtained in this work showed that MAP4343 could be a serious candidate for depression treatment, since it directly promoted neuroplasticity and enhanced cognitive performances through an original mechanism of action involving the cytoskeleton.