Åbo Akademi University

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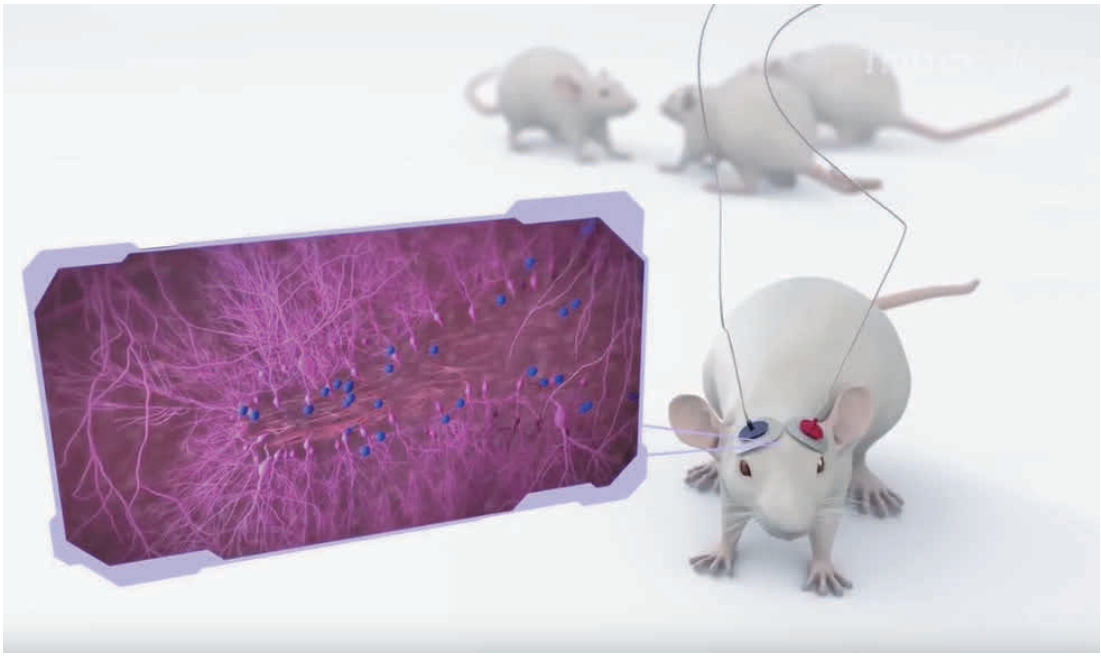
c-Jun N-terminal Kinase (JNK) Regulation of Neuroplastic Changes in Brain Associated with Anxiety- and Depressive-like Behavior



Francesca Marchisella

Francesca Marchisella obtained her Bachelor's in Biotechnology at University of Milano-Bicocca. She then obtained her Master's degree in Medical Biotechnology at University of Milano-Bicocca in 2013. She enrolled in a PhD program in January 2014 working in the laboratory of Prof. Eleanor Coffey at Turku Centre for Biotechnology, University of Turku and Åbo Akdemi University. She is currently working as a research fellow in the laboratory of Prof. Marco Riva at University of Milan.

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Francesca Marchisella

Turku Centre for Biotechnology
University of Turku and Åbo Akademi University
Department of Biosciences

Åbo Akademi University
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From the Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, Faculty of Science and Engineering, Åbo Akademi University, and Doctoral Network in Molecular Biosciences (MolBio)

Supervised by

Docent Eleanor Coffey, Ph.D. Research Director
Åbo Akademi University
Turku Centre for Biotechnology Turku, Finland

Reviewed by

Docent Iiris Hovatta, Ph.D.
Department of Psychology and Logopedics
University of Helsinki

and

Docent Marku Koulu, MD, Ph.D.
Professor of Drug Development, emeritus
Institute of Biomedicine
University of Helsinki

Opponent

Docent Paul J Lucassen, Ph.D.
Professor at Faculty of Science, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands

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ABSTRACT / ABSTRACT IN SWEDISH

Anxiety disorders and clinical depression are leading causes of disability worldwide and affect more than 300 million people of all ages, more prominently women (World Health Organization, 2017). Comorbid depression and anxiety disorders appear in up to 75% of patients, and to further complicate the picture, higher severity and chronicity of illness, reduced quality of life, treatment resistance and poor therapeutic effects are expected when comorbidity of anxiety with depression is diagnosed.

Current therapies stimulate neuroplastic changes in the brain, as a basis for their therapeutic action. In particular, it has been shown that several classes of antidepressants stimulate adult neurogenesis in the hippocampal formation, which is one brain region mostly affected by these two conditions. Nevertheless, current pharmacotherapy conveys a number of drawbacks, including delayed onset of clinical effects and debilitating side effects. Such inefficiency is most likely due to a lack of knowledge of the neuroanatomical and physiological changes occurring during the disease state. Thereby, unravelling the etiology and the pathophysiological mechanisms leading to the aberrant cerebral activity caused by the illnesses might ultimately overcome the downsides of current therapies.

Most recently, the Mitogen Activated Protein kinases (MAPs) have been linked to the development of mood disorders. In particular, the c-Jun N-terminal kinase (JNK) subfamily of MAPKs are activated by different external stressors, such as DNA damage, oxidative stress, cytoskeletal changes, infection or inflammation, and the JNK signaling has been found to be fundamental in brain developmental processes. Moreover, JNK activity modulates brain structures and neuronal morphology. Thus, generation of JNK inhibitors has been progressing as a new attractive avenue for more targeted therapies with less adverse side effects.

The work presented in this thesis tackles the role of JNK signaling in regulation of adult hippocampal neurogenesis and in the control of anxiety-like and depressive-like behavior in mice. By using *Jnk1* knockout mice and pharmacological inhibition of JNK pathway, the kinase activity was found linked to adult neurogenesis and emotional behavior in mice. More specifically, *Jnk1-/-* mice exhibited low anxiety-like and depressive-like phenotype in a battery of behavioral tests. Remarkably, genetic and pharmacological inhibition of JNK heightened proliferation, maturation and survival of the newly born neurons of the hippocampus and facilitated dendrite arborization of newborn neurons, suggesting promotion of integration of the adult born cells into the pre-existing circuitry of the hippocampus. Specific retroviral targeting of JNK in the adult born granule cells of the hippocampus produced low anxiety-like phenotype after four weeks of viral expression and decreased depressive-like phenotype eight-week post-injection. The last part of this thesis aimed to investigate synaptic changes of the adult born granule cells of the hippocampus upon

JNK kinase inhibition. Using a calcium indicator downstream of JNK inhibition, we recorded the activity of newly born neurons in awake mice. Our preliminary data indicate that JNK inhibition appears to increase adult born granule cells synaptic activity at four weeks of age in mice undergoing social interaction, and at six weeks of age in response to open field and enriched environment exposure. Altogether, the data presented in this thesis provide evidence on JNK modulation of the neurogenic niche of the hippocampus and of the dentate gyrus synaptic activity. Furthermore, these findings highlight the JNK pathway as a putative novel drug target against anxiety and mood disorders.

FÖRORD

Ångeststörningar och klinisk depression är globalt en av de främsta orsakerna till funktionsnedsättning. Dessa sjukdomar berör mer än 300 miljoner människor i alla åldrar och är vanligare hor kvinnor än hos män (Världshälsoorganisationen, 2017). Depression och ångeststörning samexisterar hos upp till 75 % av patienter. Vanligt är då att sjukdomen är allvarligare och mera kronisk, med lägre livskvalitet och sämre terapirespons som följd.

De nuvarande läkemedlens terapeutiska effekt baserar sig på att de stimulerar neuroplastiska förändringar i hjärnan. Man har visat att flera klasser av antidepressiva läkemedel stimulerar neurogenes i vuxen ålder i hippocampus – ett av de hjärnområden som mest påverkas av dessa sjukdomar. Tyvärr har de tillgängliga läkemedlen flera nackdelar, som att det tar lång tid efter att behandlingen påbörjats innan läkemedlet börjar ger effekt, och att besvärliga biverkningar är vanliga. Läkemedelsbehandlingens ineffektivitet beror troligen på otillräcklig kunskap om de neuroanatomiska och fysiologiska förändringar som sjukdomstillståndet förorsakar. Genom att klarlägga etiologin och de patofysiologiska mekanismer som leder till avvikande hjärnaktivitet förorsakad av sjukdomarna, kunde det vara möjligt att komma över problemen med nuvarande terapier.

De mitogenaktiverade proteinkinaserna (MAPK) har nyligen visats ha ett samband med utvecklandet av affektiva störningar. Speciellt c-Jun N-terminal kinas (JNK) underfamiljen av MAPK aktiveras av olika yttre stressfaktorer så som DNA-skada, oxidativ stress, förändringar i cytoskelettet, infektion eller inflammation. JNK-signalering har också upptäckts spela en fundamental roll för hjärnans utvecklingsprocesser. JNK-aktivitet modulerar dessutom hjärnstrukturer och nervcellernas morfologi. Utveckling av JNK-blockerare har föreslagits som en möjlig väg till mera fokuserad terapi som har mindre biverkningar.

Arbetet som presenteras i den här avhandlingen tacklar JNK-signaleringens roll i regleringen av vuxen neurogenes i hippocampus och i ångest- och depressionsliknande beteenden hos möss. Genom att undersöka Jnk1-knockout möss eller farmakologiskt blockera JNK-signalering visades kinasaktiviteten vara kopplad till vuxen neurogenes och emotionella beteenden hos möss. Jnk1-/- möss uppvisade minskat ångest- och depressionsliknande beteende i ett batteri med beteendetester. Påfallande nog ledde genetisk och farmakologisk blockering av JNK till ökad celldelning, mognande, överlevnad och dendritförgrening hos hippocampus nyfödda nervceller. Tillsammans tyder detta på att blockering av JNK främjar de nyfödda nervcellernas integrering i de redan existerande nervcellskretsarna i hippocampus. Blockering av JNK specifikt i de nyfödda nervcellerna i hippocampus med hjälp av injektion av retrovirusvektorer ledde till minskat ångestliknande beteende fyra veckor efter virusinjektionen och minskat depressionsliknande beteende åtta veckor efter

virusinjektionen. Den sista delen av avhandlingen hade som mål att undersöka synaptiska förändringar hos de nyfödda nervcellerna i hippocampus efter blockering av JNK. Med hjälp av en calciumindikator nerströms efter JNK-blockering studerade vi aktiviteten hos nyfödda hippocampusnervceller i vakna möss. Våra preliminära resultat indikerar att blockering av JNK verkar leda till ökad synaptisk aktivitet i de nyfödda granulära nervcellerna hos möss under beteendetest då mössen samverkar socialt eller placeras i en stimulerande omgivning. Sammanfattningsvis visar resultaten som presenteras i avhandlingen att JNK modifierar den neurogena nischen och aktiviteten i gyrus dentatus i hippocampus. Resultaten lyfter också fram JNK-signalering som ett möjligt mål för nya läkemedel mot ångest och affektiva störningar.

LIST OF ORIGINAL PUBLICATIONS

The work described in this thesis is based on the following original publication and manuscript that are referred to in the text by their Roman numerical.

I) Mohammad H*, **Marchisella F***, Ortega-Martinez S*, Hollos P, Eerola K, Komulainen E, Kulesskaya N, Freemantle E, Fagerholm V, Savontous E, Rauvala H, Peterson BD, van Praag H, Coffey E T. (2016). JNK1 controls adult hippocampal neurogenesis and imposes cell-autonomous control of anxiety behaviour from the neurogenic niche. *Mol Psychiatry*. 2018 Feb;23(2):487. https://doi.org/10.1038/mp.2016.203

*equal contribution

II) **Marchisella F**, Hollos P, Tiwari N, John J, Fagerholm V, Inoue M, Kim C, Ramakrishnan C, Deisseroth K, Coffey E. Hippocampal circuit regulation of mood controlled by adult born granule cells. *Manuscript in preparation*

Additional publications produced during my doctoral studies that do not relate to the subject of this thesis are listed below:

III) Hollos, P., **Marchisella, F.**, & Coffey, E. T. (2018). JNK Regulation of Depression and Anxiety. *Brain Plasticity*, 1–11. https://doi.org/10.3233/BPL-170062

IV) Marchisella, F., Coffey, E. T., & Hollos, P. (2016). Microtubule and microtubule associated protein anomalies in psychiatric disease. *Cytoskeleton*. https://doi.org/10.1002/cm.21300

V) Komulainen, E., Zdrojewska, J., Freemantle, E., Mohammad, H., Kulesskaya, N., Deshpande, P., Marchisella, F., Mysore, R., Hollos, P., Michelsen, K. Mågard M, Rauvala H, James P and Coffey, E. T. (2014). JNK1 controls dendritic field size in L2/3 and L5 of the motor cortex, constrains soma size, and influences fine motor coordination. *Frontiers in Cellular Neuroscience*, *8*. https://doi.org/10.3389/fncel.2014.00272

ABBREVIATIONS

5-HT	serotonin
AAV	adeno-associated virus
abGCs	adult born granule cells
ACC	anterior cingulate
ACTH	adrenocorticotropic hormone
ADs	antidepressants
AHN	adult hippocampal neurogenesis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
aNPCs	neural progenitor cells
Ara-C	Arabinoside-cytosine
ATP	adenosine triphosphate
AVP	arginine vasopressin
BDNF	brain derived neurotrophic factor
BDZ	benzodiazepines
BLA	basolateral amygdala
BrdU	bromodeoxyuridine
CA1	Cornu Ammonis 1
Ca2+	free calcium ion
CAG	chicken actin gene
CMS	Chronic mild stress
CNS	central nervous system
COMT	catechol-O-methyltransferase
CRH	corticotrophin-releasing hormone
CRHR1	corticotropin-releasing hormone receptor 1
DA	dopamine
DCX	Doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
DSM-5	Diagnostic Statistical Manual of Mental Disorder
ECT	Electroconvulsive shock therapy
EE	enriched environment
EPM	elevated plus maze
ERK	extracellular signal-regulated kinase
FP	Fiber photometry
FRET	fluorescence resonance energy transfer
FST	Forced swim test
GABA	γ-aminobutyric acid
GAD	generalized anxiety disorder

GCL	granule cell layer
GCs	mature granule cells
GECI	genetically encoded calcium indicator
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GRs	glucocorticoid receptors
HC	hippocampus
HPA	hypothalamo-pituitary-adrenal axis
IL	infralimbic
IPCs	intermediate neural progenitor cells
JIP1–4	JNK-interacting proteins
JNK	c-Jun NH2- terminal kinase
L/D	dark-light test
LTD	Long-term depression
LTP	long-term potentiation
LV	Lentiviral vectors
MAOIs	Monoamine oxidase inhibitors
MAPKs	Mitogen-activated protein kinases
MAPs	microtubule-associated proteins
MDD	major depressive disorder
ML	molecular layer
MLV	Moloney murine leukaemia virus
mPFC	medial Prefrontal Cortex
MRs	mineralocorticoid receptors
NA	noradrenaline
NAT	noradrenaline transporter
NeuN	Calretinin and Neuronal Nuclei
NIH	novelty-induced hyponeophagia
NLS-JBD	nuclar localizing sequence JNK binding domain
NMDA	N-Methyl-D-aspartate
NSF	novelty-suppressed feeding
NT-3	neurotrophin-3
OCD	obsessive compulsive disorder
OF	open field test
PFC	prefrontal cortex
PL	prelimbic
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PSD95	postsynaptic density 95
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus

RNA	ribonucleic acid
SAPK	stress-activated protein kinase
SDAMs	Serotonin-Dopamine Activity Modulators
SERT	serotonin transporter
SEZ	subependymal zone
SGZ	subgranular zone
SI	Social interaction
SNRIs	Serotonin/noradrenaline reuptake inhibitors
SSRIs	Selective serotonin reuptake inhibitors
SVZ	subventricular zone
TCAs	Tricyclic antidepressants
TMS	Transcranial magnetic stimulation
TST	Tail suspension test
UV	ultraviolet
VSVG	vesicular stomatitis virus

1. REVIEW OF THE LITERATURE

1.1 Mood and Anxiety disorders

The compelling question behind the research topic covered in this work is why is it important to focus on mood and anxiety disorders in our current society? Neuropsychiatric disorders are chronic illnesses, often times highly debilitating, due to a combination of behavioral symptoms and cognitive impairments. The severe state is further complicated by the comorbidity of two or three conditions simultaneously, as it is the case for anxiety disorders and major depressive disorder. It has been reported that almost one in two American citizens will deal with a mood disorder at some point in their life, with the first onset occurring as early as childhood or adolescence (Kessler *et al.*, 2005). As it had been previously predicted (Murray and Lopez, 1997; World Health Organization, 2017) major depression has become a leading cause of disability worldwide (Friedrich, 2017), hence there is a clear need to look for new ways to ameliorate patients´ quality of life and, at the same time, to ease the big financial burden that this illness demand on society. One first step toward these goals should be implementing early evaluation of individuals considered at risk to develop either anxiety or depression. More specifically, persons coming from familiar contexts in which one or both parents reported a history of psychiatric illness (Havinga *et al.*, 2017). Secondly, we need to unravel the etiology underlying the disease and the pathophysiological mechanisms that lead to the pathological state. Moreover, we need to search for ways to overcome the drawbacks of current therapies for anxiety and depression.

In this context, the present work focused on gaining a deeper understanding of the alterations that occur in the brain of mice manifesting anxiety-like and depressive-like phenotype. We sought to evaluate new therapeutic approaches with the overall aim of "translating" findings into meaningful health outcomes.

1.1.1 Mood disorders

By definition, the main feature of mood or affective disorders is mood disturbance in patients.

In the most recent edition of The American Psychiatric Association's Diagnostic Statistical Manual of Mental Disorders (DSM-5), mood disorders are classified as following:

- Elevated mood: mania or hypomania

- Depressed mood: major depressive disorder (MDD), furtherly sub-categorized into different sub-types of depressive disorders presenting less severe symptoms
- Persistent depressive disorder (dysthymia)
- Premenstrual dysphoric disorder
- Depressive disorder due to substances or other medical conditions
- Bipolar disorders: characterized by mood swings between mania and depression

This project mainly focuses on clinical depression, which is manifested in patients with neurovegetative symptoms such as changes in circadian rhythms, increased or loss of appetite and fatigue. Additionally, persistent, empty, low and irritable mood, guiltiness or worthlessness thoughts, abnormalities in the reward system (anhedonia) and cognitive impairments with loss of attention and concentration might be present. The worse cases present suicidal thoughts (Chand and Givon, 2017). This set of symptoms are also identified as primary disabilities, to which secondary disabilities may be combined and include other chronic illnesses (for instance diabetes, cancer and cardiovascular diseases) that depressed patients are more likely to develop (Dean and Keshavan, 2017).

Table n.1 illustrates symptoms that must be present in order to make a diagnosis of major depression. Currently, no diagnostic marker or laboratory test is available to assist a diagnosis of major depressive disorder.

Table 1. Diagnostic Criteria for Major Depressive Disorder and Depressive Episodes. Adapted from (American Psychiatric Association, 2013)

Criteria for Major depression Disorder		
At least 2 wks of low mood or a loss of interest or pleasure in daily activities		
Mood exhibits a change from the person's baseline		
Impaired **functional domain**:	Moderately Impaired	Severily Impaired
1. Familiar and social	Quiet, negative and oppositional	Withdrawn, won't talk, brusque, angry, aggressive
2. Occupational / educational	Grades/work performance deteriorating, missing/cutting class or work, decreased effort, moderate academic or work stress	Failing performance, missing school or work, doesn't care about work, oppositional, argumentative, high academic or work stress
Specific **symptoms,** at least 5 of these 9, present nearly every day:		
Depressed mood (e.g., feels sad or empty) **or irritable** most of the day	**Change in activity**: Psychomotor agitation or retardation	
Decreased interest or pleasure in most activities, most of each day	**Faitgue and Concentration**: diminished ability to think or concentrate, or more indecisiveness	
Significant weight change (5%) or change in appetite	**Guilt/worthlessness:** Feelings of worthlessness or excessive or inappropriate guilt	
Change in sleep: Insomnia or hypersomnia	**Suicidality:** Thoughts of death or suicide, or has suicide plan	

1.1.2 Etiology of depression

MDD is an extremely complex disorder and it is commonly accepted that a combination of genetic, epigenetic, environmental factors and their interaction underlie the development of depression (Klengel and Binder, 2015).

1.1.2.1 Hypothesis of depression

Since the concept of mental illness began to be perceived as a proper brain dysfunction in the early 20th, several theoretical strategies have been postulated to relate the wide array of neurobiological variables to the development of depression. From the early 1960's it became accepted that a neurochemical imbalance underlies the pathophysiology of mood disorders (monoamine hypothesis), and this theory led to

the development of a specific class of antidepressants, the Selective Serotonin Reuptake Inhibitors. Further evidence supported the theory that the interaction between environmental factors, chronic stress, and genetic vulnerability might contribute to the genesis of depression by influencing neurotrophin signaling (neurotrophin hypothesis, Duman and Monteggia, 2006). Impaired neurotrophin signaling leads to defective neuroplasticity, among which, lowered neurogenesis which has also been more recently proposed as a pathophysiological mechanism for depression (neurogenesis hypothesis, Hanson, Owens and Nemeroff, 2011). In addition, the glutamatergic system has been suggested as a primary mediator of pathophysiology for depression (glutamatergic hypothesis, Sanacora, Treccani and Popoli, 2012a). To connect the effects of stress, serotonin, and neurotrophins on excitatory synaptic transmission, the excitatory synapse hypothesis has been postulated (Thompson *et al.*, 2015). Another model that has been gaining more attention in the last few years is the two-hit model of depression (Irwin and Opp, 2017; Peña *et al.*, 2017). This model posits that a first "hit" can occur early in life (embryonic or perinatal period) and by altering the neurodevelopmental trajectory it heightens sensitivity to stressful events later in life. Environmental factors are generally considered as "hits", and these can be a viral infection during the mid-gestation period or obstetric complications at birth or underweight at birth. Interestingly, birth and growth in large cities has been reported to play a role in the development of depressive disorders (and other neuropsychiatric diseases), since urbanization negatively affects social stress processing in humans (Lederbogen *et al.*, 2011). Furthermore, poor quality family dynamics (e.g. negative interactions, lack of support, parental criticism) are reported to be a source of psychosocial stress which can lead to the onset of major depression and they strongly correlate with depressive symptoms (Chae *et al.*, 2012). The second environmental "hit" can then occur later in life. If it is encountered during adolescence, it might interfere with the physiological process of synaptic pruning that takes place around that time and that is decisive for correct late brain development (Selemon, 2013). Disproportionate elimination of synapses and loss of synaptic plasticity result in pathological wiring of neural circuits, which might aid to develop depression (Keshavan *et al.*, 2014). It has also been reported that depression rates rise in adolescence (Andersen and Teicher, 2008), as it is a period of accentuated opportunity for exposure to triggering factors such as addictive drug abuse, bullying, mourning of a close relative, etc. (Nabeshima and Kim, 2013; Schmitt *et al.*, 2014). When encountered during adulthood, typical environmental hits are major stressful events such as sudden death of a close relative or environmental catastrophe.

Nevertheless, as mentioned at the beginning of this paragraph, major depressive disorder arises from a complex interaction between hereditary and environmental factors. Twin studies have shown that genetic factors account for about 40% of the variance in risk of MDD, while non-shared environmental effects account for the

remaining 60% (Kendler *et al.*, 2006). In monozygotic twins, discordant for major depression, a higher number of traumatic life events and the endurance of stressful experience in time were more commonly reported in the affected twin. Examples of traumatic stressors comprise the end of a romantic relationship, discontent marriage, divorce, traumatic brain injury with significant consequences on cognitive function, occupational stress, job loss and failure. It is thought that genetic risk factors for MDD might influence sensitivity to the depressogenic effects of environmental challenges (Flint and Kendler, 2014a). For instance, gene x environment interaction can induce a dysregulation of the stress response system which affects depression vulnerability. This topic will be addressed in more detail in the section right below.

1.1.2.2 The role of stress in development of depression

Stress has been defined as a feeling associated with "experiences that are challenging emotionally and physiologically" (McEwen, 2007). Stress is a known risk factor for the development of depression, as largely reported in recent years (Caspi *et al.*, 2003; Cohen, Janicki-Deverts and Miller, 2007; Mothersill and Donohoe, 2016; Belleau, Treadway and Pizzagalli, 2018; Bleys *et al.*, 2018; Richter-Levin and Xu, 2018). During the 1950's, it was observed that patients undergoing chronic treatment with cortisone-based anti-inflammatory drugs showed significant mental and cognitive deficits, referred as "steroid psychosis" (Brody, 1952). Later on, in 1968, Bruce McEwen's pioneering paper demonstrated that the rodent brain has the highest density of receptors for corticosteroids, proving that the brain is susceptible to the effect of glucocorticoids, and particularly, in a structure involved in learning, memory and affective behavior (e.g. the hippocampus) (McEwen, Weiss and Schwartz, 1968).

Today we know that stress engages neural and endocrine transmission between the brain and other systems, such as cardiovascular or immune system and such communication is instrumental for screening potentially threatening situations in real life. Activation of the hypothalamic-pituitary-adrenal axis (HPA) is a hallmark of stress response (Fig.1). Exposure to a stressor induces secretion of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus. PVN is a structure belonging to the limbic system and implicated in endocrinal response. CRH and AVP act on the anterior pituitary, which induces the secretion of adrenocorticotropic hormone (ACTH), that is in turn responsible for stimulating the adrenal cortex of the adrenal glands to produce corticosteroids (referred to as cortisol in humans and corticosterone in rodents), the ultimate product of the HPA-axis. Corticosteroids can cross the blood-brain barrier and bind to glucocorticoid receptors (GRs), which, as said, are widely expressed across the brain and particularly enriched in the hypothalamic CRH neurons and pituitary corticotropes. GRs also bind mineralocorticoid receptors (MRs) that are specifically expressed in the limbic areas, especially in the hippocampus. Both

these receptors signal intracellularly. Ligand binding of receptor induces a nuclear translocation of the complex leading to the initiation or repression of transcription of more than two hundred genes that can influence brain plasticity, cell survival or neuronal remodeling (van Bodegom, Homberg and Henckens, 2017). Of note, cortisol concentrations regulate cortisol own secretion by a negative feedback mechanism that involve GRs and MRs activation in different brain regions and in the anterior pituitary. The negative feedback is key to shut down the HPA axis response to stress, as well as for optimal secretion of cortisol at baseline (i.e. during unstressed situations) (Gjerstad, Lightman and Spiga, 2018).

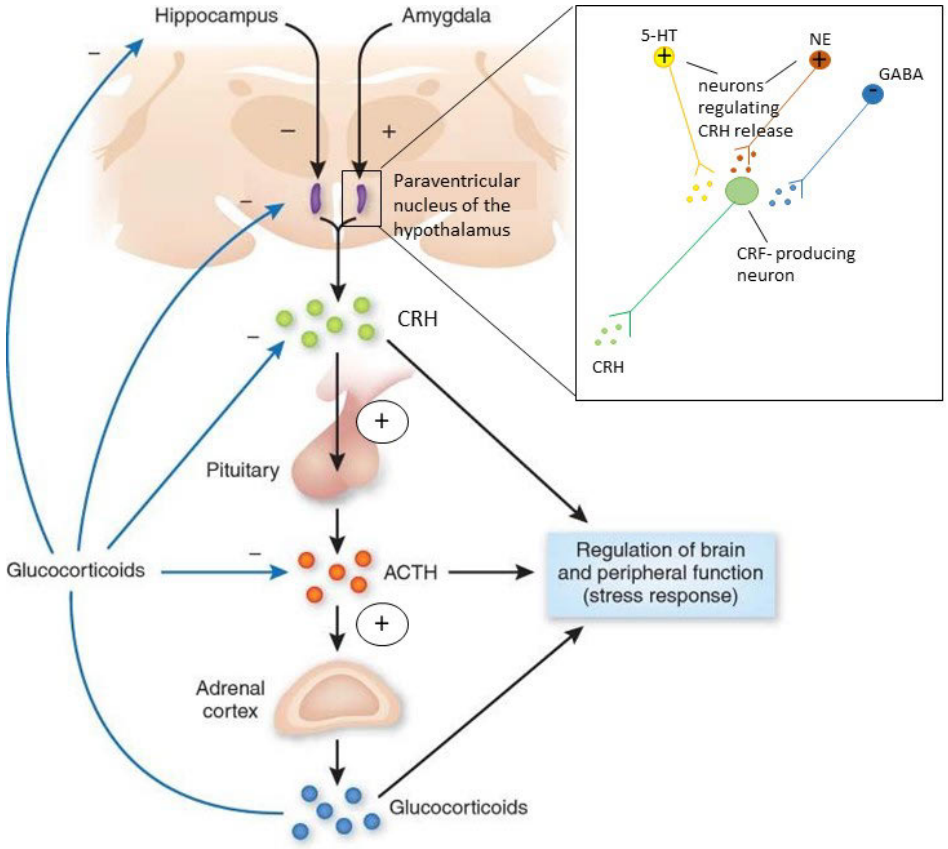

Figure1. The major components of the stress response mediated by the hypothalamic–pituitary–adrenal (HPA) axis. Stress can induce a population of neurons in the hypothalamus to produce and release corticotropin-releasing hormone (CRH). Other neurons releasing serotonin (5-HT), norepin-ephrine (NE), γ-aminobutyric acid (GABA) can also regulate CRH release (insert). CRH is transported to the anterior pituitary gland, an important endocrine gland where it stimulates production of adrenocorticotropic hormone (ACTH), and other stress-related hormones. Then, ACTH stimulates cells of the adrenal glands to produce and release the stress hormone cortisol. When cortisol levels reach a certain level, CRH and ACTH release diminishes. Adapted from: (Hyman, 2009; Stephens *et al.*, 2012)

Furthermore, increased cortisol level typically initiates the "fight or flight response", which can be categorized in two stages (Gelfuso *et al.*, 2014). Stage 1 consists of a short-term response that triggers the activation of the sympathetic system: it induces more adrenaline production leading to increased heart rate and level of alertness. Stage 2 is a long-term response, which involves activation of the HPA axis, resulting in metabolic alterations.

Several lines of evidence have shown over the years that chronic overproduction of stress hormones produces impairments in attention, memory, and emotion processing in humans (Lupien *et al.*, 2009). However, the interactions between GCs and the brain areas are complex and a combination of neurotoxic effects and genetic vulnerability ultimately influences the development of depression (Lupien *et al.*, 2018).

Remarkably, MDD patients display disruption of the negative feedback of the HPA axis, which determines the perpetuation of elevated levels of glucocorticoids (Boku *et al.*, 2017). Other deviations of the HPA axis such as, hypersecretion of CRH from the paraventricular nucleus of the hypothalamus, enlarged adrenal glands, hypercortisolemia and decreased suppression of cortisol in response to dexamethasone have all been reported in depressed patients. Similarly, chronic levels of cortisol and a hypersensitive HPA axis, with consequent cortisol release upon exposure to a minor stress can be observed in MDD subjects (Dean and Keshavan, 2017). Conversely, corticosterone administration to rodents causes depressive-like phenotype and it is used to model depression in preclinical settings (McEwen, 1999; Airan *et al.*, 2007).

From a physiological standpoint, encountering of an acute stress stimulates adaptive responses that lead to mechanisms of neuronal plasticity, including dendritic growth, synaptogenesis and transcriptional regulation. Being able to flexibly calibrate stress responses to new environments is important to properly recover from external challenges or stressors. This feature is known as *resilience*, and it seems to depend on previous experience during development and adulthood (McEwen, Gray and Nasca, 2015).

As we will examine in more detail below, acute stress also boosts the differentiation of the neuronal stem cell pool of the hippocampus into newly born neurons, that has been shown to enhance cognitive performance in rats (van Praag, 2005). The process of neurogenesis is involved in stress response and anxiety- and depressive-like behavior (Santarelli *et al.*, 2003a; David *et al.*, 2009; Snyder *et al.*, 2011). This indicates that adult born neurons could provide the plasticity required to adapt to new environments (Glasper, Schoenfeld and Gould, 2012).

On the other hand, it has been shown that severe or chronic stressors lead to disturbances in the maintenance of regular stress response, which promotes the development of depression. Additionally, it has been shown that chronic stress

represses proliferation of new neurons in the adult hippocampus (Tanti and Belzung, 2013a). However, hippocampal neurogenesis is required to buffer the stress response via negative control feedback on the HPA axis (Snyder *et al.*, 2011).

In summary, even though the entire molecular mechanism of depression remains undefined, two main messages could be derived from this section. First, chronic stress is thought to be a major player in the etiology of depression, with adult neurogenesis possibly being the link connecting stress to depression. Thereby, in preclinical settings, paradigms applying chronic stress to rodents are considered reliable animal models of depression since they encompass the main behavioral features of this illness and the animals respond to antidepressant treatments (Qiao *et al.*, 2016). Secondly, for clinical purposes we need to clarify the mechanisms underpinning vulnerability and resilience to stress, which are encoded in genes and shaped by the family environment. Early interventions should be aimed to modify the neurodevelopmental trajectories of individuals who have been exposed to major stress in order to avoid later onset of depression, more specifically by acting on stress response strategies of these individuals (Lupien *et al.*, 2018).

1.1.3 Pathophysiology of depression

It has recently been hypothesized that vascular lesions near the hippocampus (HC), amygdala and prefrontal cortex (PFC) interfere with the neural networks involved in emotional responses. Thus, they may play a role in the pathophysiology of late-onset depression (Taylor, Aizenstein and Alexopoulos, 2013). However, the historical monoamine hypothesis points to the neurotransmitter availability involving an imbalance of serotonin (5-HT) and noradrenaline (NA) activity. This started with the observation that antihypertensive drugs induced depressive episodes in susceptible individuals by decreasing the amount of monoamines at synaptic level (Tissot, 1975; Manji, Drevets and Charney, 2001).

The monoamine hypothesis is consistent with the mechanism of action of antidepressants, and current treatments for depression primarily encompass drugs targeting the monoaminergic system. As detailed in the section below, the first-generation of antidepressants were the tricyclic antidepressant (TCA) and monoamine oxidase inhibitor (MAOI) medications, which act by restoring regular neurotransmitter availability in the synaptic cleft by either inhibiting the neurotransmitter reuptake or by reducing its metabolism. Second-generation of antidepressants include selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs), that are known to exert effects on neurotransmitters such as serotonin, noradrenaline, or dopamine in the central nervous. While second-generation antidepressants did not improve efficacy with respect to first-generation (Mulrow *et al.*, 1999), they present a relatively favorable side effect profile. Third-generation antidepressants, such as venlafaxine, reboxetine,

nefazodone and mirtazapine have a variable mode of action that refines the action of first- and second-generation agents on the central monoaminergic systems (Olver, Burrows and Norman, 2001) (see section 5.1.4). However, it has been almost fifty years since a new mechanism of action for antidepressants (ADs) has been introduced, even though scientific efforts have been made. Moreover, current ADs require at least four weeks before the onset of their therapeutic effect and the condition of numerous weeks of chronic treatment before the full effect can be observed. This still represents a big drawback of current therapeutic treatment for depression (Willner, Scheel-Krüger and Belzung, 2013). Therefore, even though disturbances in the monoaminergic system appear to be crucial features of mood disorders, they most likely denote more upstream abnormalities. Indeed, the increase of intrasynaptic levels of serotonin and/or noradrenaline is only an initial effect of drug treatment, but a cascade of downstream effects is thought to be ultimately responsible for the actual therapeutic effect (Manji, Drevets and Charney, 2001). Changes in gene transcription and neuronal plasticity are the most likely mechanisms influenced by AD treatment (Hyman and Nestler, 1996; Duman, 2004; Racagni and Popoli, 2008; Andrade and Rao, 2010; Serafini, 2012; Harmer and Cowen, 2013; Hayley and Litteljohn, 2013).

More studies have suggested dopamine (DA) and glutamate system alterations in the pathophysiology of depression (Dean and Keshavan, 2017). Indeed, anhedonia and amotivation, which are symptoms of depression, seem to arise from diminished firing of dopaminergic neurons in the ventral tegmental area, a region associated to reward, upon prolonged exposure to stressors (Grace, 2016). Glutamate is the most abundant excitatory neurotransmitter in the brain and precursor of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). It is crucially involved in synaptic transmission and plasticity, and perturbations of the glutamatergic system have been linked to major depressive disorder. More specifically, alterations in the mechanisms controlling clearance and metabolism of glutamate, and morphological maladaptive changes in several brain areas modulating cognitive-emotional behaviors have been described (Sanacora, Treccani and Popoli, 2012b). Defects in neural plasticity have also been linked to the onset of depression and, as mentioned above, they might have their primary cause in endogenous alterations of levels of neurotrophic factors, such as brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). These proteins are involved in remodeling serotonergic neurons, as well as, promoting their function. Both human and animal studies have shown that antidepressant treatment boosts BDNF levels, which correlate to the onset of therapeutic effects (Bondy, 2002).

Perturbations of the sleep-wake regulation are also reported in subjects with MDD and they are used as diagnostic criteria for MDD (Luca, Luca and Calandra, 2013). Furthermore, some symptoms of depression display diurnal differences such as mood, psychomotor activity and recalling abilities of positive and negative experiences. At the same time, a study conducted on healthy young subjects, showed

that moderate variations of the sleep-wake cycle had precise effects on improving mood, while manipulations of circadian rhythms with light therapy or sleep deprivation show an antidepressant effect in subjects suffering from depression, suggesting that circadian aberrations could also underlie the pathophysiology of depression (Hasler, 2010).

In this section, I summarized current hypotheses for the pathophysiology of depression, which also covers information for the development of new therapeutic strategies. Indeed, 50% of patients are currently unresponsive to treatments (Akil *et al.*, 2018), which calls for more studies on this subject. In the next section, we will examine the state of the art of therapies for major depression.

1.1.4 Treatment for depression

To date, the most effective treatment for depression is a combination of medical and cognitive therapy, which is linked to heightened quality of life and significant alleviation of symptoms. Electroconvulsive shock therapy (ECT) is considered for cases with severe symptoms and drug-resistant depression and it has been associated to cognitive side effects.

In general, this disease is characterized by relapses that cannot be prevented with current drugs, though responsiveness to medications reduces the risk of new full blast episodes of depression.

As mentioned above, most common medications used to treat depression are:

- Selective serotonin reuptake inhibitors (SSRIs): including Fluoxetine (Prozac), Paroxetine (Paxil) which are the elective treatment, characterized by low toxicity and abuse potential. As name suggests, SSRIs selectively block 5-HT reuptake through the pre-synaptic serotonin transporters (SERT).
- Serotonin/noradrenaline reuptake inhibitors (SNRIs): are used for patient not responding to SSRIs. These are dual inhibitors of the reuptake of serotonin and NA on SERT and noradrenaline transporter (NET).
- Atypical antidepressants: commonly used for pharmaco-resistant depression.
- Serotonin-Dopamine Activity Modulators (SDAMs) were initially developed because of multitude of serotonin receptors subtypes. In general, receptor binding of the drug modulates serotonin reuptake.
- Tricyclic antidepressants (TCAs): including Imipramine (Tofranil) and Desipramine (Norpramin), associated to more serious side effects and substantial toxicity in overdose. They act by blocking of 5-HT and noradrenaline reuptake.
- Monoamine oxidase inhibitors (MAOIs): also effective in an extensive range of anxiety disorders. These compounds are active on monoamine oxidase enzyme, inhibit the breakdown (deamination) of monoamine

neurotransmitters (mainly serotonin and noradrenaline), and subsequently increase their concentrations in the brain.

Radical therapeutic interventions encompass:

- Neurosurgical treatments: involving lesions in the frontal cortex, caudate nucleus, internal capsule, the prelimbic white matter, and the anterior cingulate cortex (Read and Greenberg, 2009).
- Deep brain stimulation: targeting medial frontal cortex and the internal capsule (Mayberg *et al.*, 2005).
- Transcranial magnetic stimulation (TMS): which consists of repeated stimulation of a targeted area of the brain through the intact scalp. Pulses produce a magnetic field that alters the electrical activity of the stimulated area. Dorso-lateral prefrontal cortex is currently considered the gold standard stimulation approach in depression treatment (Chung, Hoy and Fitzgerald, 2015).

Currently, responsiveness to AD treatment cannot be anticipated. This prolongs therapy time and increases costs and uncertainty of therapeutic strategies. Therefore, much effort is being put to develop genetic markers of predictability of therapy response that could drive clinicians more towards a 'personalized' medicine approach (Carrillo-Roa *et al.*, 2017).

Even though, it is widely known that antidepressants increase serotonin and/or noradrenalin availability at the synaptic cleft, by either inhibiting the reuptake or inhibiting the decomposition, we still seem to lack the true explanation for the therapeutic effect. Therefore, it is of relevance to understand the molecular mechanisms underlying the efficacy of different classes of ADs. To this end, a number of studies on preclinical models of depression have been examining the molecular signature of these compounds (Santarelli *et al.*, 2003a; Morais *et al.*, 2014; Patrício *et al.*, 2015; Breitfeld *et al.*, 2017). Chronic administration of fluoxetine stimulates neurogenesis (Kodama, Fujioka and Duman, 2004; Encinas, Vaahtokari and Enikolopov, 2006; Marcussen *et al.*, 2008), synaptogenesis and synaptic plasticity in the adult hippocampus, cortex and amygdala (Popova, Castrén and Taira, 2017). Moreover, it has been seen that AD medication restore to original size the smaller volume of hippocampus that has been found in MDD patients (Boku *et al.*, 2017). Section 5.1.9 will further elucidate the aspect of brain structural and morphological changes that are observed in subjects affected by depression.

1.1.5 Anxiety disorders

The word anxiety derives from the Latin *angere* that means to choke or strangle. Indeed, patients with anxiety experience cognitive symptoms of feeling increased apprehension, unease, excessive worry or nervousness and anticipation of fear about

an imminent event. These symptoms are also accompanied by other physiological symptoms such as dizziness, sweaty, nausea, shaking, higher heart rate, shortness of breath, chest pain and the notorious choking sensation. Thereby, anxiety disorders are characterized by excessive fear combined to behavioral disturbances deriving from those fears. *Fear* is defined as the emotional response to any real and perceived threat, whereas *anxiety* is fear while anticipating those threats. Also, while fear is accompanied by an acute heightening of the sympathetic nervous system, anxiety displays more of muscle tension and alert priming for future threats (Craske *et al.*, 2017).

Table n.2 summarizes the anxiety disorders illustrated in the DSM–5. Phobias are diagnosed more often than other disorders and with the earliest onset already in childhood. Typically, though, anxiety disorders manifest earlier in age than other neuropsychiatric disorders (Stein *et al.*, 2017). It is reported that 1% of all disability adjusted life years is lost worldwide because of anxiety manifested in the form of panic attacks, post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder (OCD). Of note, the chapter on anxiety disorders in DSM-5 does not include PTSD and OCD in the list, rather, they have been placed in new and separate chapters entitled "Trauma- and Stressor-Related Disorders" and "Obsessive-Compulsive and Related Disorders", respectively (WHO and Organization, 2004; Kupfer, 2015). Moreover, anxiety disorders are highly comorbid with one another and with other mental disorders (see below) and their course is usually chronic-recurrent. Nevertheless, it has been estimated that patients start medication for anxiety disorders at least a decade after onset (Stein *et al.*, 2017). Thereby, there is a need for better and early diagnosis of anxiety symptoms to alleviate the socio-economic burden that anxiety creates globally. Epidemiology assessments revealed that in 2010 anxiety disorders caused 390 years lived with disability per 100000 persons, most prevalently in women between 15 and 34 years (Stein *et al.*, 2017).

Table 2. Anxiety disorders and relative descriptions, from (American Psychiatric Association, 2013)

Anxiety disorders - DSM 5	
Separation Anxiety Disorder (SAD)	Persistent and excessive fear or anxiety about harm to attachment figures, loss or separation from attachment figures. The symptoms include nightmares and physical symptoms
Selective Mutism	Consistent failure to speak in social situations where there is an expectation to speak even though the individual speaks in other circumstances, can speak, and comprehends the spoken language. The disorder is more likely to be seen in young children than in adolescents and adults
Specific Phobia	Fearfulness or anxiety about specific objects or situations, which are avoided with intense fear and anxiety. The fear, anxiety, and avoidance is almost always immediate and tends to be persistent out of proportion to the actual danger posed by the specific object or situation. There are different types of phobias, for instance animal or blood-injection-injury
Social Anxiety Disorder	Marked or intense fear or anxiety of social situations in which individuals could be the subject of scrutiny. The individual fears that he/she will be negatively evaluated in such circumstances. He/she also fears being embarrassed, rejected, humiliated or offending others
Panic Disorder	Panic attacks are abrupt surges of intense fear or extreme discomfort that reach a peak within minutes, accompanied by physical and/or cognitive symptoms (e.g., palpitations, sweating, shortness of breath, fear of going crazy or dying). They can occur unexpectedly with no obvious trigger, or they may be expected, such as in response to a feared object or situation. Recurrent unexpected panic attacks induce the individual to persistent concern and worry about having another panic attack. Subjects display changes in their behavior linked to the panic attacks which are maladaptive, such as avoidance of activities and situations to prevent the occurrence of panic attacks.
Agoraphobia	Fearfulness of the following circumstances: using public transportation, being in open spaces, being in enclosed spaces like shops and theaters, standing in line or being in a crowd, or being outside of the home alone. The individual fears and avoids these situations because he/she is concerned that escape may be difficult or help may not be available in the event of panic-like symptoms, or other incapacitating or embarrassing symptoms
Generalized Anxiety Disorder (GAD)	Persistent and excessive worry about various domains, including work and school performance that the individual finds hard to control. The person also experiences physical symptoms including restlessness or feeling keyed up or on edge; being easily fatigued, difficulty concentrating or mind going blank, irritability, muscle tension and sleep disturbance
Substance/Medication-Induced Anxiety Disorder	Anxiety symptoms are due to substance intoxication or withdrawal or to medical treatment
Anxiety Disorder Due to Other Medical Conditions	Anxiety symptoms are the physiological consequence of another medical condition, such as: hypothyroidism, hypoglycemia, and hyperadrenal cortisolism; cardiovascular disorders: congestive heart failure, arrhythmia, and pulmonary embolism; respiratory illness (asthma, and pneumonia); metabolic disturbances (B12 df porphyria); neurological illnesses: neoplasms, encephalitis, and seizure disorder

1.1.6 Etiology of anxiety

The etiology of anxiety disorders is believed to be multifactorial, with significant interactions between gene and environmental factors and their interaction (stressful or traumatic events). However, a twin study reported a primary role for the heritability component in developing one form of anxiety disorder (Hettema, Neale and Kendler, 2001). No high-risk genetic variance has been associated to anxiety disorder yet, however a few candidate genes have been proposed: corticotropin-releasing hormone receptor 1 (CRHR1) and catechol-O-methyltransferase (COMT) identified for susceptibility to panic disorder (Craske *et al.*, 2017)

1.1.7 Pathophysiology of anxiety

Currently, a complete pathophysiological mechanism for anxiety has not been elucidated. It is believed that anxiety states arise from a coordinated activity of several brain areas involving the release of different neurotransmitters and neuromodulators, all of which act together and are controlled by local and distant synapses (Nuss, 2015).

The main players are the excitatory serotonergic (Gordon and Hen, 2004) and noradrenergic (Blier and Mansari, 2007) neurotransmitter systems, and the inhibitory system (GABA) (Lydiard, 2003). Indeed, pharmacologic (selective serotonin reuptake inhibitors and benzodiazepines) treatments targeting such systems show effectiveness for many patients suffering from anxiety.

Stressful factors can trigger swift changes in the availability of neurotransmitters (Mora *et al.*, 2012) and it has been showed that reduced availability of serotonin and elevated levels of noradrenalin lead to a hyper-functioning sympathetic nervous system, which translates into peripheral symptoms manifestation (increased heart rate, sweating, visceral symptoms) (Yamamoto, Shinba and Yoshii, 2014). A growing body of evidence suggests that anxiety disorders result from maladaptive changes in the highly interconnected neural circuits that govern emotional behaviors. Major areas implicated are amygdala, bed nucleus of stria terminalis (BNST), ventral hippocampus (vHPC) and prefrontal cortex (PFC) (Calhoon and Tye, 2015). Moreover, the locus coeruleus and lateral tegmental area are dense in noradrenergic fibers projecting to the cortex and limbic system, determining emotional behavior outcomes (Kalk, Nutt and Lingford-Hughes, 2011; Beas *et al.*, 2018). Similarly, serotonergic neurons project from the Raphe nuclei to the frontal cortex and limbic system regulating emotional responses (Gelfuso *et al.*, 2014). With newly developed technology, we are able to gain more information on the neural circuits underlying fear, fear extinction, anxiety and related defensive behaviors and we can construct a comprehensive and integrated view on which individual regions are more relevant to adaptive behaviors. For state-of-the-art knowledge on this topic see (Tovote, Fadok and Lüthi, 2015).

Rapid learning is a crucial process that has always played a role during evolution because it enables an organism to survive aversive environments, avoiding or properly responding to threatening circumstances. Consequently, generalization of fear learning is also equally adaptive, as it allows anticipation of threats not specifically encountered before. However, the system becomes maladaptive when excessive generalization induces fear responses that are improper for specific situations, and they hinder actions that are more rewarding. For instance, fear in a novel environment could be protective, but, in rodents, it could induce freezing behavior and decrease exploratory behavior preventing effective food search, while in humans it could affect social behavior and pleasurable activities. Thereby, a fine balance of fear generalization is needed, and it involves cognitive abilities of predicting the likelihood of encountering danger and reward within the environment (Cameron and Schoenfeld, 2018). Anxiety disorders may arise from a decrease in rewarding activity owed to disproportionate fear (Dunsmoor *et al.*, 2011; Luyten *et al.*, 2011; Lissek, 2012). Importantly, imaging studies in subjects with generalized anxiety disorder (GAD) have reported neural differences in brain areas

associated with the emotion dysregulation, cognitive impairment, and reward processing. In particular, individuals with GAD revealed heightened activity of amygdala and inferior frontal gyrus when presented negative images during an emotion regulation task, suggesting deficits in emotional reactivity (Fitzgerald *et al.*, 2017). Moreover, a study involving a decision-making task demonstrated that subjects with GAD present with decision-making deficits, and excessive worry over everyday problems which underlie lowered correlation between prediction error and activity within the ventromedial prefrontal cortex and striatum (White *et al.*, 2017). More importantly, structural magnetic resonance revealed increased volumes in amygdala and putamen in GAD patients (De Bellis *et al.*, 2000; Schienle, Ebner and Schäfer, 2011; Liao *et al.*, 2013), as well as, altered gray matter volumes in the thalamus, hippocampus, insula, posterior cingulate, and superior temporal gyrus (De Bellis *et al.*, 2002; Strawn *et al.*, 2013; Moon and Jeong, 2017). Resting-state fMRI has been employed to investigate real time brain function in clinical settings. As the name suggests, it does not involve task stimulation for the subject and outputs are considered stable and reliable. Thereby, resting-state fMRI studies have shown decreased functional connectivity between amygdala and prefrontal cortex in adults (Etkin *et al.*, 2009; Hilbert, Lueken and Beesdo-Baum, 2014), while adolescents with GAD displayed disturbances in amygdala connectivity with medial prefrontal cortex, insula, and cerebellum (Roy *et al.*, 2013). Of note, abnormal connectivity is considered as a longitudinal biomarker of symptom changes in GAD (Makovac *et al.*, 2016). Thereby, brain structure abnormalities and functional deficits during anxiety state may also contribute to the pathophysiology of this illness. Interestingly, the pharmacological mechanism of benzodiazepines treatment has also shed light on possible disturbances of the inhibitory GABA system, which is the key inhibitory neurotransmitter in the central nervous system (CNS) (Chaki, Okubo and Sekiguchi, 2006). The next section will examine in more details the mechanistic effects of anxiolytic therapies.

1.1.8 Treatment for anxiety disorders

Consistent with the treatment of mood disorders, patients with anxiety disorders typically undergo a combination of psycho- and pharmaco-therapy. The former involves detection and modification of maladaptive rational schemes underlying anxiety manifestation through cognitive-behavioral therapy. Interestingly, a more recent approach, although still less effective than cognitive-behavioral therapy, is mindfulness which, through integration of meditation and breathing movements, aims to bring the individual's attention on present moments, recognizing and accepting feelings (Craske *et al.*, 2017). Pharmaco-therapy consists of drugs that affect mainly the serotonergic, adrenergic and glutamatergic neurotransmission, with SSRIs and SNRIs being the first-choice treatment, associated to a few and transient side

effects, differently from TCAs and MAOIs, less prescribed and linked to heavier adverse effects (Craske *et al.*, 2017).

The primary treatment used to alleviate symptoms of anxiety involved the use of sedating medications, barbiturates and benzodiazepines (BDZ) that both enhance GABAergic transmission. Low doses of barbiturates influence GABAA receptors increasing GABA-induced chloride currents. On the other hand, high concentrations open the channels in the absence of GABA. Additionally, evidence shows that barbiturates exert neuroprotective effect by blocking glutamate excitotoxicity (Gelfuso *et al.*, 2014). On the other hand, BDZs have become the most prescribed drugs in the modern world due to their quick therapeutic effect and prolonged half-lives. However, commonly reported side effects include fatigue, dizziness, decreased cognitive performances and dependency after long-term treatment (greater than six months). Interestingly though, they can be used in combination with SSRIs/ SNRIs for the first few weeks required by the antidepressants to produce effects. Due to high prevalence of comorbidity between anxiety and depression (see below), while SSRIs are able to exert a double effect, BDZ do not alleviate depression symptoms (Bandelow, Michaelis and Wedekind, 2017).

BDZs are allosteric modulators agonists of GABAA receptors, structurally coupled to a chloride channel. During physiological state, chloride channels open upon binding of GABA to GABAA receptor and produce hyperpolarization of neuronal membranes. Consequently, decreased frequency of action potentials leads to an overall depression of brain activity that alleviates anxiety symptoms. BDZs bind the extracellular domain of the alpha-subunit of GABAA receptor for which they have high affinity. This increases chloride conductance and results in anxiolytic effect (Gelfuso *et al.*, 2014).

Even though it has been known since the 4th century from Plato that physical exercise is essential for mental health, the practice to prescribe physical training alone or along with pharmaco-therapy has only recently gained more popularity. Habitual physical activity correlates with better mental health and improvement of anxiety and depression symptoms (Ströhle, 2009). Therapeutic effects of exercise have also been linked to its ability to boost hippocampal neurogenesis. Animals living in enriched environments including a running wheel display increased number of new neurons in the hippocampus (Kobilo *et al.*, 2011). In addition, exercise facilitates the recruitment of newly born cells for strengthening synaptic connections, producing an overall effect of well-being (Lee and Baek, 2017).

1.1.9 Structural and morphological abnormalities in brain associated with mood disorders

As briefly mentioned above, the limbic system encompasses a set of brain structures whose connectivity produces emotional expression. Hippocampus, amygdala,

anterior and medial thalamus, cingulate gyrus, and related visceral control areas in the hypothalamus and brainstem belong to the limbic system (Price and Drevets, 2010). Alterations in this network have been reported in MDD patients (MacQueen and Frodl, 2011). Figure 2 depicts such regions, while functions and dysfunctions of the network will be discussed in section 5.3.

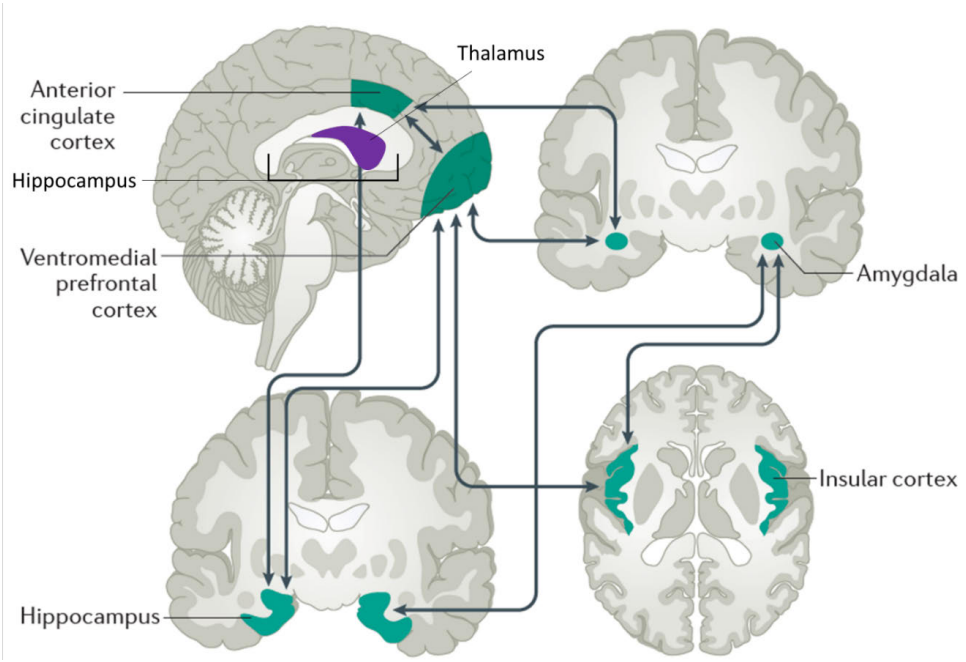

Figure 2. Major brain regions involved in the regulation of emotions and that are affected in mood disorders. In this model, amygdala activity is modulated through bidirectional connections to the ventromedial prefrontal cortex and the anterior cingulate cortex, along with functional crosstalk between these regions and the hippocampus. This model is consistent across species and with observations in patients. Representative brain images shown illustrate the human brain. Adapted from: (Craske *et al.*, 2017)

The advent of neuroimaging techniques has led to the observation that patients diagnosed with depression, PTSD and schizophrenia display decreased size and loss of function of some of the brain regions mentioned above (PFC and HC) (Sheline, Gado and Kraemer, 2003a), while amygdala displays increased functionality (heightened HPA-axis activity, fear and anxiety behavior (Duman and Duman, 2014). Moreover, 4-6 % smaller hippocampal volume has been consistently reported in patients affected with major depression. Such structural change correlates with the temporal duration of the condition and pharmacotherapy is able to revert the loss of volume in the hippocampus (MacQueen and Frodl, 2011; Duman, 2014). One hypothesis ascribes the volumetric loss of the hippocampus to the morphological

changes of hippocampal neurons: shortening length of dendrites and decreased number and density of spines. An additional aspect determining the shrinkage of the hippocampus could be the decrease of neurogenesis in the hippocampal dentate gyrus (DG). Both these hypotheses account for the latency of response to antidepressants (Boku *et al.*, 2017).

Furthermore, literature reports reveal that the underlying alterations are also to be sought at the cellular level. Specifically, neuronal cell body size appears to be decreased in the PFC and hippocampus of post-mortem tissue of depressed subjects, and remarkably, no change in cell number is detected (Stockmeier *et al.*, 2004). This could account for the observed loss in grey matter. Moreover, loss of synapses in the PFC has been reported in depressed subjects and murine models of depression, which has been hypothesized to produce an aberrant connectivity with consequent alteration of control of mood and emotion in depression (Duman, 2014). Considering that depression *per se* is considered a stress-related condition and animal models of depression involve administration of chronic stress, consistent findings show alterations in number and function of spine synapses upon stress exposure (for a review see Duman & Duman 2014). The molecular mechanism leading to these changes may be due to decreased levels of BDNF or reduced mTORC1 signaling pathway components which correlates with lower expression of synaptic proteins with consequent impaired number and function of spine synapses (Li *et al.*, 2010). Additionally, post-mortem tissue from depressed subjects display low number of glia cells in cortical regions, predominantly in the PFC and cingulate cortex.

1.1.10 Comorbidity of depression and anxiety disorders

"Depression that is not complicated by comorbidity is the exception, not the rule" (Hirschfeld, 2001).

Clinicians report that comorbid depression and anxiety disorders appear in up to 75% of patients and rates exceed comorbidity of either of the two with other common medical illnesses such as hypertension, diabetes, or asthma (Hirschfeld, 2001; Tiller, 2013).

Remarkably, original diagnosis with major depression increases the likelihood (up to 8-fold) of a co-occurring anxiety disorder, but if original diagnosis is anxiety disorder, patients have up to 62 fold increased risk to develop comorbid major depression within twelve months. Mechanistically, this might occur because initial environmental stressor (emotional trauma, financial stress) triggers clinical anxiety which, in turn, amplifies chronic stress producing constant, elevated levels of cortisol that eventually lead the onset of depression (Hirschfeld, 2001).

Interestingly, clinical manifestation of comorbidity appears as one of four possible scenarios. Figure.2 exemplifies these scenarios. Briefly, patients usually

exhibit full-blown state of either anxiety or depression, and subsyndromal levels of the other. This complicates differential diagnosis, which is essential for effective therapy (see below). Less frequently, patients display both full-blown conditions or subclinical forms of both diseases (Hirschfeld, 2001).

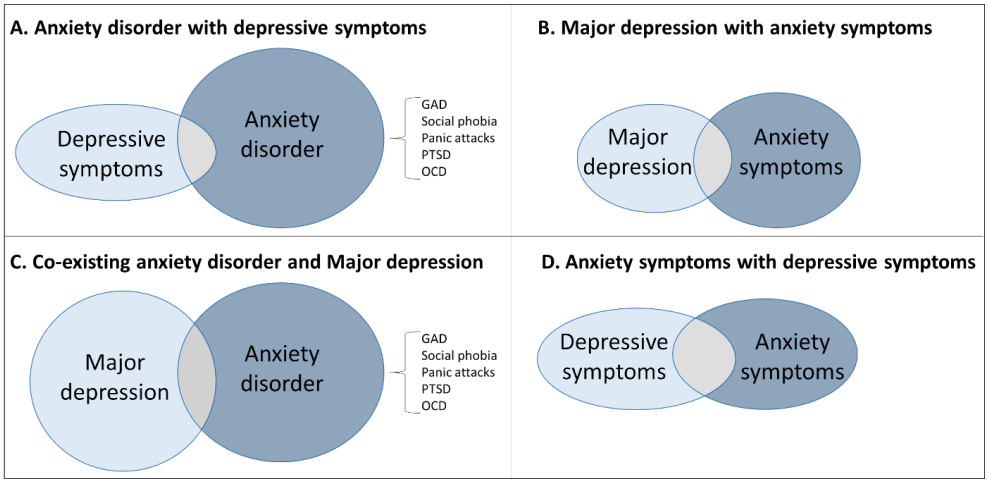

Figure.3: In this representation, possible scenarios of comorbidity of anxiety and depression are depicted. **A:** Subjects with anxiety disorder can exhibit depressive symptoms at a subsyndromal level. **B:** Subjects with major depression exhibit anxiety symptoms at a subsyndromal level. **C:** Subjects exhibit full-blown state of both disorders. **D:** Patients exhibit subsyndromal levels of both disorders. Adapted from (Hirschfeld, 2001).

Furthermore, higher severity and chronicity of illness, reduced quality of life, treatment resistance and poor therapeutic effects are expected when comorbidity of anxiety with depression is diagnosed. Consequently, medications for comorbid anxiety and depression requires specific psychopharmacological variations as compared to treating either condition alone. SSRIs are the most preferred treatment when uncomplicated depression comorbid with a spectrum of anxiety disorders (Coplan, 2015).

Lastly, the reason why anxiety is so highly predisposing to the onset of depression might be due to specific features that these disorders share and, as briefly mentioned above, comorbidity the two disorders complicates differential diagnosis in clinic. Anxiety disorders and major depressive disorder show common genetic and environmental risk factors; so, could anxiety disorders be considered as initial and alternative manifestations of depressive-like phenotype?(Kaufman and Charney, 2000). More studies are needed to better elucidate this hypothesis, for both clinical and pre-clinical purposes. Indeed, there is an unmet need in neuroscience of reliable animal models of anxiety and depression that could clearly differentiate features of these two conditions. From a scientific standpoint, it is convenient to decompose

anxiety- and depressive-like phenotypes into endophenotypes in laboratory animals, to better control the disease and link genetic loci to symptomatic manifestations (Gould and Gottesman, 2006). Even though, ideally, one model should reflect one disorder and one test one readout (Harro, 2017). "Depression-related" behavioral tests for rodents (more details can be found in the next section) tend to include anxiety-like traits as an endophenotype, which not necessarily reflects a clinical condition and it can lead the researcher to wonder about the existence of a clear splitting line between equivalent animal models (Harro, 2017). The next section will more deeply dissect popular animal models of anxiety and depression.

1.2 Animal models in neuropsychiatry

Animal models of diseases are an extraordinary tool in research that helps to represent a given disease or to model its development in "controlled" conditions. This leads to a better understanding of the etiology and enables to search for higher treatment efficacy.

Nevertheless, in order to authentically reproduce as many symptoms of disease as possible, any animal model should have the following characteristics (Geyer and Markou, 1995):

- Construct validity: pathophysiological hallmarks of disease are consistent with humans
- Etiological validity: disease is established by the same means, such as environmental factors
- Face validity: symptomology parallel to that observed in humans
- Predictive validity: responding to current treatment approaches in humans

The extent to which we can reproduce psychiatric conditions in animals is limited to plausible features of diseases such as impaired social behavior, memory, emotion, and executive function. Symptoms such as suicidal thoughts, hallucinations, delusions, sadness and guilt cannot be translated to animals given their inability to speak. Most animal models of neuropsychiatric diseases have been conveniently developed in rats or mice, which present neurobiological dissimilarities compared to humans and an overall reduced capacity to process complex psychological aspects (Cryan and Holmes, 2005; Nestler and Hyman, 2010). However, they provide the opportunity to easily design and generate new animal models not only starting from a scientific discovery that translates into a safe and effective treatment for the patient (forward translation), but also starting from information gathered from humans (reverse translation) to develop an improved new animal model (Belzung, 2014; 'T Hart, 2015). Aspects related to gender dichotomy should also be considered: robust evidence indicates women as more susceptible to stress-related psychiatric disorders (Holden,

2005) and sex differences are reflected in animal models of MDD (for a review see (Dalla *et al.*, 2010). Hence, there should be a higher interest in developing animal models with greater female prevalence. Lastly, despite the fact that animals are just a partial reproduction of the full spectra in humans, animal models are currently still an invaluable and essential source of information on mechanism of diseases for us (Nestler and Hyman, 2010).

The process of generating animal models can involve:

- Genetic manipulations: including selective breeding, genetic engineering (knock-ins or knock-outs lines), viral targeting and delivering to specific brain regions
- Pharmacological manipulations: also including cannula implantations and catheterization
- Anatomical lesions: altering brain physiological integrity and producing specific phenotypes
- Environmental manipulations: involving the use of restricted housing conditions and application of stressors
- Optogenetic manipulation: involving selective alteration of brain circuitry

It should also be highlighted that a model of disease always includes a manipulation (genetic or external) that triggers the development of the disease, and a readout (behavioral test or imaging session). Differently, a test only aims to measure the readout, but it is not a model of the disease. For instance, this has become evident when acute administration of SSRIs induced anxiety in several tests and these drugs did not retain the fast sedative effect that characterizes the anxiolytic drugs (Bodnoff *et al.*, 1989; Griebel *et al.*, 1994) Conversely, chronic treatment with SSRIs produced anxiolytic responses in the hyponeophagia test (Dulawa *et al.*, 2004)(see below). However, this led to the understanding that ADs can still be effective to treat anxiety disorders, while highlighting the difference between anxiety tests and anxiety disorder models as tools to develop improved therapies (Harro, 2017).

In conclusion, this section described the general approaches currently used to generate animal models of anxiety and depression. In the following sections, we will examine typical tests used to screen depressive-related and anxiety-related phenotypes in rodents.

1.2.1 Depression related models:

Chronic mild or chronic unpredictable stress (CMS) refers to the application of a set of repeated, mild physical stressors (for example, restraint or food deprivation), or random change of environmental conditions (circadian rhythms disruptions, exposure to cold temperatures, presentation of sudden noises or intense light sources)

for a prolonged period of minimum two weeks. The rationale behind this model is the uncontrollable reality of being exposed to novel and chronic stress which is a well-known trigger in the development of MDD in humans (Nestler, Gould and Manji, 2002; Nollet, Le Guisquet and Belzung, 2013). When normal rodents are subjected to this range of stimuli, they develop anhedonia (face validity), measured as decreased sucrose (or saccharin) consumption, furtherly explained later in this section (Katz, 1982; Papp, Willner and Muscat, 1991). This deficit can be reverted by a wide spectrum of antidepressant medications (predictive validity) (Papp, Moryl and Willner, 1996). The importance of this model relays on the fact that animals undergoing CMS paradigms display brain structural changes (e.g. decreased hippocampal volume) and neurochemical alterations (impaired serotonergic system), very frequently observed in human patients as well (Hill *et al.*, 2012).

Chronic glucocorticoid administration consists of chronic exogenous exposure to corticosterone, based on reported alterations in the HPA axis in MDD patients. Animals eventually display anatomical changes (reduced hippocampal volume), behavioral changes (anhedonia) and neurochemical changes all reversed by antidepressant treatment (Sapolsky, 2001; Hellsten *et al.*, 2002; Sterner and Kalynchuk, 2010).

Early life stress, such as *maternal separation* and *social isolation*, respectively involve deprivation of contact with maternal support or conspecifics during early post-natal days, which represent a critical period for adaptive development of cognitive and social abilities. These paradigms derive from the observation (as mentioned earlier) that adverse insults during early-life, such as child neglect and abuse prompt vulnerability to the development of depression during adulthood. Of note, vulnerability does not result in depressive-like state *per se*, but it increases the risk of developing the condition upon further exposure to additional stressors. Both paradigms produce neuroendocrine abnormalities such as disrupted HPA-axis and altered neurotransmitter levels, all reversible by antidepressant medications or environmental enrichment (Pryce, Rüedi-Bettschen and Dettling, 2005; Cui *et al.*, 2006; El Khoury *et al.*, 2006; Brenes, Rodríguez and Fornaguera, 2008). Furthermore, early life challenges are also associated to increased development of anxiety disorders and are a further translational tool that can be reproduced in the laboratory to mimic trends in the human population (Cryan and Holmes, 2005).

1.2.2 Anxiety-related models:

Ultrasonic Vocalizations (USV): involves quantitative measurements of highly specific and well-studied ultrasonic call that pups (younger than 14 days) emit when removed from their mothers, littermates, and nest. Vocalizations can also be prompted by controlled stimuli and can be analyzed without further distress of the pups. This paradigm has been used as an animal model of anxiety-like and can be

blocked by benzodiazepine and anxiolytics (Dichter, Brunelli and Hofer, 1996; Scattoni, Crawley and Ricceri, 2009).

Genetic models of anxiety-like behavior represent an invaluable tool to investigate the neurobiological basis of anxiety and strategies used to generate new models range from knockout to more sophisticated approaches such as tet-on/off and Cre-lox, as well as siRNA-mediated gene knock down. Genetic models of anxiety behavior have been extensively listed elsewhere (Flint, 2003; Cryan and Sweeney, 2011), however few examples are given below:

LAB/HAB: this acronym stands for high anxiety behavior (HAB) and low anxiety behavior (LAB) and it consists of a selective inbreeding strategy for outbred mice (CD-1) or rats (Wistar). Animals tested for elevated plus maze and light–dark box (description of tests will be given below) reported increased anxiety-like behavior for HAB animals and decreased anxiety-like behavior for LAB animals (Salomé *et al.*, 2002; Kromer, 2005; Landgraf *et al.*, 2007).

5HT1$_A$ system: considering the neurochemical imbalance in the serotonergic system, a single gene manipulation at the serotonin receptor 5HT1 level has been targeted with effective development of anxiety-like and depressive-like traits in animals, observed when tested for typical anxiety- and depressive-related tests (Holmes, 2001; Holmes *et al.*, 2002).

GABA system (including: GAD65 knockout, γ2, γ2L subunit knockout): single gene manipulation targeting the GABAA receptor has been targeted and animals show increased anxiety-like behavior when tested for anxiety-relevant tests (Finn, Rutledge-Gorman and Crabbe, 2003).

To validate animal models of anxiety-like and depressive-like phenotype, *behavioral tests* are widely used, but as already said, they are not animal models. Rather, tests were developed to measure the time that animals spend active or passive while a brief stress is given. More specifically, the stress is usually unavoidable and leads to a state of *behavioral despair.* Acute antidepressant treatment improves time of active responding in forced swim test and tail suspension test (Nestler and Hyman, 2010). A large variety of tests measuring anxiety-like and depressive-like behaviors are available nowadays. This is connected to the advantage of performing a battery of behavioral tests to elaborate reliable and consistent data. Moreover, tests have been classified according to the neural functions that they are able to detect (Harro, 2017).

An in-depth description of the most relevant animal tests for anxiety-like and depressive-like behavior and of choice for our studies is given below.

1.2.3 Depression related tests:

Forced swim test (FST) and Tail suspension test (TST), both aim to measure behavioral despair. Briefly, FST consists of a cylinder filled with tepid water, whereas TST consists of an apparatus where mice are suspended by the tail. In both tests,

animals tend to initially adopt an escape-directed behavior, after which, they assume an immobile posture, likely, after terminating active coping to the stressful stimuli. A single dose of AD can increase mobility time, while non-antidepressant compounds have been screened and showed no effect on immobility time. Circuitries and systems behind the behavioral and pharmacological effects of these tests are still needed to elucidate their validity and employment (Slattery and Cryan, 2012). Indeed, it remains controversial whether these tests replicate depression-like symptoms in animals or they are simply acute testing protocols for screening compounds with antidepressant properties. Additionally, traditional ADs such as imipramine, desipramine, and amitriptyline show effectiveness after a single dose, whereas a delay of 4-6 weeks is required until the therapeutic effects start to appear in humans. Such downside poses doubts as to whether these tests are sensitive enough to detect the real neuromodulator actions that AD agents induce in the nervous system or, rather, they are limited to detect only immediate effects. Besides, the risk of false positives is heightened in these tests, since drugs like stimulants decrease immobility but are not antidepressants (Nestler and Hyman, 2010). Nonetheless, while they are performed during subacute conditions (30 minutes post drug injection), they are strongly reliable in predicting the therapeutic potential of screened compounds. Furthermore, these tests are low cost, present inter-laboratory reliability and specificity and, the neural circuits underlying depression and antidepressant response in humans are shown to be recruited during FST sessions (O'Leary and Cryan, 2013; Slattery and Cryan, 2014).

Anhedonia is defined as inability to experience pleasure while performing a pleasurable activity (eating, sexual or social activities, hobbies or exercise) (Romer Thomsen, Whybrow and Kringelbach, 2015). The original definition was coined in 1896 and to date it includes several facets of reward-related aspects that are found impaired in depressed patients, such as inability to anticipate rewards or lack of motivation to pursue expected rewards. This interesting flattening of the reward circuitry in MDD has been largely investigated and, currently, it is believed that multiple neurobiological changes occur in several neuronal circuitries responsible for different functions. More specifically, PFC alterations have been linked to decision making dysfunctions, variations in the amygdala result in alterations of emotional regulation and changes in the nucleus accumbens impair motor coordination and impulsivity along with decreased dopamine levels in the reward system (Gorwood, 2008; Der-Avakian and Markou, 2012). Anhedonia is typically measured in rodents with preference tests, observing sweet solution intake (sucrose or saccharin) relative to water: lower intake is indicative of anhedonic state. However, a more sophisticated approach has also been developed and it is known as intracranial self-stimulation (ICSS). With ICSS rodents learn to press a lever or turn a wheel to receive an electrical stimulation in specific brain regions of the reward circuitry, through chronically implanted electrodes. Stimulation threshold is heightened in conditions characterized

by anhedonia (Moreau *et al.*, 1992). On the other hand, sucrose self-administration protocols have also been developed to assess motivational behaviors aimed to obtain natural rewards like food. Here, the operant behavior learnt by animals allows to self-administer a palatable food by pressing a lever. However, to examine animals' motivation to work for a reward, the progressive ratio schedule is commonly used, which consists of gradually increasing the effort needed to obtain the reward. Some animal models of anhedonia have shown reduced self-administration of sucrose in this test (for a review see Scheggi, De Montis and Gambarana, 2018). Remarkably though, in one study subjects suffering from depression do not have significantly decreased preference for sweet solutions compared to healthy controls (Berlin *et al.*, 1998), calling for caution when interpreting data for translational purposes.

Social interaction scores the time spent in social contact or investigation with a conspecific. When interaction time increases without affecting locomotor activity, measures are reliable. Originally, this was designed as readout for anxiety-like behavior since male and female rats that scored high interaction time also displayed more exploratory behavior in classic tests of anxiety-like behaviors (File and Hyde, 1978). However, social interaction is now also accepted as a test mirroring social withdrawal of depressive state (Krishnan and Nestler, 2008). Social impairment could be paired to anhedonia as measurements of reward-related behaviors for more reliability of data. Elimination of aversive appetitive stimuli is a plus for this test, along with the fact that by varying illuminating conditions it is possible to modify baseline anxiety-like levels of the animals, which is beneficial for screening of new compounds (Bailey and Crawley, 2009).

As mentioned in the previous section, many MDD subjects are diagnosed with anxiety disorders. These two conditions are highly comorbid but different neural circuitries seems to be involved (Nestler and Hyman, 2009). Similarly, behavioral tests of depression often include anxiety-like phenomena and specific conclusions on either disorder derived from these tests should be cautiously interpreted because of the hybrid of symptoms of depression and anxiety (Nestler and Hyman, 2010).

1.2.4 Anxiety-related tests:

Exploratory-based-approach avoidance tests include:

- open field test (OF)
- elevated plus maze (EPM)
- dark-light test (L/D)

These are the three most commonly used tests to assess anxiety-like behaviors in rodents and, similarly to depression related tests, they were originally designed to investigate benzodiazepine-like anxiolytic drugs. The environment provides the option to explore aversive areas (open, brightly lit, or elevated space) and scoring is

based on the preference of rodents for less anxiogenous places of the apparatus, avoiding open exposure (evolutionarily, to predators) (Crawley and Goodwin, 1980; Pellow *et al.*, 1985). Engaging in exploratory behavior is an index of anxiolytic-state and conversely, benzodiazepines administration produces increased exploratory behavior (Christmas and Maxwell, 1970; Rodgers, 1997; Gonzalez, Ouagazzal and File, 1998). Of note, these tests do not clearly differentiate between decreased avoidance due to low anxiety-like state from increased novelty seeking behaviors and the ability to inspect these tests relies on integral sensory and motor function in the animal. Thereby, scores should always be compared to measures of locomotor activity within the same test to structure the data analysis when investigating something specific to anxiety-related behavior (Cryan and Holmes, 2005).

Marble burying test is performed by placing animals in a novel cage containing colored novel marbles symmetrically positioned on the sawdust of the cage. This test is based on the typical tendency of animals to bury novel or aversive items and it has the peculiarity to involve an active behavior, as opposed to the aforementioned passive avoidance tests of anxiety-like behavior. The act of burying of the marbles is considered as anxiety-like behavior and the precise number of buried marbles is scored during trials. Administration of anxiolytics reverts burying (Treit, Pinel and Fibiger, 1981; Njung'e and Handley, 1991; Czech and Quock, 1993). However, it has long been argued that this test is rather a measure of obsessive–compulsive disorder (Witkin, 2008). The current view in the field argues that although the test retains a potential utility to model or test for anxiety- or compulsive-like behavior. However, as it has been applied so far, the test has reported inconsistent and contrasting data, making it overall of no translational value (de Brouwer *et al.*, 2018).

Hyponeophagia-based models include:

- novelty-induced hyponeophagia (NIH)
- novelty-suppressed feeding (NSF)

These are generally performed in a novel environment for the animal, who is either food deprived and presented with a pellet food (NSF), or is satiated and a highly palatable and familiar food is given (NIH). In both scenarios, the latency to feed and/or the amount of eaten food is calculated. The model is based on the anxiogenic effect of novel cage that suppresses eating. Rodents are challenged with feeding desire in contrast to their natural tendency of avoiding novel environments (Dulawa and Hen, 2005). Benzodiazepines and chronic treatment of antidepressants are able to reduce the latency to feed conferring predictive validity to these models (Bodnoff *et al.*, 1988).

1.3 Technological advances to study neurocircuitry of anxiety and mood disorders

Generation and experience of emotion is a process of tight collaboration between several encephalic structures, among which hypothalamus and other visceral elements that regulate expression of emotions (Price and Drevets, 2010). As we have previously mentioned, neurochemical imbalances have been largely proposed to represent the etiopathophysiology of anxiety and depression but rather recently, evidence suggest that mood regulation goes beyond the chemistry behind. Indeed, current investigations focus on the aberrant connectivity and information processing within neural networks linked to emotional responses and diseases state.

The advent of sophisticated technologies and diagnostic tools that combine genetic, neuropathological and imaging studies revealed that dysfunctions of the medial prefrontal network and the limbic regions underlie anxiety disorders and mood disorders. Altered neurophysiological activity, receptor pharmacology, and gene expression within these structures contribute to the symptomatology of mood disorders (Drevets and Price, 2008).

Relevant to mood disorders, within the limbic regions, the amygdala strongly projects to the medial prefrontal cortex and the hippocampus (Ressler and Mayberg, 2007; Rauch and Drevets, 2009; Price and Drevets, 2010). Furthermore, as mentioned, outputs to visceral control areas in the hypothalamus are embedded within this network. Amygdala is primarily involved in emotion generation and automatic evaluation, while medial prefrontal cortex plays a crucial role in cognitive regulation of emotion. Imaging studies in patients have consistently found high activity in the amygdala, upon exposure to emotional stimuli (Siegle *et al.*, 2002; Surguladze *et al.*, 2005). Conversely, pharmacotherapy with ADs is able to revert the amygdalar activation (Sheline *et al.*, 2001), indicating an inhibitory pathway from prefrontal cortex to downregulate the amygdala (Johnstone *et al.*, 2007). On the other hand, reports highlight significantly low prefrontal–amygdala connectivity changes in depressed subjects performing cognitive tasks (Thomas and Elliott, 2009), suggesting that depression may arise from boosted bottom-up response to emotional stimuli and impaired top-down control of emotional responsiveness (Dannlowski *et al.*, 2009). Most likely, this is due to inactive prefrontal cortex (Erk *et al.*, 2010) and hypometabolism (Rigucci *et al.*, 2010) and it is rescued by antidepressant medication (Mayberg *et al.*, 2000).

A recent body of evidence in animals has shed light on how the hippocampus influences the activation of groups of neurons in the PFC during emotion regulation (Godsil *et al.*, 2013). The monosynaptic unidirectional projection of neurons from the hippocampal formation to the PFC is named H-PFC pathway, which is highly sensitive to stress and it plays a crucial role for interactions with the amygdala.

Findings in rodents link the H-PFC pathway in facets of executive function and in contextual processing that aid emotional regulation. Anatomically, the H-PFC pathway stems from the CA1 region of the hippocampus, with the strongest projections deriving from the ventral hippocampus.

The fibers run ipsilaterally through the fimbria/fornix system, and stop in the infralimbic (IL), prelimbic (PL), and anterior cingulate (ACC) areas of the PFC. The knowledge on general connectivity of the hippocampus, amygdala and PFC arises from rat and monkeys. Consistent sets of data employing diffusion tensor imaging report that the H-PFC pathways share similarities between humans and monkeys (Ongür and Price, 2000). Excitatory glutamatergic pyramidal neurons characterize the H-PFC pathway, whose terminal synaptic connections are on both principle neurons and GABAergic interneurons of the PFC (Carr and Sesack, 1996; Tierney *et al.*, 2004). The pathway has been shown to transmit contextual information from the HC to the PFC during behaviors anticipating potential danger and inhibiting fear when threat has diminished. Such signaling is important to inform the mPFC whether amygdalar responsiveness to fear should be suppressed, since both the HC and mPFC are reciprocally connected with the amygdala. This function represents highly adaptive behavior and it is associated to proper emotional regulation (Orsini *et al.*, 2011). On the other hand, exposure to psychological stress induces short, medium, and long-term changes within the hippocampus and mPFC, according to the intensity and duration of stress (Rocher *et al.*, 2004). More specifically long-term stress disrupts neuroplasticity within the H-PFC pathway (Rocher *et al.*, 2004; Judo *et al.*, 2010). Abnormalities in the H-PFC pathway converge in multiple psychiatric disorders, such as schizophrenia, major depression, and PTSD, which could explain the shared symptomatology of cognitive impairment and emotional dysregulation among these disorders (for review (Godsil *et al.*, 2013).

Altered gray matter volume (Murray, Wise and Drevets, 2011), as mentioned above, are found in the hippocampus and amygdala (Sheline, Gado and Price, 1998) of individuals affected by anxiety and major depression. Moreover, individuals who displayed larger hippocampal volumes before undergoing pharmacological AD treatment were responsive to therapy and showed full remission, in contrast to those with smaller hippocampal volumes which had increased susceptibility to relapse (Frodl *et al.*, 2008; MacQueen *et al.*, 2008). Smaller size of these structures implies reduced neuronal complexity and connectivity, most likely due to the effect of stress, which can be counteracted by antidepressant action (Santarelli *et al.*, 2003b). In addition, antidepressant drugs stimulate the birth of new neurons in the physiological niche of the hippocampus, the dentate gyrus, hence increasing the pool of mature cells that can be ultimately recruited to strengthen and restore proper neuronal connectivity (Malberg *et al.*, 2000). Simultaneously, AD therapy promotes dendritic arborization and synaptic plasticity (Fujioka, 2004) in the DG. However, the ADs

action on hippocampal plasticity requires a few weeks to occur, which is thought to explain the apparent delay required by medications to exert their therapeutic effect (Castrén, 2005).

In preclinical settings, conclusions from rodents can be useful to untangle mood and anxiety disorders, however, the brain structures examined are only homologues to humans and they could vary in many and significant ways (Murray, Wise and Drevets, 2011). Moreover, the lack of technologies accessing the intact brain has reduced thus far our possibilities to study brain circuitries underling specific behaviors. However, the latest technologies have been developed to spatio-temporally modulate networks that control complex and/or maladaptive behaviors in animal models. Thereby, we currently have excellent tools to manipulate projection activity of neuronal populations involved in specific networks of interest and from which derive important information on structural mapping. Optogenetics is one of these new technologies through which bacterial opsin genes, which encode for photosensitive ion-conductance regulators or biochemical-signaling protein, are inserted into viral vectors and delivered into specifically targeted areas of the brain. More specifically, opsins excite or inhibit neural projections when light is delivered through a fiber-optic. Thereby, neural activity can be manipulated in distinct neuronal populations and projections while observing the effects of these manipulations on animal behavior (Deisseroth, 2014). One important application of this approach, in the context of anxiety, has revealed that excitation of basolateral amygdala (BLA) cells with axons that project to the central nucleus of the amygdala terminals produced an acute, reversible anxiolytic effect in freely behaving mice, tested for OF and EPM (Tye *et al.*, 2011). A recent study investigated the optogenetic control of the tripartite circuitry of ventral hippocampus -medial prefrontal cortex and basolateral amygdala. Authors found that direct projections from the ventral hippocampus to the medial prefrontal cortex are essential for anxiety-like behavior and the inhibition of this circuitry is purely anxiolytic and induces relative safety state in tested mice (Padilla-Coreano *et al.*, 2016).

In the context of depression, optogenetics was used to apply bursts of laser stimulation to the medial prefrontal cortex of mice previously subjected to chronic social defeat stress who displayed depressive-like phenotype, resulting in an antidepressant effect and indicating a key role of medial PFC in pathophysiology and antidepressant response in major depression (Covington *et al.*, 2010). Moreover, optogenetics was applied to induce depolarization of cells with a projection between the medial prefrontal cortex and the dorsal raphe nucleus (characterized by serotonergic neurons), while rats were tested for FST. The study shows that when this population of cells was activated, rats modified their behavior towards active-coping (swimming or climbing) mode, demonstrating how medial PFC is instrumental in

controlling decision pathway refinement and implementing this response (Warden *et al.*, 2012).

A further development of optogenetics has led to the use of genetically encoded calcium indicators (GECIs), *in vivo* sensors of neuronal activity that enable to monitor changes in cytosolic calcium levels resulting from opening of voltage-gated Ca2+ channels in a population of cells of choice and intra-cellular compartments. This approach allows to record the activity of a projection *in vivo*, coupling GECI emission to an optic fiber and a photodetector for digitalization of the acquisition (Akerboom *et al.*, 2012). In the following section, I will discuss the evolution of GECI into an innovative technology that allows recording of genetically defined activity of cell populations, which is of relevance for the work of this thesis.

1.3.1 Fiber photometry (FP)

Elucidating connections between neuronal networks and behaviors poses a great challenge for modern neuroscience. The function of the nervous system is based on *electrical activity of neurons*; hence, several systems currently enable a direct recording of the cells in a given brain area and/or during specific conditions of interest. However, extreme accuracy is needed when biological events are to be detected to explore the mechanisms underlying neuropsychiatric disorders in humans.

Electrophysiology, has been improved to the point that it currently allows recording of the activity between brain regions from thousands of cells, while animals are awake and freely-moving animals (Viventi *et al.*, 2011). Typically, the electrode is implanted in the animal's brain and the signal coming from a single cell or the collective activity of a brain region (according to the diameter of the electrode) is detected. Nonetheless, a major drawback of such approach is the absence of cell-type specificity because recording is an indiscriminate acquisition from all cells in proximity of electrode tip. Moreover, it still lacks the ability to target deep brain structures in freely moving animals. Hence, the advent of optical reporters, combined with the possibility of expression in transgenic animals, has met the need of investigating the brain with good accuracy (Packer, Roska and Häuser, 2013). Thereby, these reporters are used to study a variety of cell dynamics, namely the intracellular concentration free calcium (Chen, Trevor J Wardill, *et al.*, 2013) and neurotransmitters, including serotonin and glutamate (Hires, Zhu and Tsien, 2008; Nguyen *et al.*, 2010; Yamauchi *et al.*, 2011) or dopamine (Patriarchi *et al.*, 2018). Reporter proteins have been engineered to be fluorescent (Naumann *et al.*, 2010): they absorb a photon and then emit a photon at a longer wavelength. A great advantage of fluorescent reporters is that they provide a great contrast on a background, making them suitable for imaging. Additionally, fluorescence signal can be stably detected over months, due to the long-term labeling of cells (Looger and Griesbeck, 2012).

Of interest for this work is the measurement of free Ca2+, which is implicated in several intracellular processes. Particularly, in postsynaptic neurons, the intracellular calcium concentrations reflect neuronal activity because voltage-gated and calcium-permeable channels open in response to action potentials, which results in an influx of free calcium into the excitable cell. Moreover, calcium transients in postsynaptic cells mediate events of activity-dependent synaptic plasticity. Conversely, in presynaptic cells, action potentials come with the release of calcium stocks from cytoplasmic organelles, such as the endoplasmic reticulum, which enables exocytosis of synaptic vesicles for release of neurotransmitters into the synaptic cleft. This explains how our ability to monitor calcium in neurons can be a powerful tool, and for this reason, a wide variety of calcium indicators have been studied, produced and improve (Grienberger and Konnerth, 2012), until arriving to what we have available today, which will be described more in detail in the next section.

The main application of indicators is to study deeper structures in mammals' brain. Popular laboratory animal models, such as rats and mice have brain thicknesses of roughly 5 mm and 10 mm, respectively (Paxinos *et al.*, 2004; Khazipov *et al.*, 2015). Visible light emitted by indicators is strongly scattered by brain tissue (J. Mobley, T. Vo-Dinh, 2014), which produces an attenuation of signal throughout the tissue. Hence, since imaging with visible light is currently possible for structures of approximately 50 μm depth (Scanziani and Häusser, 2009), a number of approaches such as 2-photon imaging, endoscopes or imaging with a multimode fiber have been developed to improve the limit of brain depth. However, these approaches require the animal to be restrained during the imaging session, preventing the animal's behavior to be examined.

Fiber photometry is a revolutionary change to these approaches, and it offers real time measurement of calcium transients from selected cell types, while animals are freely moving or performing behavioral tasks (Gunaydin *et al.*, 2014). In addition, fiber photometry comes with the advantage to be expandable to multiple brain regions in the same animal (Kim *et al.*, 2016). Furthermore, a recent development is an integrated wireless photometry system, which avoids restriction of types of behavior measurable due to fiber patch cords impediments, and motion artifacts that complicate analysis of recorded traces (Luyao Lu *et al.,* 2017).

Calcium-based fiber photometry uses chronically implanted optic fibers to shine excitation onto a virally labeled population of neurons expressing a calcium indicator. The light excites the GECI and the resulting fluorescence is recorded. The emitted fluorescence is assumed to reflect the summed neural activity within the entire transduced population. A representation of the classic setup for awake and behaving animals is shown in Fig. 4. It comprises the excitation light source that delivers light into the optical fiber, a dichroic mirror, which separates excitation and emitted fluorescence, and a photodetector to which the emitted fluorescence is finally

conveyed. Additionally, the system includes a fiber-optic rotary joint to avoid tangling of the fiber cord, the optical cannula implanted into the animal's brain, which is approximately 300 μm in diameter, and the connecting fiber-optic patch cords. The light source, optical components, and photodetector are positioned on the benchtop, while the fiber-optic patch cords follow the animal on its task, deliver excitation light and carry emitted light back. Recordings are expressed as baseline-referred changes in fluorescence intensity, ΔF/F. Implementation of genetic targeting techniques (viruses, promoters, transgenic animals) facilitate targeting of specific cell-type, connectivity-dependent, and projection-based expression of the fluorescent proteins (Packer, Roska and Häuser, 2013).

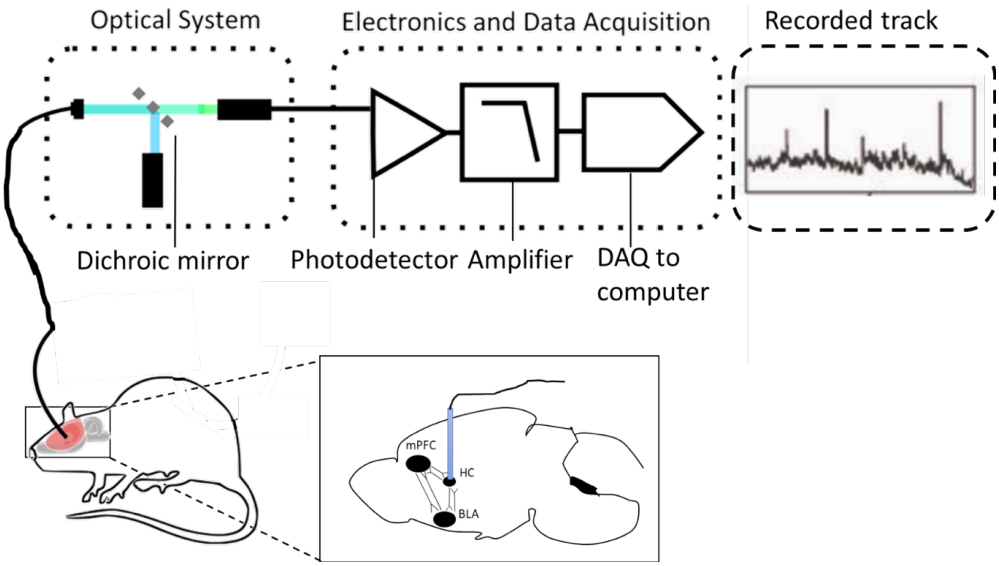

Figure 4. Graphic of a typical Single Fiber Photometry System.

It must be said, though, that fiber photometry also comes with some complications that should be considered when performing experiments. A list of the most critical ones is discussed below. Excitation of a fluorophore in living tissue can induce photobleaching of the reporter, endogenous fluorophore excitation, overheating of the tissue with consequent damage and even cell death due to phototoxicity. In addition, due to the scattering nature of the brain tissue, the tip of the optical cannula must be near the cells expressing fluorescence, or the signal will not be detectable. In this regard, phototoxicity and photobleaching can be avoided by employing a detection scheme that is highly sensitive to fluorescence, along with larger-diameter cannulas to improve acquisition of fluorescence. Recorded traces that contain artifacts are another source of drawbacks for FP. However, excitation of the tissue at two wavelengths with discrete modulation properties can overcome this issue. The first

wavelength is around the indicator's peak excitation, while the second is at a point where the emitted fluorescence does not result from bound Ca2+. This is indicated as *isosbestic point* and the whole arrangement produces a calcium signal and a control fluorescence signal (Lerner *et al.*, 2015). Furthermore, data processed subtracting the control from the calcium signal produce an additional correction for artifacts in the traces. Lastly, to avoid excessive tissue damage and the consequent acquisition of unreliable data, the cannula must be implanted in the correct region of interest, according to stereotactic coordinates. On top of this, an innovative approach that has been previously described enables to split the implantation surgery in two phases: during the first phase animals are injected with viral vectors encoding for the fluorescence indicator. Few days or weeks after, which allows the expression of the fluorescent protein, animals undergo a second surgery during which the fiber photometry system is used to detect the area with the highest fluorescence within the injected region of interest, which will be the exact spot where the optic cannula will be implanted (Grienberger *et al.*, 2012).

1.3.2 Genetically encoded calcium indicators for real-time in vivo analysis

GECIs have been largely studied and improved during the last years to reach higher accuracy in reporting neuronal synaptic activity. Indeed, an influx of calcium in the cell is correlated with an action potential in the neuron.

Before GECIs advent, the most used calcium indicators to monitor intracellular calcium responses were: i) aequorin, a bioluminescent calcium-binding photoproteins (Ashley and Ridgway, 1968). ii) Arsenazo III, a synthetic molecule, whose absorption spectrum changes as a function of bound calcium (Brown *et al.*, 1975). iii) fluorescent calcium indicators, such as Fura-2, which were generated as a hybridization of highly calcium-selective chelators like EGTA or BAPTA with a fluorescent chromophore (Tsien, 1980). All of these carry both benefits and drawbacks, and the choice of which to use must be assessed according to the type of experiment, the calcium signals to measure and the imaging equipment available. For instance, an advantage of using aequorin is high signal-to-noise ratio combined to a wide dynamic range. Thus, it allows monitoring changes in the cytosolic calcium concentration from 10−7 to 10−3 M (Grienberger and Konnerth, 2012). Additionally, the use of bioluminescent calcium reporters, such as aequorin, do not involve external illumination, thereby preventing drawbacks, such as phototoxicity, photobleaching, autofluorescence, and undesirable stimulation of photobiological processes. Indeed, photobleaching of a fluorophore causes an irreversible loss of fluorescence due to the reaction of indicators with oxygen during their excitation state, with consequent generation of non-fluorescent molecule. This represents a major weakness for several experimental conditions, particularly when rapid image acquisition or long duration are performed (Bootman *et al.*, 2013). However, aequorin does not penetrate the plasma membrane of intact cells, hence, it

requires loading into single cells through micropipette, making it difficult to be delivered in specific cell types. Furthermore, low quantum yield and low protein stability usually characterize aequorin-based recording of calcium signals (Grienberger and Konnerth, 2012).

Fluorescent calcium indicators, on the other hand, are excitable by UV light (fura-2 is excitable at 350/380 nm), with the advantage that they can be used with dual wavelength excitation and enable quantitative indication of calcium concentrations in a neuron of interest, regardless the intracellular dye concentration. Another key advantage of these fluorescent calcium indicators regards the possibility of finding them in a membrane-permeable and a membrane-impermeable form, facilitating their employment in combination with several loading techniques (Grienberger and Konnerth, 2012). Indeed, the most popular method used to introduce calcium indicators into cells is as a membrane-permeant acetoxymethyl ester, which, upon calcium indicator release, yields formaldehyde and acetic acid as by-products, potentially affecting cell activity. Moreover, it has been reported that indicator loading can decrease cellular ATP content, and overloading of cells with indicator can severely compromise calcium signals. In addition, sustained irradiation of cells with UV light can cause phototoxicity and cell damage, making these indicators not compatible with long-term and repeated in vivo measurements (Bootman *et al.*, 2013).

Advantages correlated to the use of GECIs over synthetic indicators encompass the possibility of incorporating the indicators into the genome of transgenic mice, avoiding the exogenous indicator loading complicacies listed above. Moreover, GECIs allow for targeting different neuronal populations and/or subcellular locations through cell specific promoters and targeting sequences, which overcomes the unselective uptake of membrane-permeant dye esters (Lock, Parker and Smith, 2015).

The general structure of a GECI contains a calcium-binding domain, such as calmodulin, fused to a fluorescent protein that is circularly permuted and possesses calcium binding-dependent fluorescence properties. GECIs that comprise two fluorescent proteins, are instead characterized by calcium binding controls fluorescence resonance energy transfer (FRET) between the two proteins (Tian *et al.*, 2009).

One class of single fluorescent protein indicators, of specific interest for this work, is GCaMPs. These are currently the most widely used class of calcium indicators to label subgroups of neurons with engineered fluorescent proteins that fluoresce according to changes in intracellular calcium concentration. More specifically, when calcium is present, it binds the calmodulin domain bringing it in contact with a linker peptide (M13). Hence, these come in close proximity to the chromophore from the circularly permuted GFP that undergoes a conformational change producing a boost of fluorescence of 515 nm. GCaMPs have been rapidly evolving to overcome limits of low sensitivity and slow kinetics, with the most recent version being GCaMP6. This

sensor was developed using structure-based mutagenesis targeting the interface between permutated GFP and calmodulin and the M13/calmodulin interface, which influences more the calcium affinity. Newly generated indicators were subsequently screened in neurons because of their remarkably fast calcium dynamics and low peak calcium accumulations (Girven and Sparta, 2017).

Thereby, GCaMP6 indicators are highly sensitive to changes in calcium concentrations and the family encompasses three alternative isoforms of increasing speed of kinetics: GCaMP6, 6s (slow), 6m (medium), and 6f (fast), with the most sensitive in detecting single action potentials having slower kinetics. Lastly, these tools allow either imaging of large groups of neurons and small synaptic compartments, with the possibility of repeated imaging sessions, a part with each other (Chen, Trevor J. Wardill, *et al.*, 2013).

As briefly mentioned above, GECIs are focally injected to the region of interest through viral vectors, which are carefully chosen according to needs, such as, length of the transgene, route of delivery, tropism (cell-type specificity), efficiency of transduction, stability of gene expression, and immunogenicity (Davidson and Breakefield, 2003). The next section will examine the most commonly used viral vectors to deliver transgene in the brain.

1.4 Viral targeting of central nervous system

Lentivirus and adeno-associated virus (AAV) vectors are largely used to deliver constructs to the brain in the preclinical context because of their ability to transduce non-dividing cells. These types of viruses have the advantage of inducing low inflammation and a widespread, stable, long-term expression and high levels of transgene. Lentiviral vectors (LVs) hold a maximum capacity of 10 kb exogenous DNA which will integrate permanently into the genome of target cells. On the viral particle, the envelope protein is key for cell-specificity and the mechanism of infection. Ordinarily, the glycoprotein G from vesicular stomatitis virus (VSVG) is the envelope protein used. Moreover, due to the ability of VSVG-pseudo-typed LVs to transduce all neural cell types, the use of specific promoters is required to guide the expression of the transgene. AAV vectors, on the other hand, include several serotypes which infect defined populations of cells, as well as, both dividing and non-dividing cells. In addition, AAVs rarely integrate, and can accommodate transgene constructs up to 5 kb. This represents a limitation of the viral delivery method, and consequently, only relatively small promoters can be used (Davidson and Breakefield, 2003).

Of specific interest for this work, we turn our attention to another class of Lentivirus, Retroviruses, derived from Moloney murine leukaemia virus (MLV), which hold limited applications as vectors for the brain, since they preferentially infect dividing cells. Nonetheless, this category of vectors is extensively used to target

neurogenesis (see below) in rodents' brain. This tool allows for specific targeting of the newly born neurons, which can be easily visualized and functionally analyzed (van Praag *et al.*, 2002). Although, efficiency of transduction relies on integration site into the genome of the target cell and on the level of differentiation of the cells. Retroviruses infect host cells with a defined mechanism: first, they bind to receptors expressed on the target cell's plasma membrane. Consequently, the fusion of the viral membrane and the host cell membrane occurs, and the transgene enters the host cell. The retroviral genome is replicated inside the cell by reverse transcriptase, which generates DNA from the viral RNA genome. If the target cell enters mitosis at this moment, the nuclear membrane is disrupted, allowing retroviral integrase to insert the viral DNA into the host genome, at a site that holds high transcriptional activity. Thereby, the reporter transgene will replicate in the infected host cells and will be delivered to their progeny. Typically, transgenes are expressed under the control of a specific promoter. The most commonly transgenes used to target neurogenic cells are: chicken actin gene (CAG) to target adult born granule cells of the hippocampus, glial fibrillary acidic protein (GFAP) to target the neural stem cell pool, Nestin to target the amplifying cell pool, and Doublecortin (DCX) to target the neuroblast pool (Enikolopov, Overstreet-Wadiche and Ge, 2015).

The following section will dissect in more detail the process of neurogenesis in the adult brain, with specific relevance for neuropsychiatric disorders.

1.5 Adult Neurogenesis

The discovery that new neurons are continuously generated in the adult brain resets a century of scientific theory, which stated that the production of functional neurons only occurs during embryonic development. The New York Times first published about this revolutionary news in 2000, shading light on the field of neurogenesis (Ming and Song, 2011). Though, the first attempt to demonstrate that the brain is able to renew itself throughout life goes back to 1965, when Altman and colleagues employed autoradiography and found that neurogenesis occurs in the dentate gyrus of newborn, young and up to eight months of age rats (Altman and Das, 1965). Later, the employment of a synthetic analog of thymidine, bromodeoxyuridine (BrdU), allowed it to be incorporated into the DNA of dividing cells, establishing the presence of neurogenesis in the adult brain (Corotto, Henegar and Maruniak, 1993; Luskin, 1993; Seki and Arai, 1993). Since then, the field has grown enormously, also considering the advent of more sophisticated technologies to target the newly born neurons specifically and trace their fate over time. To date, neurogenesis is known to be conserved across mammals: from rats to non-primates to humans (Kaplan and Hinds, 1977; Cameron *et al.*, 1993; Kuhn, Dickinson-Anson and Gage, 1996; Kempermann, Kuhn and Gage, 1997; Eriksson *et al.*, 1998; Kornack and Rakic, 1999;

Gould *et al.*, 2001; Spalding *et al.*, 2013). Rodents produce about 9,000 new cells each day in the dentate gyrus, meaning that about 6% of the total granule cell population is renewed every month (Cameron and Mckay, 2001). As for humans, on the other hand, it is remarkable how Spalding et al., took advantage of aboveground nuclear bomb tests from 50 years back to assess neurogenesis in humans and model its dynamics. Indeed, nuclear bombing during the World War II heightened atmospheric levels of the radioactive carbon-14 isotope (14C), which decayed gradually over time. From postmortem tissue of patients, the authors first measured atmospheric 14C concentration in the DNA of hippocampal neurons, then defined the age of the cell population and finally revealed that there is a turnover rate of approximately 700 new neurons per day in each hippocampus during adulthood, thus proving that neurogenesis occurs in humans (Spalding *et al.*, 2013). Interestingly, however, the debate as whether adult neurogenesis really occurs in humans remains hot. Indeed, in 2018 two almost simultaneous contrasting articles were published and revived the controversy. In particular, the first one reported that DG neurogenesis rapidly decreases with progressing age and no markers for neural progenitors or immature neurons could be detected in post-mortem tissue from subjects older than 13 years (Sorrells *et al.*, 2018). In contrast, the second one found that hippocampal neurogenesis does occur in adult humans and it does not regress with aging (Boldrini *et al.*, 2018). Boldrini et al. used a stereology approach to estimate cell numbers across the DG in post-mortem tissue of individuals ranging from 14 to 79 years. This leaves an open discussion (Lucassen *et al.*, 2019) as to whether adult neurogenesis contributes to human cognition, and evidences are still strong to discard the idea that the course of human adult neurogenesis does occur throughout the lifespan (Kempermann *et al.*, 2018). Most likely what makes these two papers contrasting with each other is the methodology that was followed. The way brain tissue is preserved influences the staining and cells visualization. Sorellas et al., used flash-frozen tissue samples, which might have contributed to lower the sensitivity of imaging protocol. On the other hand, Boldrini et al., used fixed samples, and it is commonly accepted that fixation preserves proteins and cell architecture better and more reliably.

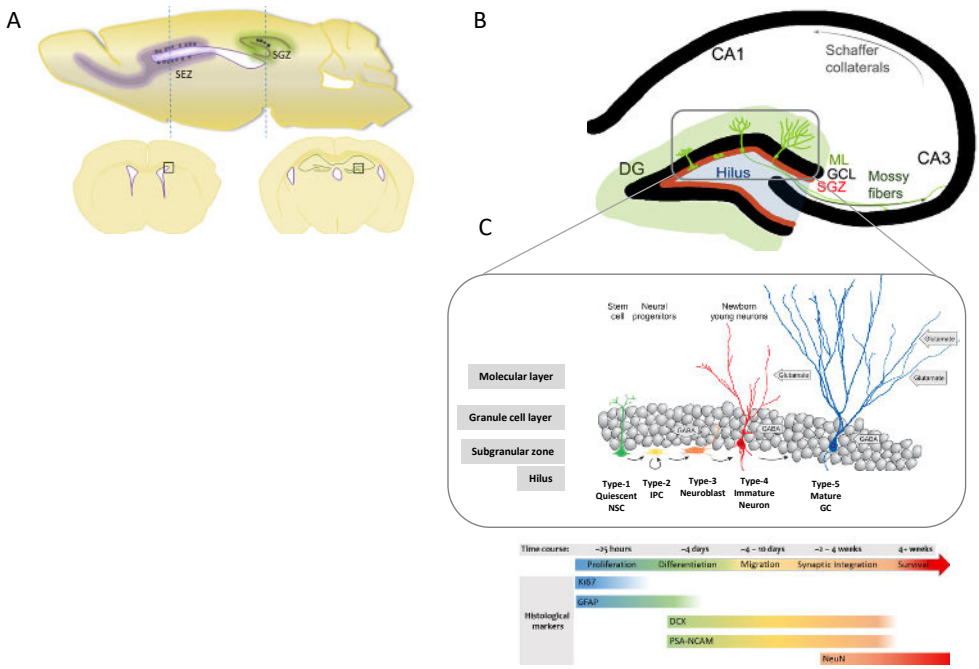

Figure 5. Schematic representation of adult neurogenesis. A: mouse adult brain, in sagittal (top) and coronal views (down), showing the two neurogenic niche: subependymal zone (SEZ) and the subgranular zone (SGZ). Adapted from (Stolp and Molnár, 2015) **B:** Schematic depiction of the anatomical organization of adult DG, comprising the molecular layer (ML), the granule cell layer (GCL), the subgranular zone (SGZ) and the hilus. Newly born neurons are found within the SGZ. Adapted from (Vadodaria and Jessberger, 2013) **C:** A higher magnification view of the boxed region shown in B, revealing neurogenesis phases in the DG (top) and histological marker proteins (down) that specifically identify different populations of newly born neurons in the DG. Adapted from (Bischofberger, 2007; Mahmoud, Wainwright and Galea, 2016).

The neural stem cell population from which new neurons are constantly generated in the process of neurogenesis reside in two main niches, which have been identified in the mammalian bran. These are: the subependymal (SEZ, or subventricular zone SVZ) at the edge of the ventricle, and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Reynolds and Weiss, 1992; Gage, 2000) (Fig. 5, panel A). From the anterior part of the SVZ, neuronal stem cells undertake the rostral migratory stream to the olfactory bulb and give rise to new interneurons (Lois and Alvarez-Buylla, 1994), while the neuronal progenitors cells of the SGZ give rise to new dentate granule cells.

SVZ and SGZ dock adult neural progenitor cells (aNPCs) from which astrocytes and amplifying neural progenitor cells (IPCs) arise, and further develop into immature and mature granule cells (GCs). Several mechanisms of tight control of proliferation allow these regions not to undergo depletion of the neurogenic aNPC pool. More specifically, regulation occurs to maintain a certain number of actively proliferating cells, also in balance with quiescent aNPCs (Christian, Song and Ming, 2014).

The whole process of neurogenesis is depicted in Fig. 5, panel C, and it can be divided into the following steps: proliferation, differentiation, migration, maturation and final integration of the newly born adult cells into the established neuronal circuits. Each of these phases is characterized by distinct cell morphology and expression of marker proteins, which have been extensively studied. Initially, the stem cell pool consists of quiescent type 1 radial glia-like cells expressing markers like Ki67 and GFAP (in addition, Nestin and Sox2), whose cell body resides in the SGZ, while the processes extend into the molecular layer. From these, type 2 highly proliferating daughters arise and commit to the neuronal lineage. They begin to express Polysialylated-neural cell adhesion molecule (PSA-NCAM), DCX and Prox1 (specific of granule cells development) markers until they further mature to reach the post-mitotic stage, which is characterized by the expression of Calretinin and Neuronal Nuclei (NeuN) (Kempermann *et al.*, 2004). Interestingly, the number of NeuN-positive neurons has been reported to peak at very early time points, though it lowers within a few days because of high apoptosis that eliminates most of the newly born cells (Biebl *et al.*, 2000; Kuhn *et al.*, 2005). Therefore, only the surviving cells will be able to make functional connections and play a role in the existing circuitries.

Remarkably, the newborn cells display hyperexcitability and enhanced synaptic plasticity during specific developmental stages (Schmidt-Hieber, Jonas and Bischofberger, 2004). During type 2 cell phase, cells receive GABAergic synaptic input that is excitatory due to the high content of chloride in the cytoplasm. GABA comes from the surrounding interneurons that release it locally. GABA-induced depolarisation/excitation is required for further maturation and integration of these cells (Ge, Eyleen L K Goh, *et al.*, 2006). In later stages of development, a decrease in intracellular concentrations of chloride ion allows GABA to become inhibitory. Following sustained maturation phase, few extra weeks are necessary for the adult-born neurons to exhibit comparable electrophysiological properties as mature granule cells (Ming and Song, 2011). During this late stage period, cells exhibit increased synaptic plasticity characterized by a lower threshold to induce long-term potentiation (LTP) (Schmidt-Hieber, Jonas and Bischofberger, 2004). It has been proposed that such hyperexcitability plays a critical role in the dentate gyrus where memory formation processes occur (more details below), or, it facilitates adaptation responses to new environment. The debate is still open, but the common assumption that can be traced thus far is that presumably, these properties allow for a long-term function (Kempermann, Song and Gage, 2015).

Considering that the focus of this work is anxiety and mood disorders, and that decreased hippocampal volume is an hallmark for major depression in humans and rodents (Malykhin and Coupland, 2015), we focused on hippocampal neurogenesis in the dentate gyrus. The section below will examine more in detail neurogenesis

occurring in the hippocampus and its functional and dysfunctional role in the adult brain and neuropsychiatric diseases.

1.5.1 Adult hippocampal neurogenesis in the adult brain

The hippocampus is a brain structure belonging to the limbic system and it is essential for short-term and long-term memory formation, spatial memory and navigation. It contains three subfields: area CA1, area CA3, and the dentate gyrus Fig. 5, panel B. While area CA1 and CA3 mediate memory processes (Nakazawa *et al.*, 2002, 2003, 2004), the dentate gyrus is of interest for this thesis and, it has been functionally linked to spatial pattern separation, the process that enables to encode similar memories into separate and non-overlapping events (Leutgeb *et al.*, 2007). The dentate gyrus is characterized by a V-shaped layer of densely packed, glutamatergic cells, named granule cells, with sparse synaptic activity (Neunuebel and Knierim, 2012). These cells have round cell bodies with spiny apical dendrites that outspread into the overlying molecular layer. Because of their low firing probabilities only a small proportion of mature granule cells is activated by inputs at any given time, resulting in sparseness of DG representations—also known as "engrams"—, that are considered instrumental for generating non-overlapping responses to diverse experiences, in so doing creating individual memories (Gonçalves, Schafer and Gage, 2016).

The granule cell population of the dentate gyrus is characterized by a continuous production throughout life; more specifically, the population starts to arise during gestation when new neurons residing in the ventricular zone migrate through the hippocampal rudiment to populate the developing dentate gyrus. Subsequently, during early postnatal period progenitor cells migrate into the subgranular zone and continue to proliferate giving rise to the granule cell layer. Lastly, the subgranular zone continues to be the source of granule cells also during adulthood (Altman and Bayer, 1990).

The DG molecular layer receives inputs from the septum, entorhinal cortex, mossy cells, and GABAergic interneurons. The two edges of the "V" of the DG mark the hilus, in which inhibitory GABAergic interneurons and excitatory glutamatergic mossy cells are found. The joining portion between the GCL and the hilus is the subgranular zone, the known niche of where the neural stem cell pool resides and from which new neurons develop in the adult mammalian brain (Amaral, Scharfman and Lavenex, 2007). The hippocampal circuit is characterized by a closed loop that processes sensory stimuli. Information travels through medial and lateral perforant pathways from the entorhinal cortex to the dentate gyrus, subsequently to CA3 pyramidal cells via mossy fiber axons of granule cells, then to CA1 pyramidal cells via Schaffer collateral projections of CA3 neurons, and lastly to the subiculum and back to the entorhinal cortex (Christian, Song and Ming, 2014).

Hippocampal neurogenesis is known to be a process subjected to regulation in vivo. A list of endogenous and exogenous factors regulating neurogenesis to different aspects has been shaped. Factors reported to boost neurogenesis include antidepressant compounds (Malberg *et al.*, 2000; Santarelli *et al.*, 2003b), exercise (van Praag, Kempermann and Gage, 1999), environmental enrichment (Kempermann, Kuhn and Gage, 1997) and learning (Gould *et al.*, 1999). Factors which contribute to suppress hippocampal neurogenesis comprise stress (Gould *et al.*, 1997, 1998), aging and pathological state (Apple, Solano-Fonseca and Kokovay, 2017). Moreover, neurotransmitters, hormones, proteins, epigenetic regulation, microglia and immune system can control the proliferation, differentiation, or survival of newborn neurons (de Miranda *et al.*, 2017). However, it is important to highlight that mechanisms of regulation occur at the level of neurogenic niche, such as the DG, which is a dedicated microenvironment that encompasses peculiar factors supporting the aNPC. Indeed, transplantation of these cells outside of the neurogenic niche, produces loss of ability of self-renewal and commitment to neuronal lineage (Suhonen *et al.*, 1996).

As far as morphological development of hippocampal neurogenesis goes, progressively during the first 2weeks cells extend dendrites and axons (known as mossy fibers) toward the molecular layer and project axons through the hilus toward the CA3 to form synapses with CA3 pyramidal cells. Furthermore, just one primary dendrite with multiple branches is seen within 7 days followed by rapid development between 7 and 17 days and terminating with reduced growth for at least two months. Spine density has been observed during the third week. However, complete integration of the newly born neurons into the existing dentate granule cell layer requires several months (Zhao, 2006). So, the question is when are these new neurons able to participate and modulate the hippocampal circuitry to impact on brain function and behavior? Interestingly, adult-born neurons show functional glutamatergic synaptic inputs and output at 14 days, suggesting that they can already contribute to neural processing during immature stages (Christian, Song and Ming, 2014).

The aforementioned intrinsic nature of hyperexcitability is due to the absence of solid GABAergic inhibition of immature neurons and this critical period is protracted for three to six weeks after birth (Ge et al., 2007). This might allow the newly born cells to have an active role in experience-induced plasticity. In addition, since a functional separation along the septo-temporal axis of the hippocampus has been extensively described, adult hippocampal neurogenesis has been linked to respective functions. Indeed, the dorsal hippocampus is implicated in cognitive functions and spatial navigation, while the ventral hippocampus is involved in affective behavior responses (Tanti and Belzung, 2013b). Furthermore, behavioral studies have reported that adult born granule cells (abGCs) play a role in stress and anxiety-like behavior (Snyder *et al.*, 2011; Mohammad, Marchisella, Hollos, *et al.*, 2016), in responses to

antidepressant treatments (Santarelli et al., 2003b) and in pattern separation (Clelland *et al.*, 2009; Sahay *et al.*, 2011). Remarkably, more recently, Danielson et al. recorded for the first time the activity of abGCs in vivo, using GCaMP imaging and providing a functional characterization of identified abGCs in behavioral pattern separation. The authors found that abGCs younger than 6 weeks fire at a higher rate than the mature population, though being less spatially tuned (Danielson *et al.*, 2016).

1.5.2 Neurogenesis and/or neuroplasticity in emotional responses

Neurogenesis during adulthood is a form of experience-dependent neuroplasticity through which new neurons integrate in the brain (Nottebohm, 2002). Impaired adult hippocampal neurogenesis has been associated to pathophysiology of depression, and a number of studies reported antidepressant treatment to affect levels of neurogenesis in both rodent models and human studies (Miller and Hen, 2015). However, this only remains a theory of pathophysiology of depression since the literature appears inconsistent on results found investigating the link between neurogenesis and depression. However, it can be generally deducted that:

Several animal models of depression and anxiety present altered neurogenesis. These include repeat restraint stress (Pham *et al.*, 2003), chronic unpredictable mild stress (Lee *et al.*, 2006) social defeat stress (Van Bokhoven *et al.*, 2011), social isolation (Dranovsky *et al.*, 2011), and corticosterone administration (Cameron and Gould, 1994). In addition, maternal separation model of anxiety and depression in rat shows both increased anxiety-like behavior in adulthood (Wigger and Neumann, 1999) and decreased adult hippocampal neurogenesis (Mirescu, Peters and Gould, 2004). Also, studies in non-primates are consistent, but due to technological challenges, human evidence is still lacking exhaustiveness (Gould *et al.*, 1998; Perera *et al.*, 2011; Boldrini *et al.*, 2018; Sorrells *et al.*, 2018).

Number of adult born neurons is consistently boosted by antidepressants in animal models of stress-induced depression and more specifically, fluoxetine treatment (Malberg *et al.*, 2000), electroconvulsive treatment (Madsen *et al.*, 2000) and lithium (mood stabilizer) (Chen *et al.*, 2000), constitute solid foundation for this hypothesis. It is well-known that ADs are able to modulate neurogenesis at distinct stages: stimulation of proliferation, speed up of maturation and encouragement of survival are the most affected, but action on differentiation is scarce (Eliwa, Belzung and Surget, 2017). Furthermore, despite the hippocampus does not present a uniform structure, monoaminergic ADs influence adult neurogenesis equally all along the dorso-ventral axis (Tanti and Belzung, 2013a). Animals with depleted neurogenesis exhibit impaired cognitive functions significant to depression, but a direct link has not yet been elucidated (Miller and Hen, 2015).

New drug avenues aiming to restore impaired neurogenesis is promising in animal models and, in clinical settings, it has been proposed that an initial screening

of patients based on their real impairment of neurogenesis should be assessed first in order to increase chances of positive responsiveness to the therapy (Miller and Hen, 2015).

Studies specifically linking hippocampal neurogenesis to anxiety disorders are also inconsistent, which poses a solid challenge to neuroscientists. In particular, it has been shown that physical exercise stimulates adult hippocampal neurogenesis (AHN), alleviating depressive-like symptoms in preclinical models (Kempermann, 2002) and cognitive functions in rodents and MDD patients (Babyak *et al.*, 2000; van Praag, 2005). However, reports on the effects of physical exercise on anxiety in both humans and rodents have been contrasting. Some studies have described reduced anxiety (Greenwood *et al.*, 2003; Fulk *et al.*, 2004; Duman *et al.*, 2008). Others heightened anxiety (Burghardt *et al.*, 2004; Van Hoomissen *et al.*, 2004; Leasure and Jones, 2008; Fuss *et al.*, 2010); one study found both increased and decreased anxiety-like behaviors (Binder *et al.*, 2004) and finally, some found no effect at all on anxiety (Pietropaolo *et al.*, 2006). Such contrast might be due to several external factors that contribute to differentiate the effect of exercise on anxiety. For instance, the amount of physical activity, the housing conditions of animals, the either voluntary or forced running paradigm that is applied.

The suppression of hippocampal neurogenesis has also been investigated in order to better shed light on the relationship between neurogenesis and anxiety-like behavior, still though with pronounced discrepancies in results. X-ray irradiation is an established method to ablate hippocampal neurogenesis, and it is performed by directing low doses of X-rays directly on the head of animals, to which neurogenic cells show extreme sensitivity (Mizumatsu *et al.*, 2003). In 2010, a group showed that irradiated mice did not develop running-induced anxiety-like behavior, indicating a direct role for neurogenesis in the development of exercise-induced anxiety-like behavior (Fuss, Nada M.B. Ben Abdallah, et al., 2010). Consistently, in another study irradiated animals developed cognitive deficits, but deletion of neurogenesis was not sufficient alone to induce anxiety-like behavior (Saxe *et al.*, 2006). It is also worth highlighting that these findings seem counterintuitive to the beneficial effects of running and physical exercise seen in humans. However, few variables should be considered when directly extrapolating data from rodents, such as: running distances performed by rodents do not reliably mirror the exercise paradigms that are proved to be therapeutic for mood and anxiety disorders in humans, since unlimited voluntary wheel running paradigms are typically applied. Furthermore, excessive exercise has been reported to have negative impact (Peluso and Guerra de Andrade, 2005), as well as, exceeding neurogenesis (Saxe *et al.*, 2007). Hence, moderate exercise is the recommended therapy in depressed subjects, typically combined to pharmacological approaches.

Contrarily, a conditional deletion of BDNF/TrkB signaling in a model of mouse impaired survival of newly born hippocampal neurons, determining anxious phenotype in mice (Bergami, Berninger and Canossa, 2009). On the other hand, a partial knockdown of neurogenesis, obtained by modifying Activin levels of expression or over-expressing the pro-apoptotic protein Bax in neuronal precursors, respectively, led to increased anxiety-like behavior (Ageta *et al.*, 2008; Revest *et al.*, 2009). Moreover, while the anxiolytic/antidepressant effect of fluoxetine is abolished when ventral newborn neurons are removed (Wu and Hen, 2014), the anxiolytic effect of benzodiazepines is not mediated by neurogenesis per se, though it contributes indirectly to the activity of hippocampal network implicated in the control of anxiety-like behavior. Taken together, these information lead to the idea that defaults at the hippocampal circuitry level might influence its ability to adapt to external inputs, such as, physical exercise, environmental enrichment and medication. In turn, drugs modulate the rate of neurogenesis, with a propagation of impairment to structures downstream the hippocampus, such as prefrontal cortex and amygdala, associated with mood behavior (Bergami, Berninger and Canossa, 2009).

Interestingly, can neurogenesis loss be considered a good thing happening while in challenging environments? Perhaps. Since it has been postulated that the overall effect of inducing anxio-depressive behavior can be seen as a way to shift the behavior towards a more cautious attitude, when challenges are unpredictable in a given environments (Cameron and Schoenfeld, 2018), further providing evidence for a role of neurogenesis in adaptive behavior. However, additional research in this area is still needed.

On the other hand, other neuroplasticity mechanisms different from neurogenesis and involving volumetric changes of crucial brain regions (hippocampus, prefrontal cortex and amygdala) and apoptosis of hippocampal neurons have been called into question. It has been hypothesized that targeting of these latter may reveal to be a more effective strategy to guide recovery from anxiety and depression. The term neuroplasticity defines our brain's capability of undergoing neuronal modifications in response to external stimuli; an evolutionary mechanism that has led to a better adaptation to new environments (Liu *et al.*, 2017) and, a new hypothesis that changed the accepted idea of seeing the brain as a static organ (Serafini, 2012). Typical neuroplastic changes involve strengthening or weakening synapses and modifications of neural circuits. In physiological state, the brain-derived neurotrophic factor (BDNF) plays a crucial role in supporting survival of existing neurons and stimulating the new neural cells and synapses to grow and differentiate. Remarkably, serum levels of BDNF are found lowered in subjects with MDD (Monteleone *et al.*, 2008) suggesting a likely involvement of BDNF in the pathophysiology of depression. Furthermore, when BDNF is knock-out in the dorsal

dentate gyrus of the hippocampus, depressive-like behavior is elicited in rats, suggesting a link between neuroplasticity and depression (Taliaz *et al.*, 2010).

Decreased volume of HC in depression has been mentioned several times already in the previous sections, but, in the context of impaired neuroplasticity, numerous mechanisms have been proposed to explain this phenomenon. These include neuronal and glial remodeling, neuronal and glial death, suppressed adult neurogenesis and elevated levels of glucocorticoids, which modulate cell morphology and cause dendritic atrophy and neurotoxicity in the HC, which is considered a major target site because of prominent expression of GRs (Sapolsky, 2000; Malykhin and Coupland, 2015). Thereby, the model hypothesized here is that chronic exposure to stress induces stabilized high levels of glucocorticoids; these act at the level of hippocampal formation where mineralocorticoid and glucocorticoid receptors are abundant. The principle function of these receptors is to maintain basal HPA tone and to control the negative feedback of glucocorticoid release during a stress response. Hence, this explains the peculiar vulnerability of HC to effects of stress and its facilitation of inducing depressive-like state (Meaney, Sapolsky and McEwen, 1985). Robust evidence has shown that the application of chronic stress to animals creates reductions in expression of hippocampal proteins associated with neural plasticity, reduction of neuronal complexity and spine density (Watanabe, Gould and McEwen, 1992; Shors, Chua and Falduto, 2001), as well as, reduction of connectivity and neurotransmission due to loss of glia cells (Andrade and Rao, 2010).

In addition, stress-induced neuroplastic changes also affect the other relevant areas involved in the neurocircuitry of emotions: the prefrontal cortices of animal models of depression also display loss of dendritic spines, atrophy of the dendritic tree, loss of synapses, decreased number and size of glia (Pittenger and Duman, 2008; Marsden, 2013). Post-mortem analysis of brain tissue from depressed subjects also exhibits a lower neuronal and glial cell (both in number and size), and an overall decrease in cortical thickness (D'Sa and Duman, 2002). Simultaneously, a global increased volume of the amygdala has been described in either stressed animals and patients with MDD, a modification causing structural and functional impairments in this region (Serafini, 2012).

Of note, molecular mechanisms resulting in neuroplasticity dysfunctions have been speculated. Evidence suggest that both N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors activation plays a key role in regulating neuronal modifications, especially in the DG. Indeed, glutamate activity is influenced by elevated glucocorticoids levels and modulates most of the structural modifications of neurons (Serafini, 2012). At the same time, several lines of evidence have proposed an NMDA glutamate receptor antagonist: ketamine, as a promising avenue for treatment of depression (for a review see (Andrade, 2017)). Ketamine seems to have a unique pharmacological profile, since

a single, subanesthetic dose promotes a rapid antidepressant effect (within hours compared to 3-4wks for ADs), anti-suicidal effects, and, remarkably, it is effective in treatment-resistant patients. Though its long-term adverse effects are still not clear and, more importantly, its mechanism of action has not yet been elucidated in the context of depression (Hayley and Litteljohn, 2013).

Subsequently, an effect of antidepressants reverting the stress-induced neuroplastic changes has been described, supported by robust literature (Pittenger and Duman, 2008). ADs reverse hippocampal shrinkage and significantly improve cognitive functions associated to hippocampal activity (Dranovsky and Hen, 2006). Consistently, in animal models of chronic mild stress, restored neuronal plasticity in the HC and PFC, but not neurogenesis, produced a rescue of behavioral phenotype (J. M. Bessa *et al.*, 2009). Moreover, it has been speculated that ADs induce a neural adaptation in the limbic structures, stabilizing the negative feedback inhibition of the HPA axis, which has been seen to improve mood scores either before or concomitantly to alleviation of symptomatology in depressed patients (Ising *et al.*, 2007).

Globally, antidepressants first have an acute effect on monoamine metabolism, which, in turn, activates processes of plasticity that gradually conduct to improved information processing in the brain circuitries that are relevant in emotional regulation. Neurogenesis, selective neural elimination, increased dendritic complexity and retraction of axons and dendrites, synaptogenesis and pruning, are all modifications promoted by ADs action and require time to develop and mature, which can explain the delayed onset of the clinical effects of antidepressants (Castrén, 2005).

In this section, I attempted to summarize the ongoing controversy of whether a specific form of neuroplasticity (adult hippocampal neurogenesis) is crucial for pathophysiology of depression and efficacy of antidepressant medications. Alternatively, all the modifications underneath the term "neuroplasticity" are crucial for understanding the molecular mechanisms underlying the diseases and could be considered as a hallmark for neuropsychiatric disorders (like neurofibrillary tangles and amyloid plaques for Alzheimer's disease or loss of dopaminergic neurons in Parkinson's disease).

The next section will examine the Mitogen-activated protein kinases (MAPKs), of interest for this work, starting from a general background to a narrower description of specific involvement in neuropsychiatric disorders.

1.6 Mitogen-activated protein kinases

The enzymatic reaction of adding phosphate groups to substrate proteins is catalyzed by protein kinases and the event induces a functional change on the substrate protein.

Phosphorylation is a key, molecular event characterizing signaling pathways. These cell pathways elaborate external stimuli through a series of reactions inside the cytoplasm, inducing a response that allows cells and tissues to adapt to extracellular environment.

MAPKs are a family of serine– threonine protein kinases known to control various cellular activities such as proliferation, differentiation, apoptosis, survival, inflammation, transformation and innate immunity. Alterations of the pathway have been extensively studied and linked to the pathogenesis of several diseases going from cancer to neurodegenerative disorders or inflammatory diseases (Kim and Choi, 2015).

MAPK pathways are known as "three-tiered": the signaling starts with a top tier of kinases (MAP3Ks) which, through phosphorylation events send inputs down to a middle tier of kinases (MAP2Ks) that in turn activate the last and lower tier of MAPKs. This latter subfamily of proteins act as effectors phosphorylating downstream substrates to prompt cellular responses (Zeke *et al.*, 2016).

In mammals, the identified MAPKs comprise extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun NH2- terminal kinase (JNK). Multiple isoforms for each of these proteins exist and are found shared among the cascades, thus enabling cross-talk and coordinated integration of external stimuli.

1.6.1 General background on c-Jun NH2- terminal kinase

The JNK family will be of most interest for this thesis. JNK was originally identified as stress-activated protein kinase (SAPK) due to its responsiveness to stress insults such as DNA damage, oxidative stress, cytoskeletal changes, infection or inflammation, but then it was renamed "JNK" to highlight its role in phosphorylation and activation of c-Jun transcription factor.

JNK pathway is classically found active in cancer, heart disease, axon degeneration (Geden and Deshmukh, 2016), neurodegenerative disease (Alzheimer´s disease, Parkinson´s disease, Multiple sclerosis, Huntington´s disease) obesity and insulin resistance (Pal, Febbraio and Lancaster, 2015). Research has been actively progressing towards the generation of new JNK inhibitors as a new attractive approach for more targeted therapies with less undesirable side effects (Koch, Gehringer and Laufer, 2015).

The MAPK tier requires scaffold proteins to coordinate the physical association of constituents of the pathway in order to reach effective signal transduction. Scaffold proteins lack any catalytic activity and the described structure involves several modular protein-protein interaction domains, which facilitate specific tethering of substrate with its enzyme (Good, Zalatan and Lim, 2011). The JNK scaffold proteins that have been described comprise the JNK-interacting proteins (JIP1–4), plenty of SH3s (POSH), β-arrestin, IκB kinase complex-associated protein (IKAP), JNK-

binding protein 1 (JNKBP1/MAPKBP1), and WDR62 (Cohen-Katsenelson *et al.*, 2013).

Phosphorylation at Thr183 by MKK4 and at Tyr185 by MKK7 activate JNKs. Active JNKs can phosphorylate very many substrates such as the cytoskeleton (e.g. microtubule-associated proteins, Tau, Neurofilament), at the mitochondria (e.g. Bad, Bim) and in the nucleus (ATF, c-Jun, p53) (Eleanor T Coffey, 2014; Zeke *et al.*, 2016).

Three JNK genes have been found encoded in the mammalian genome: JNK1 (MAPK8), JNK2 (MAPK9) and JNK3 (MAPK10). All three genes are expressed in the human and mouse brain, with a preponderance of mRNA expressed in the neocortex, hippocampus, thalamus and midbrain. The size of the kinases is about 400 amino-acids. Haeusgen et al., mapped human JNK1, JNK2 and JNK3 genes on chromosome 10, 5 and 4, while in mice the three genes are mapped on chromosome 14, 11 and 5 respectively, and on chromosome 16,10 and 14 in rats (Haeusgen, Herdegen and Waetzig, 2011). Ten alternative transcripts are generated for JNK gene; JNK1 and JNK2 have four transcripts whereas JNK3 has 2 transcripts. The C-terminal lobe sequence of the kinase domain encompasses the splicing site and the process gives rise to two similar kinases, called the α- and β-isoforms (Zeke et al., 2016).

JNK1 and JNK2 are widely detected in most tissues, whereas JNK3 is largely expressed in the nervous system, heart and testis (Waetzig and Herdegen, 2004; Kim and Choi, 2015).

1.6.2 JNK in the developing brain

The pattern of expression of JNK genes varies significantly spatiotemporally from development to adulthood: JNK1 and JNK2 expression starts at embryonic day 8 (E8), while JNK3 becomes detectable at E11. *Jnk1* mRNA levels have been shown to decay postnatally, though they are still detectable during adulthood in the olfactory region and DG (Antoniou and Borsello, 2012; Eleanor T Coffey, 2014).

The importance of JNK in the nervous system has been revealed using approaches of *Jnk*-knockout mice and JNK inhibitor. More specifically, JNK signaling has been found to be fundamental in brain developmental processes (Eleanor T Coffey, 2014).

A proper cortical development is essential to give rise to a functional neuronal connectivity; Westerlund et al., identified JNK1 as a regulator of neuronal migration required for proper cortical layer formation during development (Westerlund *et al.*, 2011). Later on, a study reported that *Jnk1* and *Jnk1/2*-null embryos display slower and abnormal migration of cortical interneurons during development. This indicated a novel role for the JNK pathway in cortical interneuron migration (Myers *et al.*, 2014), and interneuron dysfunction have been linked to a serious impairment of brain activity and to neuropsychiatric diseases such as schizophrenia and autism (Marín, 2012).

Moreover, during development axon pruning eliminates excessive axons and/or dendrites without causing neuronal death. This process is important for regular brain wiring in vertebrates and invertebrates. In flies, it was recently shown, that JNK is necessary for axon but not dendrites pruning of mushroom body γ neurons (Bornstein *et al.*, 2015). By deleting the upstream regulator of JNK, MKK7, Yamasaki et al., were able to find JNK pathway implicated in regulating axon elongation during corticogenesis (Yamasaki *et al.*, 2011), followed by another study that revealed JNK1 as major player of axon guidance in the developing nervous system (Qu *et al.*, 2013). *Jnk* single knockout mice *(Jnk–/–)* do not present any obvious abnormality during initial development, which suggests forms of compensation from the other JNK isoforms. Although, it has been shown that in *Jnk1–/–* mice the anterior commissures degenerate in young animals to disappear in adults, which could suggest JNK1 may be involved in neuronal survival. Indeed, anterior commissures degeneration is in fact due to a deficiency in phosphorylation of microtubule-associated proteins (MAPs) which induces a shortening of neuronal microtubules that in turn prompts a progressive neurite degeneration (Karin and Gallagher, 2005).

Energy homeostasis is disturbed in *Jnk1–/–* animals that display altered hormone secretion and feeding patterns and data indicate that normal activity of JNK1 in the brain is essential for regular metabolism (Karin and Gallagher, 2005). Of note, a possible mechanism of JNK1 involvement in energy balance has been recently dissected by Martínez-Sánchez et al (Martínez-Sánchez *et al.*, 2017).

Jnk2–/– mice are perfectly viable and do not display any development malformation. Though, JNK2 is necessary for the proper differentiation of precursor CD4+ T cells into effector Th1 cells (Yang *et al.*, 1998).

Jnk3–/– mice display heightened apoptosis of hippocampal neurons and are resistant to treatment with excitotoxic agents suggesting a role of JNK3 in death response (Yamasaki, Kawasaki and Nishina, 2012). Furthermore, these single knockout animals are also a model of resistance to cerebral ischemia (Pirianov *et al.*, 2007) and more generally, JNKs are shown to exert a neuroprotective effect downstream Neuregulin-1β activation, in a model of middle cerebral artery occlusion/reperfusion in rats (Ji *et al.*, 2017).

Interestingly, JNK1/JNK3 and JNK2/JNK3 double knockout mice are viable, while JNK1/JNK2 double knockout mice are embryonically lethal: they die at E11.5 because of failings in neural tube morphogenesis closure (Zeke et al., 2016).

1.6.3 JNK in the adult brain

JNKs are evolutionarily highly conserved from yeast to humans and JNK signaling in the brain is known to be constitutively activated (Waetzig, Zhao and Herdegen, 2006). This is explicative of how crucial JNK signaling is for adult physiological brain functions such as synaptic plasticity, which is the essential basis of learning and

memory formation. By exploring the phenotype of knockouts of JNK isoforms and the effect of JNK inhibitor (DJNKI-1) infusion in the hippocampus of mice, it was found that JNKs are specifically linked to associative learning (Sherrin *et al.*, 2010).

LTP and Long-term depression (LTD) are forms of synaptic plasticity that induce reinforcement or weakening of synaptic strength in response to external stimuli. The requirement of MAP kinases including p38, JNKs and ERKs in synaptic plasticity in the hippocampus has been extensively shown (Thomas and Huganir, 2004). Moreover, the inhibition of JNK signaling has been proposed to be promoting LTP indirectly by impairing LTD, boosting synaptic plasticity (Yang *et al.*, 2011) and the phosphorylation of the c-Jun N-terminus is necessary for LTP in the hippocampus, suggesting a possible mechanism of how JNK mediates modulation of synaptic plasticity (Seo *et al.*, 2012). The lack of LTD has been described in *Jnk1-/-* mice and JNK inhibitor treated rats (Li *et al.*, 2007), while JNK2 is associated to mechanisms of memory formation, being required for late phase LTP (Chen *et al.*, 2005). To further elucidate the mechanism by which JNKs contribute to synaptic plasticity, a pioneering study showed that NMDA glutamate receptors activate JNKs (Mukherjee *et al.*, 1999) and synaptic plasticity is controlled by NMDAR activation, calcium influx and AMPAR recruitment. Later it was described that the absence of JIPs affects NMDARs function (Kennedy *et al.*, 2007) and more recently it was defined that JNK controls NMDA dependent glutamate release at presynaptic level (Nisticò *et al.*, 2015). Therefore, it seems that this complex process requires the joined interaction of at least three partners: NMDAR, JIP1 and JNK. Additionally, among the aforementioned identified substrates of JNKs, many are key regulators of synaptic plasticity. PSD95 and GluR2 (AMPAR subunit) are phosphorylated by JNKs (Kim *et al.*, 2007; Ahn and Choe, 2010). In aged mice, JNK pathway is essential to promote neurite outgrowth, a process important for neuronal differentiation and on which repairing mechanisms of nerve system damage rely, as well as learning and memory formation (Park *et al.*, 2017). JNKs appear to be conserved regulators of motor transport, which is necessary for neuronal function, in Drosophila and mammals; besides, JNK signaling is shown to repress autophagy, in neuronal cells (Yamasaki, Kawasaki and Nishina, 2012). More recently, it has been reported that suppression of JNK activity disrupts normal circadian rhythms and is essential for preservation of neuronal activity in adult brain (Yamasaki *et al.*, 2017). Implications of JNK in the maintenance of cytoskeleton of neuronal cells are also known, and specifically interaction of JNK1 with downstream substrate MAP2 is essential in defining dendritic architecture for a functional synaptic connectivity in the adult brain (Bjorkblom, 2005; Waetzig, Zhao and Herdegen, 2006).

1.6.4 JNKs in anxiety and depression

A number of genetic studies have long associated JNK to neuropsychiatric disorders because of its relevance in modulating brain structures and neuronal morphology. For

example, genome-wide association studies applied to human evidence show strong correlation between microduplications at 16p11.2 and susceptibility to schizophrenia (Morris and Pratt, 2014) and autism (Weiss *et al.*, 2008), given that a MAP kinase kinase kinase (TAOK2) that mediates the activation of p38 and JNK has been found located within the duplicated region. Furthermore, two case reports of intellectual disability describe a *de novo* chromosome translocation resulting in a truncated JNK3 protein with partial loss of function (Shoichet *et al.*, 2006; Kunde *et al.*, 2013) (Shoichet *et al.*, 2006; Kunde *et al.*, 2013). More recent evidence shows that inhibition of JNK activation by neuronal p38α reduces anxiety-related behavior in mice (Stefanoska *et al.*, 2018).

Genetic studies specifically aimed to detangle association between JNK and anxiety or depression have not yet reached a stage where findings can be considered reliable (Flint and Kendler, 2014b). Recently, the transcription factor *FoxO1,* that is a downstream substrate of JNK in neurons (Xu *et al.*, 2011), was shown to be significantly predisposing to adult depression in individuals that had been exposed to early life trauma (Cattaneo *et al.*, 2018).

In a mouse model of acute stress in which exposure to cold is followed by rewarming, JNK activity has been found upregulated in the hippocampus and prefrontal cortex, structures relevant to emotional responses (Zheng *et al.*, 2008). Similarly, an acute model of forced swimming, significantly activated JNK in hippocampus, prefrontal cortex and amygdala, along with upstream activators of JNK pathway, which were upregulated even to a greater extent (Shen *et al.*, 2004).

Also, different studies revealed that following a period of chronic stress, animals displayed heightened JNK levels in these same brain regions, when tested for forced swim test, tail suspension and restraint (Adzic *et al.*, 2009; Galeotti and Ghelardini, 2012). Furthermore, when a cohort of animals subjected to chronic social defeat stress was screened for susceptible and resilient units, remarkably, JNK activity was higher in the brain of animals susceptible to stress (Rosa, Pesarico and Nogueira, 2017).

Stress produces elevated levels of glucocorticoids, which correlate to depressive phenotype in rodents (Warren *et al.*, 2013). In humans, patients with depression display increased cortisol levels (Rao *et al.*, 2008), even higher when comorbid with anxiety (Vreeburg *et al.*, 2009). Furthermore, significantly higher levels of GR phosphorylation at serine 226 is seen in MDD patients and JNK modulates GR function (Wang *et al.*, 2005), at serine 226 (Itoh *et al.*, 2002). Hence, JNK inhibition is expected to reduce glucocorticoids toxic effect and in turn, it will ameliorate symptoms in MDD patients (Jovicic *et al.*, 2015)

Of note, traditional herbal medicine with antidepressants properties was found to decrease JNK expression in the hippocampus tissue of rats exposed to a paradigm of unpredictable chronic mild stress (Li *et al.*, 2012).

In conclusion, in this section I described how JNKs are important kinases in brain and how their activity has been linked to susceptibility to neuropsychiatric disorders in both humans and rodents, in particular to depressive-like behavior. Previous study has shown that JNKs display elevated activity in brain in the absence of stress compared to other tissues such as heart, liver, kidney, spleen, indicating that CNS-expressed JNKs also retain non-stress related functions (Coffey *et al.*, 2000). Therefore, taken together these data signify that JNK inhibition in brain could be a novel, powerful strategy to improve pharmacological treatment of depression.

The next section will explain the state of the art of pharmacological targeting of JNK in neuropsychiatric disorders.

1.6.5 JNK pathway as a novel pharmacological target

Pharmacological blocking of JNK pathway has been long studied in the context of cancer, due to its critical role in key cellular functions (Bubici and Papa, 2014).

A first class of compounds are the ATP-competitive JNK inhibitors, which are designed upon the highly conserved ATP-binding site. These are able to block all JNK isoforms, lacking specificity, thus they could consequently trigger off-target effects, not suitable for clinical purposes. The SP600125, anthrapyrazole, was the first small molecule reported to show neuroprotective effects in animal models of Alzheimer's (Braithwaite *et al.*, 2010) and stroke (Shvedova *et al.*, 2018). In the context of neuropsychiatric disorders, SP600125 was used to investigate the role of JNK in the behavioral, hormonal and neurochemical effects of an acute psychological stressor (wet bedding + restraint) and it was seen that JNK inhibition exerted a neuroprotective effect by reversing some behavioral and neurochemical effects induced by the application of the acute stressor (Clarke *et al.*, 2012). Nevertheless, SP600125 inhibits not only JNK1, JNK2 and JNK3 isoforms with equal potency, but also kinases upstream in the JNK pathway, ERK and p38 pathways, thereby inducing a loss of control of its action in more complicated systems than *in vitro* (Bogoyevitch *et al.*, 2004).

A second class of inhibitors, that still lacks solid information on applicability in clinics, targets the docking sites of JNK substrates, or the regulatory protein site of the JNK, such as, JIPs. JIPs are scaffold proteins that regulate JNK pathway, they retain specificity for particular isoforms of JNKs, though the effects of these inhibitors in the context of anxiety and depression has not been tested. However, these helped extrapolating the concept that inhibition of JNK activity depended on either the ability of JIP overexpression to inhibit JNK activity in mammalian cells or its ability to maintain JNK in the cytoplasm preventing its nuclear translocation, which blocked phosphorylation and activation of downstream transcription factors, such as c-Jun.

Later, it was revealed that these peptides aim to a region not directly in the active site of the kinase. Furthermore, through comparisons of two isoforms of the JIP/IB

scaffold protein sequences a stretch of 18 –20 conserved amino acids was identified and then coupled to a cell-penetrating peptide sequence of 10 amino acids derived from the human immunodeficiency virus TAT protein, in order to confer spontaneous cell uptake of the peptide (Bonny *et al.*, 2001). To improve intracellular stability the peptide was synthesized in the reverse sequence from D-amino acids rather than L-amino, even though this change reduced JNK inhibitory efficacy of approximately 15- to 20-fold, revealing the complexity of simultaneous improvement of delivery, stability and inhibitory efficacy (Bogoyevitch *et al.*, 2004). This specific peptide is known as DJNKI-1 and is the inhibitor of choice for this work. Mechanistically, it blocks protein-protein interaction preventing the access of the kinase to its targets through an allosteric modulation on JNK. DJNKI-1 has recently been validated in preclinical and clinical settings without undesirable side effects in a number of degenerative diseases, such as traumatic hearing loss (Wang *et al.*, 2007) and ischemic stroke (Borsello *et al.*, 2003; Wiegler *et al.*, 2008; Benakis, Bonny and Hirt, 2010).

In summary, JNKs are connected to a plethora of diseases and they are essential for a variety of cellular functions. Thereby, while targeting the JNK pathway looks promising in fighting a variety of diseases such as cancer, inflammatory diseases, diabetes, neurodegenerative diseases, stroke and neuropsychiatric disorders (Manning and Davis, 2003), the challenges regarding potential side effects upon systemic inhibition of JNK remain, such as induction of tumorigenesis (Cui *et al.*, 2007). Inhibitors for different components of the JNK pathway would allow to obtain higher specificity and potentially decrease the risk undesired effects since JNK isoforms exert redundant functions inside the cells. Thereby, isoform-specific inhibitors are likely to be better tolerated. In conclusion, identifying which isoforms play predominant roles in anxiety disorders is the key to help pinpointing specific and more efficacious therapeutic targets.

1.6.6 JNK mediated regulation of dendritic development and spine density

The development of functional neural networks involves the correct navigation of axons to their targets and the expansion of complex dendritic arbors that integrate multiple synaptic inputs. This process of dendritic development is controlled by a combination of intrinsic and extrinsic factors, which act on neuronal cytoskeleton reorganization to regulate dendritic growth, branching and orientation (Jan and Jan, 2003). The reshaping of dendritic morphology and dendritic spines is the mechanism underlying synaptic plasticity in the adult brain, and alterations in dendritic spines (more than in dendritic tree) are linked to brain trauma, addictive behavior and in neuropsychiatric disorders, as well as learning and memory (Frankfurt and Luine, 2015).

Dendritic spines are small protrusions that cover the dendritic tree and represent specialized subcellular compartments where excitatory synapses are formed, hence extending the neuron surface area of neurotransmission. Interestingly, the density of spines fluctuates from extremely spiny pyramidal neurons in the cortex and hippocampus (Nimchinsky, Sabatini and Svoboda, 2002; von Bohlen und Halbach, 2009) to the quite sparse spine density of neurons in the hypothalamus (Frankfurt and Luine, 2015). Typically, the dendritic spine density ranges from 0.2 to 3.5 spines per 1 μm of dendrite, considering the neuron type, age, and position along the dendrite as well as the methodology applied for counting (Sala and Segal, 2014). The cytoskeleton of dendritic spines contains mostly filamentous actin, which spreads from the base of the spine to the postsynaptic density.

Moreover, dendritic spines develop during the consolidation of the synapse, and they mature from filopodial extensions from the dendritic shaft into:

- Mushroom: most abundant subcategory, characterized by a large bulbous termination head and a short neck, with the diameter of the head being greater than the diameter of the neck. These are believed to be key in memory processes and to carry more biochemical signals (Bourne and Harris, 2007)
- Stubby: characterized by no apparent neck and small bulbous head and with length and width equal
- Thin: characterized by a small bulbous head plus a thin, long neck

Interestingly, different spine types elicit different functions and modifications in the ratio of these spines may produce a sensitive effect on neuronal excitability and function. Indeed, while thin spines are considered learning spines, the large, mature, and less motile mushroom spines embracing larger and stronger synapses are memory spines with the function of maintenance of neuronal networks and long-term memory. Moreover, the mushroom neck is a central structure for the spine because it prevents calcium exchange between the spine head and dendrite shaft, which controls proper synaptic transmission and may be neuroprotective, avoiding excitotoxicity to the dendrite and neuron by confining excessive influxes of calcium within the neck (Segal, 1995).

Dendritic spines are extremely dynamic during development (with stubby and thin being the most prevalent type) and also in the mature nervous system. Spine formation, turnover and morphology is continuously adjusted according to the inputs from the external environment, in the form of synaptic plasticity. Furthermore, both LTP and LTD can exert a bidirectional control of the size of a spine through regulators of the actin cytoskeleton, expanding the spine volume or inducing spine shrinkage, respectively (Okamoto *et al.*, 2004).

LTP and LTD characterize the cellular basis of hippocampal-dependent learning and memory. In 2009, Xu et al., monitored how spines of pyramidal neurons in

the motor cortex were affected in training mice with a motor learning task. Interestingly, the motor activity rapidly prompted the formation of new spines, and many of these new spines persisted for weeks and months after training, with mice performing the motor task at best positively correlate with the magnitude of new spine formation (Xu *et al.*, 2009).

In the context of neuropsychiatric disorders, dendritic spine pathology has long been associated with disease state (Penzes *et al.*, 2011) and structural and molecular remodeling of dendritic spines in the hippocampus, prefrontal cortex, amygdala, and nucleus accumbens have been extensively reported during depression, with antidepressant treatment reversing such changes (for a review see Qiao *et al.*, 2016).

Enhancement of neuronal plasticity is key for adaptive intracellular changes during the normal stress response to an acute challenge, which stimulates dendritic growth, synaptogenesis, and neuronal protein translation, while severe or chronic stressors can disrupt the ability of the brain to maintain its normal stress response, facilitating the development of major depression. Mechanistically, it is thought that the increased levels of glucocorticoid are responsible for suppressing the production of new neurons in the hippocampus which leads to decreased dendritic spine density and synapse number, with consequent memory impairment (McEwen, 2005a).

In particular, dendritic retraction or atrophy, which involves both reduction in total dendritic length and a simplification of dendritic arbors, has been shown in the dendrites of CA3 pyramidal neurons of the HC following unpredictable and chronic stress. Such effects were also shown to recover along with the stress induced depression-like behaviors following stress-free period (for a review see (Qiao *et al.*, 2016). Stress-derived spine density shifts on CA3 pyramidal neurons are influenced by stressor types, animal species, sex, and the length of stress. Contrasting literature reports either a decrease (Magarin˜os and McEwen, 1995; Stewart *et al.*, 2005; Conrad *et al.*, 2012), an increase (Sunanda, Rao and Raju, 1995; Sandi *et al.*, 2003), or no change (Magariños *et al.*, 1996) in spine density in the dendrites of male rat CA3 pyramidal neurons. On the other hand, chronic stress-induced changes in spine density in CA1 pyramidal neurons are less described and seems to follow a sex-dependent fashion (Qiao *et al.*, 2016). Interestingly though, post-mortem studies in humans revealed a reduction in the dendritic arborization and dendritic spines in the hippocampus of patients that suffered from anxiety and depression (Soetanto *et al.*, 2010).

Decreased volume of mPFC found in subjects affected by depression is consistent with decreased expression of synaptic-function-related genes and loss of synapses in post-mortem tissue (Kang *et al.*, 2012). On the other hand, animal studies reveal retraction of apical dendrites of pyramidal neurons and spine loss in the mPFC following chronic stress are accompanied by cognitive impairments (Qiao *et al.*, 2016). As changes in amygdalar volume are also implicated during depressive state, increased spine density and enhanced dendritic arborization in the amygdala have been reported in animals who

underwent chronic stress paradigms (Duman and Duman, 2014). Regulatory mechanisms underlying dendritic spine alterations upon stress application are not well understood yet, though the typical end result is a lowered expression of synapse-related genes, which is also confirmed by tissue analysis from post-mortem brains of depressed patients (Kang *et al.*, 2012). As briefly mentioned above, glucocorticoids are involved in such effects, as well as, neurotrophic factor signaling, such as BDNF or the mTORC1 pathway, whose components of cascades are found downregulated, or the whole signaling is reduced (Duman and Duman, 2014). Remarkably, monoaminergic antidepressants or ketamine and physical exercise have been shown to revert these pathophysiological alterations, revealing targeting of synaptic plasticity an effective therapeutic direction (Duman and Duman, 2014; Chen *et al.*, 2017).

The JNK pathway is associated to psychiatric disorders and intellectual disabilities implicating synaptic structure abnormalities (Eleanor T Coffey, 2014). JNK is also crucial in controlling physiological processes of neuronal plasticity and regeneration in the adult brain. More specifically, JNK regulates synaptic plasticity through the direct phosphorylation of synaptic target proteins. PSD95 is an abundant synaptic scaffolding protein directly phosphorylated by JNK on Ser295 residue, which increases the synaptic accumulation of PSD95 and reduces NMDA-induced AMPA receptor internalization (Kim *et al.*, 2007). In addition, JNK phosphorylates the Ser447 residue of delta-catenin, which decreases dendritic branching (Edbauer *et al.*, 2009). JNK is reported to accelerate AMPA receptor recycling by direct phosphorylation of GluA2L and GluA4 (Zhu *et al.*, 2005; Thomas *et al.*, 2008a). In neurodegenerative conditions, such as Alzheimer's disease, it has been found that JNK mediates synaptopathy facilitating a substantial removal of AMPA and NMDA receptors from the postsynaptic density region, resulting in defective LTP and LTD, as well as, dendritic spines loss (Sclip *et al.*, 2013, 2014).

Whether or not JNK regulates synaptopathy in the context of anxiety and depression is not known and it will be of interest for this work elucidating whether JNK inhibition affects dendritic arborization and spines in the neurogenic population of adult born granule cells of the hippocampus, leading to lower anxiety-like behavior and depressive-like phenotype.

2. OBJECTIVES

My PhD project aimed to establish whether the JNK pathway is a novel pharmacological target to control neuroplastic changes in mouse brain leading to anxiety-like and depressive-like behavior.

The starting point of this project was based on our previous works, which identified JNK1 as regulator of several neuronal processes and morphology. The goal of my study was to investigate whether the inhibition of this kinase activity influences neuroplasticity in the adult mice brain, and whether such changes underlie behavioral effects in the context of neuropsychiatry. Furthermore, I was intrigued to employ a cutting-edge technology, fiber photometry, to explore changes in synaptic activity of the newly born neurons, upon JNK inhibition. In summary, the main goals of this thesis were:

1. To study whether inhibition of JNK alleviates anxiety-like and depressive-like symptoms in mice upon regulation of 4 stages of neurogenesis (proliferation, differentiation, maturation and survival)

2. To evaluate the effect of JNK inhibition on the baseline synaptic activity in adult born granule neurons *in vivo,* using the calcium sensor reporter (GCaMP6) to image neural activity.

3. EXPERIMENTAL PROCEDURES

The following section will briefly describe experimental procedures used throughout the work of this thesis. Detailed information regarding each experiment can be found in the original publication and manuscript (Study I and II).

3.1 Plasmid construction

For Study I, replication-incompetent MLV retroviral vectors encoding for inhibitors of cytosolic (GFP-NES-JBD) and nuclear (GFP-NLS-JBD) pools of JNK (already described (Bjorkblom, 2005)) were generated by using regular cloning technique. PCR-amplified NES-JBD and NLS-JBD sequences from pEGFP-C1 (Clontech, Mountain View, CA) were inserted into CAG- GFP vector, previously described (van Praag et al., 2002), at AgeI and DraI restriction sites to yield CAG-GFP-NES-JBD and CAG-GFP-NLS-JBD.

For Study II, replication-incompetent MLV retroviral bicistronic vectors expressing the calcium indicator GCaMP6s and the nuclear-targeted inhibitor of JNK, NLS-JBD, downstream from the fluorescent marker mCherry were engineered in the lab. The original pGP-CMV-GCaMP6slow plasmid was bought from Addgene (plasmid # 40755, deposited by Douglas Kim). The GCaMP6 coding sequence was amplified by PCR and inserted in place of mEGFP gene in the pCru5-GCCACC-mEGFP-IRES-mCherry from Clifford Wang lab (Addgene plasmid # 49226), which was previously reported to provide a higher protein expression due to a modified Kozak sequence to within the IRES (Ferreira, Overton and Wang, 2013), using EcoRI and NsiI restriction sites. mCherry was then replaced with mCherry sequence without stop codon derived and PCR-amplified from pmCherry-C1 (Clontech, Mountain View, CA). NLS-JBD sequence was amplified from pEGFP-C1 (Clontech, Mountain View, CA) vector and cloned into NotI and NheI sites of pCru5-GCaMP6s-IRES-mCherry. The entire insert containing GCaMP6s-IRES-mCherry-NLS-JBD sequence was PCR-amplified and ligated into CAG-GFP at AgeI and PmeI restriction sites to generate CAG-GCaMP6s-IRES-mCherry-NLS-JBD.

3.2 Antibodies

Name	Source/type	Supplier	Application
Phospho-JNK	Rabbit/polyclonal	Cell signaling	Western Blot
JNK	Rabbit/polyclonal	Upstate Biotechnology	Western Blot
Phospho-PSD95	Rabbit/polyclonal	Millipore	Western Blot
PSD95	Mouse/polyclonal	Millipore	Western Blot
DCX	Rabbit/polyclonal	Cell signaling	Western Blot, Immunohistochemistry
BrDU	Mouse/monoclonal	Thermoscientific	Immunohistochemistry
GFP	Rabbit/polyclonal Chicken/polyclonal	Cell signaling Abcam	Immunohistochemistry
RFP	Rabbit/polyclonal	Rockland	Immunohistochemistry
Prox1	Mouse/monoclonal	Millipore	Immunohistochemistry
Cleaved caspase -3	Rabbit/polyclonal	Trevigen	Immunohistochemistry
Ki67	Rabbit/polyclonal	Novacastra	Immunohistochemistry
NeuN	Mouse/monoclonal	Millipore	Immunohistochemistry

3.3 Special reagents

Arabinoside-cytosine (Ara-C) mitotic inhibitor and BrdU were purchased from Sigma. The cell penetrating peptide DJNKI-1, inhibitor of JNK activity was ordered from GeneCust (Laboratoire de Biotechnologie du luxembourg, SA). Hoechst 33342 dye used to visualize nuclei, and Mowiol mounting medium were from Hoechst Marion Roussel (Frankfurt, Germany). Alexa 488 and 568 dyes were acquired from Molecular probes.

3.4 Cell lines

Human embryonic kidney cells (HEK-293FT) were from Thermo Fisher Scientific. Cells were grown in high glucose Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with: 10% fetal bovine serum; 0.1 mM MEM Non-Essential Amino Acids; 6 mM L-glutamine; 1 mM Sodium Pyruvate; 1% Pen-Strep, plus 500 µg/mL Geneticin were freshly added to the complete medium every time cells were divided or plated. The cell culture was grown in an incubator with temperature set at 37°C and humidity set at 5% CO2.

3.4.1 Retroviral production

Viral particles were assembled by transiently transfecting HEK-293FT cells as previously (van Praag *et al.*, 2002). 10–12 × 10 cm plates were seeded the day before and when 50–70% confluency was reached, Lipofectamine 2000 (Invitrogen) was used

to transfect cells with retroviral constructs and packaging plasmids pCMV-VSVG and pCMV-GP. 5 h post transfection, transfecting media was replaced with 10ml of fresh, warmed Dulbecco's modified Eagle's medium. 48 h and 72 h, media containing virions were collected and shortly centrifuged in an Eppendorf 5804R Centrifuge (Hamburg, Germany) at 2000 r.p.m. for 3 min. The supernatant was filtered using a 0.2 µm Minisart filter (Sartorius, Göttingen, Germany) and centrifuged in a Beckman Coulter Optima L-90K Ultracentrifuge (Vantaa, Finland) for 2 h at 4 °C, using a SW32Ti rotor at 19 400 r.p.m. (RCF_{avg} 46 220). At the end of ultracentrifugation, 2 ml of sterile PBS was used to resuspend the viral pellet and a second centrifugation was repeated. 100 µl of sterile PBS was used to gently resuspend the viral pellet and 10 µl viral aliquots were snap-frozen in liquid nitrogen and stored at −80 °C until use. Viral titration was assessed in 24-well plates containing 50–70% confluent HEK-293FT. Retrovirus stocks were serially diluted ($1:10^2$ to $1:10^7$) and 10 µl volume per dilution was added to the wells, in duplicate. Plates were gently shaken and incubated at 37 °C and 5% CO_2. 48 h post-transduction, 1 × with sterile PBS was used to rinse wells and cells were fixed with 4% paraformaldehyde, pH 7.4, for 15–20 min. Wells were rinsed again 1 × with PBS and viral titre was calculated by counting the number of cells expressing GFP or simultaneous GCaMP6 and mCherry using an Olympus IX70 fluorescence microscope. Titre was expressed as number of transducing units (TU) per ml and batches ranging from 10^8 to 10^{10} TU ml^{-1}were used for *in vivo* experiments.

3.5 Animal experiments

All animal procedures were conducted in accordance with the guidelines from Finnish (62/2006 Act and 36/2006) and European (86/609/EEC, 2010/63/EU, 1986/ETS 123) directives, approved by the National Animal Experiment Board (ELLA). C57BL/6J wild type and *Jnk1-/-* mice were purchased from The Jackson Laboratoriy. For stereotactic surgeries experiments the wild type C57BL/6J were purchased from commercial vendor Harlan Netherlands and left to acclimatize for one week upon arrival, before undergoing surgery. Typically, all animals were group housed; those who underwent surgery were single housed right after operation. All mice had access to food and water *ad libitum*, and were maintained on 12hr of light dark cycle.

3.5.1 Stereotactic surgical procedures (Study I and II)

8–10-weeks C57Bl/6J male mice were anaesthetized with 4% isoflurane and transferred to a stereotaxic frame (Kopf instruments). Anesthesia was maintained with 2.5% isoflurane throughout the surgery. Before making the surgical incision, lidocaine (0.5%) was subcutaneously injected to anesthetize the injection site and 0.1 mg kg^{-1} of buprenorphine hydrochloride was injected intraperitoneally.

Viscotears eye protectant gel (Novartis, Basel, Switzerland) was applied to avoid eye dryness and the head was shaved with a scalpel to expose the scalp where a linear incision was made. The two skin edges were retracted, and the area was dried and disinfected. Thereafter, a drill was used to generate holes in the cranium according to the coordinates derived from mouse atlas (Paxinos *et al.*, 2004). Coordinates allowed targeting of a specific brain region of interest.

3.5.2 Intracerebroventricular injections (Study I)

For the acute intracerebroventricular administration of DJNKI-1, one cannula (Agnthos) was chronically implanted and cemented (Agnthos) at the stereotactic coordinates: anterior-posterior -0.6mm, medial lateral 1.5mm, dorsoventral -2.0mm to the lateral ventricle. Two screws placed close to the cannulation site provided an additional anchorage. Mice were recovered from surgery for 7 days. Thereafter, 2µl of JNK inhibitor DJNKI-1 (100 µM) or vehicle (Milli Q) were injected at a rate of 0.25µl/min, using CXMI-5 microinjection cannula (Agnthos) under isofluorane (2.5%) anesthesia and returned to their home cage until behavioral testing 6h after.

3.5.3 Osmotic minipumps implantation (Study I)

For chronic intracranial administration of the compounds (DJNK-1 inhibitor, Ara-C and vehicle) a small miniosmotic pump (Alzet model 2006, Cupertino, CA) was inserted in a pocket created subcutaneously on the back of our mice. A catheter connecting the mini pump and the cannula was pre-filled with the appropriate compound and was placed underneath the skin. The cannula was implanted unilaterally at the stereotactic coordinates: anterior-posterior -0.6mm, medial lateral 1.5mm, dorsoventral -2.0mm targeting the lateral ventricle and cement was finally used to glue the cannula on the skull. Mice were single housed after surgery to avoid accidental damage of the implant by cage mates. Additionally, saline and analgesics were injected 12 hours after surgery. The system continuously infused its content at a rate of 0.15µl/hr., for 6 weeks. Thereafter, mice underwent behavioral testing.

3.5.4 Retroviral injections and fiber optic cannula implantation (Study I and II)

For Study I, retroviruses stocks expressing CAG-GFP, CAG-GFP-NES-JBD and CAG-GFP-NLS-JBD were mixed with polybrene (10-µg ml^{-1}) and injected bilaterally (two µl per site at a rate of 0.1 µl min^{-1}). The following coordinates were used to target the dentate gyrus: anterior–posterior=−2.5 mm from Bregma; medial lateral=1.8 mm; dorsoventral=−2.0 mm and for ventral injections; anterior–posterior=−3.1 mm from Bregma; medial lateral=2.8 mm; dorsoventral=−3.1. The injection needle was kept in

the injection site for additional 20 min to avoid backflow of virus. Stitches were used to suture the animals' skin.

For fiber photometry experiments in Study II, viral solution containing CAG-GCaMP6s-IRES-mCherry or CAG-GCaMP6s-IRES-mCherry-NLS-JBD was injected at following coordinates to target the dentate gyrus: anteroposterior −2.5 mm, mediolateral 1.8 mm, and dorsoventral −2.0 mm. At each injection site, custom 400-μm-diameter, 0.48-NA fibers attached to a 1.25-mm-diameter stainless steel ferrule (Doric Lenses) were implanted into the skull using Superbond C&B Kit (PlanNet). Intraperitoneal injection of saline was administered, and mice were transferred onto a heating pad until fully awake. Behavioral testing started 2 weeks after surgery.

3.5.5 Behavioral testing

The following section will briefly introduce the behavioural tests related to anxiety and depression used for Study I and II descripted in this thesis. Behavior was assessed between 10.00 and 16.00 h. Experimenters were blind to the treatment. For fiber photometry experiment (Study II), immediately before mice started the trial, a fiber patch cord was clipped in the chronically implanted fiber-optic cannula and the left-over portion of the cord was suspended above the animal, in order for the animal to freely move within the testing arena. Moreover, mice were habituated to wearing the fiber patch cords while in their home cage 2-3 days before starting the behavior. During such habituation trials, the strongest calcium signal coming from one hemisphere was selected for the subsequent behavioral battery. Below is a list of all tests used. Specific details for each test can be found in the original publications.

1) **Open field (Study I and II)**
2) **Elevated plus maze (Study I and II)**
3) **Sucrose preference (Study I)**
4) **Forced swim test (Study I)**
5) **Social interaction (Study II)**
6) **Enriched Environment (Study II)**

3.6 Fiber photometry (Study II)

This approach yields insight into the global synaptic activity of a cell population of interest, reported by the intracellular calcium dynamics, with adequate temporal resolution (Gunaydin *et al.*, 2014; Calipari *et al.*, 2017), and it can be applied to study freely moving animal's behavior in response to a given behavioral cue.

Mainly, the system was purchased by Doric Lenses and it is constrained by light-emitting diodes (LED; 490 nm and 405 nm), which emit light to the tissue in order to generate fluorescence from the GCaMP6s protein, encoded by retroviral construct. 490 nm is the GCaMP stimulation wavelength, while 405 nm is a control channel whose wavelength generates calcium-independent events. Thereby, any fluctuation observed in the 405 nm can be removed by subtraction from the 490 nm channel before analysis, to identify only real events and exclude those derived from auto-fluorescence or fiber bending. Excitation light is delivered by a fiberoptic patch cord, which is coupled to a permanent fiberoptic cannula implant through a bronze sleeve. Moreover, such patch cord also collects emitted photons from the tissue of interest and directs them onto a photodetector. Photodetectors diverge in sensitivity and minimum detectable power. Typical fluorescence changes in FP system are in the nano-Watt range, hence detection of such low-level signals demands a photomultiplier tube (PMT). PMTs amplify photoelectrons through an internal configuration allowing secondary emission, ultimately making them extremely sensitive tools to detect all the light that impinges on the sensor. In addition, a dichroic mirror is placed into the light-source system and it separates the excitation and emission photons once interaction with the sample has taken place. PMT output is directed through a lock-in amplifier, which protects signal from artifacts generated by external cues, such as room lighting penetrating the brain tissue and detected by the optic fiber. Lastly, the signal is digitized using the manufacturer's software and incoming data is displayed in real-time.

Data analysis is then performed through custom-written Python software. Fluorescence intensity changes are calculated for each behavioral session by applying the following mathematical formula: (490 nm signal−fitted 405 nm signal)/ (fitted 405 nm signal). Identification of significant fluorescence peaks throughout the recorded track is carried out. Frequency (events per second) and amplitude (DF/F) of peaks are the parameters considered. Events that exceed the median average deviation (MAD) of the normalized data set by 2.91 are considered meaningful peak events and included in the analysis (Calipari *et al.*, 2016, 2017).

3.7 Stereology (Study I and II)

Stereology provides an unbiased estimate of quantitative metrics from histological analysis. Starting with 2-D planar sections of tissue, stereology delivers information about the 3-D structure. It can accurately estimate the number of cells or the area and volume of brain structures or regions. Representative sections of the region of interest are selected through systematic random sampling (a statistical sampling method). Then, a set of thoroughly verified rules are used to mark cells and structures in each sampling position for quantification. Stereological-based study designs were

performed with the aim to quantify changes in the number or morphology of hippocampal neurogenic cells and hippocampal volume, in both Study I and II.

3.8 Tissue processing and immunofluorescence (Study I and II)

Brain fixation was performed before processing for immunohistochemcial analysis started. Specific details on antibodies used, incubation time and dilutions can be found in the original studies. Briefly, anesthetized mice were intracardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Cryo-protected brains were frozen and cut in 40 μm sections. One in every fifth free-floating section was selected throughout the rostral–caudal extent of the hippocampus and processed with immunohistochemistry. Incubation with blocking solution was followed by primary and then secondary antibodies. Nuclei were visualized with Hoechst-33342. Lastly, sections were mounted onto glass slides and imaged using a confocal microscope with 20 ×, 40 × and 100 × objectives.

3.9 Optical fractionator analysis for unbiased adult neurogenesis quantification (Study I)

The major advantage of using the optical fractionator method to quantify the number of cells in a given structure is that it avoids counting labeled cells twice (Mouton *et al.*, 2017). Random sampling of sections was performed by picking every fifth section throughout the rostral-caudal extent of the hippocampus (Bregma −1 to −4) (George Paxinos, 2012), for a total of 8 to 12 sections per animal. Quantification of adult born neurons positive for BrdU, Ki67, DCX and cleaved-caspase 3, MLV-CAG- GFP and MLV-CAG-mCherry markers in Study I and II was completed. Ki67 and DCX immunopositive cells were counted using the optical fractionator workflow of the Stereoinvestigator (MBF Biosciences). Tissue thickness was repeatedly re-measured at different regions while counting to account for the shrinkage due to tissue processing. The final cell number estimation was calculated according the stereological equation: (N= (1/tsf x 1/ssf x 1/asf) x ΣQ)

with ΣQ being the total number of cells counted using dissector in optical fractionator;

tsf being the thickness sampling factor;

ssf being the section sampling factor;

asf being the area sampling factor.

The software automatically generates all the above parameters.

BrdU, cleaved-caspase 3, MLV-CAG-GFP and MLV-CAG-mCherry labeled cells were counted applying a modified unbiased stereology protocol. Because of the small number of cells positive for these markers, exhaustive manual counting was performed instead the optical fractionator workflow (Kempermann, 2003). Every fifth section of dentate gyrus was used for immunolabeling.

3.10 Dendrite analysis (Study I and II)

In study I, the optical fractionator was used to estimate the maturation index of DCX positive cells: DCX positive cells and DCX positive cells with dendrites were counted. The total numbers were quantified using the stereological parameters described above. The maturity index was calculated by dividing the value of DCX cells with dendrites over the total number of DCX positive neurons in the dentate gyrus. In Study II, MLV-CAG-GFP and MLV-CAG-mCherry double positive cells were manually scored for tertiary dendrites and the number obtained multiplied by 5 (because every fifth section was used).

3.11 Spine analysis (Study II)

From MLV-CAG-GFP and MLV-CAG-mCherry injected animals, one in five sections, (8 to 12 sections in total per animal) were stained with anti-GFP antibody. Three-dimensional confocal images were acquired with a 100 × objective and 4 dendritic segments were acquired per every double-positive neuron. Spines were manually categorized into thin, mushroom and stubby according to neck length and head bulbosity, using the Neurolucida software (Williston, VT, USA).

3.12 Volumetric estimation using Cavalieri method (Study I)

Volumetric changes in the granule cell layer of wild type and *Jnk1-/-* mice were estimated in Study I. An interval of one in five sections was used and stained with Cresyl violet. The anatomical contour of the granule cell layer of the detate gyrus was traced in each section with the Cavalieri workflow of Steroinvestigator software (MBF Bioscience). Automated output numbers were averaged to estimate the total volumetric differences between the genotypes.

3.13 Pearson's correlation (Study II)

Correlation analysis describes the relationship between two variables. The Pearson's correlation coefficient is the covariance of the two variables divided by the product of their standard deviations. The correlation coefficient ranges from −1 to 1. A value of

1 infers that a linear equation defines the two variables, and all data points are depicted by a line for which one variable increases when the other increases. On the contrary, a value of −1 suggests that all data points lie on a line for which one variable decreases while the other one increases. In Study II, relationship between fiber photometry (FP) peak data and behavior scores was investigated. Peak amplitudes (DF/F) or peak frequency (events per second) parameters were extrapolated from FP peak analysis. Behavior data comprise time scores (in seconds) for social interaction and elevated plus maze. Pearson correlation was calculated using Microsoft Excel and custom-written Python software to identify correlative relationship between synaptic activity changes of the newly born granule cells of the dentate gyrus, upon JNK inhibition and behavioral performance. P-value for significant correlation was set to <0.05.

3.14 Statistical analysis

Full details of statistical tests used are described in each study. Student's two-tailed t-test was applied when two conditions were compared. Two-way analysis of variance (2W ANOVA), instead, was used when more than two conditions were considered. In all the graphs, significances are reported, and error bars represent s.e.m. N-values, expressing the number of experimental animals used are reported in each figure legend.

4. RESULTS AND DISCUSSION

The results will be summarized in this section; more detailed description of the results is available in the original articles.

4.1 JNK controls adult hippocampal neurogenesis and structural changes underlying anxiety-like and depressive-like behavior in mice (Study I)

Evidence shows elevated hypothalamic–pituitary–adrenal axis (HPA) in the pathophysiology of many neuropsychiatric disorders, although not universal, along with structural abnormalities in the brain, alterations in dendritic architecture and spine density (Drevets, Price and Furey, 2008), and irregularities in the adult neurogenesis of the hippocampus (Petrik, Lagace and Eisch, 2012). Conversely, the JNK pathway has been found compromised in psychiatric disorders and intellectual disabilities (Eleanor T Coffey, 2014), but its role in adult neurogenesis, anxiety and depression has not been investigated. In this study, we sought to investigate whether JNK regulates adult neurogenesis and whether a causal relationship associates JNK's regulation of neurogenesis to improved anxiety-like and depression-like behavior in mice.

4.1.1 *Jnk1*-/- adult mice exhibit low anxiety-like and depressive-like behavior and increased adult hippocampal neurogenesis

A recent body of evidence suggests a potential role for the JNK subfamily in mood regulation (Galeotti and Ghelardini, 2012), and we have recently collected information on its involvement in models of neuropsychiatric conditions (Marchisella, Coffey and Hollos, 2016). To better elucidate such contribution, we started by testing adult *Jnk1*-deficient mice for anxiety-related behavior, exposing them to three separate and reliable tests for anxiety, i.e. the elevated plus maze, the light-dark test, and the open field test. In all these tests *Jnk1*-/- mice displayed low anxiety-like behavior compared to control animals.

More specifically, in the elevated plus maze, which is based on the rodents' aversion to explore exposed areas that lead to thigmotaxic behavior (preference for enclosed areas or nearby the edges of a confined space), the *Jnk1*-/- mice explored and entered the open arms significantly more (Study I, Fig. 1a and 1c). Consistently, they spent significantly less time in the closed arms when compared to wild type animals (Study I, Fig. 1b). Mice lacking *Jnk1*, also displayed increased head dipping and rearing behavior (Study I, Fig. 1d and 1e), which, altogether, indicate a high level of exploratory behavior and reduced anxiety-like behavior.

Next, mice were subjected to the light-dark test, which is based on the natural rodents' aversion to brightly, lit, open areas and their spontaneous exploratory instinct in novel environments. The *Jnk1*-/- mice spent significantly more time in the light box as compared to their wild type counterparts (Study I, Fig. 1f), consistently revealing low anxiety-like phenotype.

The following open field test is centered on the conflict between the rodents' innate propensity to explore novel environments and their intrinsic aversion to open spaces. Here, *Jnk1* knockouts displayed decreased latency to enter the center, while also spending more time in the center of the open field chamber, and lastly, they traveled significantly longer distances in the center (Study I, Fig. 1g, 1h and 1i). Notably, the total distance travelled by *Jnk1*-/- mice was found unaltered compared to wild type mice, in the EPM, light/dark or open field tests (Study I, Suppl. Fig. 1a, 1b, 1c and 1d). Taken together, these data robustly indicate that *Jnk1*-/- mice exhibit low anxiety-like behavior at baseline in comparison to wild type corresponding.

To examine depressive-like phenotype, we subjected our *Jnk1* knockouts animals to the forced swim test, which assesses rodents' susceptibility to negative mood, and it targets the rodents' response to the threat of drowning. Mice lacking *Jnk1* were significantly less immobile during the test, compared to wild type mice (Study I, Fig. 1j). Furthermore, in the following sucrose preference test, which is a reward-based test indicator of anhedonia and focused on the rodents' innate interest for sweet foods and solutions, *Jnk1*-/- animals tended to rapidly increase their preference for sucrose-based water compared to WT mice (Study I, Fig. 1k), indicating altogether that the genetic ablation of *Jnk1* decreases depressive-like behavior.

Hippocampal volume is decreased in patients suffering from anxiety and depression, and such reduction correlates with the severity and duration of these conditions (Campbell *et al.*, 2004; Kalisch *et al.*, 2006; Kheirbek *et al.*, 2012). Hence, we sought to investigate volumetric changes in the hippocampus of *Jnk1*-/- mice, specifically, in the granule cell layer of the dentate gyrus. We applied Cavalieri analysis which revealed a trend towards an increased volume of the dentate gyrus in mice lacking *Jnk1* (Study I, Fig.1l and 1m), consistent with the ameliorated behavioral phenotype observed in these animals.

In order to unravel JNK's role in adult hippocampal neurogenesis, we broke down the histological analysis exploring proliferation, differentiation, maturation and survival phases of neurogenesis, employing timely specific expressed markers. We started by using a gold standard method that estimates the number of proliferating cells: the thymidine analog BrdU was used to label dividing cells. Animals were injected and sacrificed 28 days after; brains were extracted and immunostained with BrdU antibody. Optical fractionator analysis revealed an overall heightened number of BrdU positive cells in the DG of *Jnk1*-/- mice, with respect to wild type corresponding (Study I, Fig.1n and 1o), also highlighting a significantly increased

number in the dorsal portion of the dentate gyrus. Next, we used the neuroblast doublecortin (DCX) to mark young adult-born neurons in *Jnk1* knockout mice, consistently finding boosted number of DCX+ cells (Study I, Fig.1p and 1q). Lastly, to investigate JNK's role in survival phase of adult neurogenesis, we estimated the total number of mature adult born neurons, which co-expressed BrdU, and the neuronal marker NeuN, concluding that the number of surviving cells was also increased in *Jnk1-/-* mice. In line with these data, when we used an antibody that specifically recognizes the cleaved form of caspase-3 to measure apoptosis, we observed a reduced number of cleaved caspase-3-positive cells in *Jnk1-/-* mice (Study I, Fig.1t and 1u). Altogether, these results suggest that increased adult hippocampal neurogenesis in the absence of *Jnk1*, might underlie the improved anxiety-like and depressive-like phenotype observed in the behavioral batteries. Indeed, about 700 new granule cells are added daily per hippocampus in human, representing around 0.004% of dentate gyrus GCs (Spalding *et al.*, 2013). In rodents, the rate of neurogenesis is higher (Cameron and Mckay, 2001) and it accounts for up to 0.06% of DG neurons in two-month-old mice (Kempermann, Kuhn and Gage, 1997; Bergmann, Spalding and Frisén, 2015). It has been established that neurogenesis in rodents can be modulated by environmental factors (Kuipers *et al.*, 2014); in particular, exposure to enriched environment (Kempermann, Kuhn and Gage, 1997) and exercise (van Praag, Kempermann and Gage, 1999; Snyder *et al.*, 2009) boost the number of neurogenic cells, whereas aversive stress lowers neurogenesis (Warner-Schmidt and Duman, 2006). It has been previously shown that heightened neurogenesis in the ventral dentate gyrus reduces anxiety-like behavior (David *et al.*, 2009; Revest *et al.*, 2009). Molecular players regulating such process are still elusive. JNK in the developing brain is primarily required for region-specific apoptosis (Kuan *et al.*, 1999). Here, we found augmented survival of neurogenic cells in *Jnk1-/-* mice, along with increased progenitor cell proliferation, suggesting a regulatory mechanism of neurogenesis mediated by JNK. More specifically, it has been proposed that stages of development are dependent on previous ones (additive) (Kempermann, 2011). Thereby, our evidence highlights that the neuroprotective effect of JNK inhibition is to provide a powerful neurogenic stimulus that improves anxiety-like behavior. Anxiety and MDD have been linked to decreased volume of the hippocampus (Deisseroth, 2014) and DG (Besnard and Sahay, 2016; Boldrini *et al.*, 2019) and we presently show a trend towards increased volume in mice lacking *Jnk1*. We observed that the 6% extent of change, although still a trend, resembled the relative decrease reported for patients affected by major depressive disorder (Frodl *et al.*, 2014), encouraging us to further examine promising antidepressant properties of JNK inhibition.

4.1.2 Acutely inhibited JNK does not exert anxiolytic and antidepressant effect in adult mice

To further investigate JNK's role in anxiety-like and depressive-like phenotype, we employed a pharmacological inhibitor of JNK, DJNKI-1 (Study I, Fig.2a top), which has been successfully tested for several conditions in preclinical animal models (cerebral ischemia, neuropathic pain, myocardial ischemia-reperfusion injury, Alzheimer's disease). In addition, DJNKI-1 is currently in clinical trial for preventing hearing loss and as a therapy for patients with intraocular inflammation (for a review see (Cicenas, 2015)). Since it is still unclear whether the inhibitor crosses the blood brain barrier, we performed intracerebral–ventricular infusion of 100μM of DJNKI-1, to administer the peptide directly to the ventricles. We tested the animals for anxiety-like behavior 6 hours after infusion. More details on the experimental design are depicted in Study I, Fig.2a, bottom.

Acute treatment with DJNKI-1 did not exert any effect on anxiety-like behavior in mice when subjected to EPM and light-dark paradigms. More specifically, animals that received DJNKI-1 inhibitor spent the same amount of time of vehicle treated counterparts, in both open and closed arms of the EPM (Study I, Fig.2d and 2e). In addition, other anxiety-related scored parameters did not reveal any effect of DJNKI-1 treatment (Study I, Fig.2f), as well as, no difference in the number of entries of both arms was found (Study I, Fig.2h). Equally, the light-dark test showed overlapping amount of time spent in both compartments by DJNKI-1 group and control mice (Study I, Fig.2i). Yet, 100 μM DJNKI-1 efficiently downregulated JNK activity (Study I, Fig.2b and 2c), at least in the hippocampus, as confirmed by immunoblotting for phospho-S295 of PSD-95 (JNK substrate). Notably, we found that inhibition was stronger in the DG than in the PFC, which might be due to lower levels of expression of JNK in the PFC compared to the hippocampus (Lee, Ekstrom and Ghetti, 2014). In animal models, the hippocampus is considered a central regulator of depressive- and anxiety-like behavior (Bannerman *et al.*, 2002, 2004; Kjelstrup *et al.*, 2002; Strange *et al.*, 2014; Anacker and Hen, 2017). Equally, an extensive body of clinical studies have shown that structural changes at many levels in the hippocampus can be observed in subjects affected by depression and anxiety (Drevets, 2000; Sheline, Gado and Kraemer, 2003b; Sawyer *et al.*, 2012; McEwen, Nasca and Gray, 2016). Neurons in the hippocampus are known to project to mPFCv (Eichenbaum, 2017), and a recent study has reported sparse projection from the anterior cingulate cortex to the hippocampus in mice (Rajasethupathy *et al.*, 2015). It has also been shown that synchronized hippocampal-PFC activity is required for normal adaptive, anxiety-like behavior (Padilla-Coreano *et al.*, 2016). Thereby, activity generated in the hippocampus might influence behavioral decisions elaborated in other cortical areas (Ito *et al.*, 2015) and maladaptive changes in circuits arising from the hippocampus are likely to contribute to anxiety-like traits. In our study, acute inhibition of JNK in hippocampal tissue was

not sufficient to induce an effect on anxiety-like behavior; therefore, we hypothesized that a more protracted process could underlie the lower anxiety-like and depressive-like traits observed in *Jnk1-/-* mice. We propose hippocampal neurogenesis as central because at least 4 weeks are required for the newly born neurons to complete their development and as it has been linked to therapeutic effects of antidepressants. More specifically, neurogenesis analysis in post-mortem brain has shown that depressed subjects treated with SSRI or TCA medications display increased neural progenitor cells in the ventral dentate gyrus with respect to untreated subjects (Boldrini *et al.*, 2009). While this has provided some evidence in humans, a definitive proof that neurogenesis is impaired in human subjects suffering from affective disorders is still elusive, as there are currently no methods to directly assess neurogenesis *in situ* in humans.

4.1.3 Chronic DJNKI-1 treatment elicits anxiolytic and antidepressant effect by boosting neurogenesis

To determine whether JNK's inhibition regulates anxiety-like behavior by affecting adult neurogenesis, we employed the mini-osmotic pumps filled with 100μM DJNKI-1inhibitor and infusion was directed to the ventricles for a 6 weeks-long treatment. Afterwards, animals underwent behavioral testing in EPM, which is a widely used paradigm to screen anxiolytic drugs (Study I, Fig.3a). We assessed the successful inhibition of JNK activity by DJNKI-1 quantifying the phosphorylation levels of JNK downstream substrate PSD95 with Western blot analysis. Phospho-JNK was reduced in the hippocampus at the end of 6 weeks, however, consistently with previous data no significant effect was detected in the prefrontal cortex (Study I, Fig.3b and 3c). Remarkably, in the elevated plus maze, animals that received the DJNKI-1 inhibitor, entered the open arms more and spent significantly more time than vehicle-treated counterparts (Study I, Fig.3d, 3e and 3f). In addition, the DJNKI-1group displayed lower anxiety-like behavior in all the other scored parameters of EPM (Study I, Fig.3g), overall showing consistent behavior with *Jnk1* knockout mice. We also evaluated adult hippocampal neurogenesis in brain tissue coming from this cohort of animals and we found an overall increase in the DCX-positive neuroblast population in DJNKI-1 treated mice (in line with previous data in *Jnk1* knockout animals) (Study I, Fig.3h, 3i and 3j). Moreover, we detected increased survival and/or maturation of neurons, estimated by counting the BrdU/NeuN-positive cells (Study I, Fig.3k, 3l and 3m), in the same group. Besides, chronic DJNKI-1 infusion decreased apoptosis in the DG, as the total number of cleaved caspase-3-positive cells was found reduced (Study I, Fig.3n, 3o and 3p). Remarkably, while the increase in the number of neurogenic cells was more obvious in the ventral DG, the neuroprotective effect of inhibitor was more similar throughout the whole structure of the dentate gyrus. Next, to corroborate that the anxiolytic effects upon pharmacological blockade of JNK are dependent on adult

hippocampal neurogenesis, we selected the mitotic inhibitor cytosine arabinoside (Ara-C) and infused it for 6 weeks via mini-osmotic pump system to entirely ablate neurogenesis in the brain of our mice. A 2% dose was carefully chosen with the aim of inhibiting cell divisions, leaving unaffected the survival of non-dividing cells. Mice underwent 6-week treatment and then were tested for behavior (Study I, Fig.4a). Consistently with previous cohort, animals who received DJNKI-1alone increased open arm entries, time spent in open arms and head dips (Study I, Fig.4b, 4c and 4d), and displayed lowered events of grooming and stretched attend postures (Study I, Fig.4e and 4f) with respect to control groups, which signified lowered anxiety-like behavior. On the other hand, animals who received the combination of DJNKI-1 plus Ara-C, failed to show any of the above effects (Study I, Fig.4b, 4c, 4d, 4e and 4f). Likewise, in the light/dark test, the DJNKI-1 group exhibited a strong trend towards heightened time spent in the light box, as well as, significantly increased latency to enter the dark compartment, when compared to control groups, though such scores were abolished for the animals receiving the combination treatment of DJNKI-1 and Ara-C (Study I, Fig.4g and 4h). As expected, Ara-C alone did not induce any anxiolytic effect in animals receiving the mitotic inhibitor. Histological examination of brain of these animals shows that the neuroblast population (DCX-positive cells) increases upon DJNKI-1 chronic infusion, and no effect is detected in the brain tissue of animals who received the combination treatment of DJNKI-1 and Ara-C (Study I, Fig.4i, 4j and 4k). Furthermore, cells that showed to be positive for the proliferation marker Ki67 were increased along the dorsal–ventral axis of the hippocampus (Study I, Suppl. Fig.2a, 2b and 2c), while AraC suppressed neuronal proliferation throughout the DG, as expected. Taken together, these data strongly imply that adult hippocampal neurogenesis underlies the anxiolytic effect of DJNKI-1 and that JNK is an important controller of anxiety-like behavior in mice. In line with this proposal, it has been shown that neuronal JNK activation is triggered by stressors that are typically associated with anxiety and depressive disorders, such as corticosterone (Qi *et al.*, 2005) and glutamate, acting via NMDA (N-methyl-D-aspartate) or AMPA receptors (Mukherjee *et al.*, 1999; Thomas *et al.*, 2008b). Moreover, glutamate and glucocorticoids are known to impair neurogenesis in both rodents and humans (Popoli *et al.*, 2012) and in animal models of depression JNK regulate glucocorticoid receptor signaling (Rogatsky, Logan and Garabedian, 1998; Adzic *et al.*, 2009). Thereby, JNK may be primarily involved in modulating the signaling events underlying affective disorders. From the molecular perspective, the mechanisms whereby JNK could affect newly born neurons' behavior leading to circuitry alterations involved in emotional response will be better investigated in Study II.

4.1.4 Morphological changes associated to JNK inhibition

The dendritic morphology of the neuron influences connectivity, excitability and synaptic integration, therefore, constituting a critical step in the progression of neurogenesis. It requires fine regulation, and aberrations affecting the dendritic tree complexity and its functionality are considered pathophysiological causes of neuropsychiatric disorders (Kulkarni and Firestein, 2012). It is known that JNKs modulate dendritic architecture (Eleanor T. Coffey, 2014), and evidence from our lab showed how inhibition of JNK1 alters the dendritic architecture *in vitro* and *in vivo* (Bjorkblom, 2005; Komulainen *et al.*, 2014). In addition, Soetanto et al., linked dendrite modifications in the CA3 region of the hippocampus to anxiety and depression (Soetanto *et al.*, 2010), most likely as an effect of stress (McEwen, 2005b). Therefore, we sought to study whether JNK inhibition increases dendritic complexity of adult-born neurons of the hippocampus.

First, from the pool of DCX-positive cells we extrapolated DCX-positive cells with dendrites to evaluate the maturation index, as previously described (David *et al.*, 2009). The maturation of newborn neurons was calculated as a ratio of tertiary DCX-positive cells over the total number of DCX-positive cells.

6-week chronic treatment with DJNKI-1 not only affected the overall number of DCX-positive cells, as previously mentioned (Study I, Fig.4i, 4j and 4k), but it simultaneously augmented the proportion of DCX-positive cells with tertiary dendrites in the ventral dentate gyrus (Study I, Fig.5a and 5c), as well as, increased maturation index in the same region (Study I, Fig.5b). The genetic ablation of *Jnk1* also increased the number of DCX-positive cells with tertiary dendrites and the maturation index uniformly throughout the hippocampus (Study I, Fig.5d, 5e and 5f). Therefore, these data demonstrate that JNK inhibition encourages dendritic growth and maturation of the adult born neurons, which may underlie the anxiolytic effects.

Next, since mature newly born granule cells project to the CA3 region, primarily exciting pyramidal neuron apical dendrites (secondarily, they excite basal dendrites), we further investigated JNK1's role in the integration of the adult generated neurons in the existing circuitry of the hippocampus. CA3 pyramidal neurons in *Jnk1-/-* brain slices were labeled with lucifer yellow dye to trace dendritic projections and Sholl analysis was performed on 3D sections acquired at confocal microscope. Significant differences were more prominent for the apical dendrites with respect to basal dendrites. In particular, higher number of intersections and length of dendrites was detected in *Jnk1* knockout animals as compared to wild type counterparts (Study I, Fig.5g, 5h, 5i and 5j). In addition, the total dendritic field length and the number of nodes per dendrite were augmented in the apical dendrites of CA3 pyramidal neurons of mice lacking *Jnk1* (Study I, Fig.5k and 5l). These data suggest that JNK1 inhibition promotes functional integration of the newborn neurons. Dentate gyrus granule cells are a key component of the trisynaptic circuit of the hippocampus. Their main

excitatory input arises from the entorhinal cortex via the perforant path axons and transmit this to CA3 pyramidal neurons via the dentate axons, the mossy fibers. From there the circuit is completed when projections from CA3, the Schaffer collaterals, convey the processed input to the CA1, which projects back to the entorhinal cortex. Indeed, the immature granule cells of the hippocampus are particularly sensitive to perforant path input (Marín-Burgin *et al.*, 2012) and especially within the second postnatal week more than 50% of the adult-born cells fail to integrate and subsequently undergo apoptosis (Dayer *et al.*, 2003). Immature newborn cells display higher excitability than resident granule cells and because of this feature they are thought to be major contributors to cognitive processes such as memory (Jaeger *et al.*, 2018), learning and pattern separation (Toda *et al.*, 2018). Furthermore, immature granule cells exert inhibitory effect on resident dentate granule cells by recruiting local GABergic inhibitory neurons, and a higher number of neurogenic cells in mice has been shown to lower the overall activity of the dentate gyrus (Marín-Burgin *et al.*, 2012; Drew *et al.*, 2016). A synaptic input represents an impulse that preserves and elaborates dendritic arbors. Thereby, the increase in apical dendritic complexity that we observe in the CA3 pyramidal neurons might be a consequence of the overall increase in neurogenesis upon JNK inhibition due to the presence of more axonal input from the adult born granule cells in the dentate gyrus.

4.1.5 Effects of JNK inhibition on the functional division of the hippocampus

A functional division within the structure of the hippocampus is believed to exist, supported by a growing convergence of reports (Tanti and Belzung, 2013a). In particular, along the septo-temporal axis of the hippocampus, the dorsal region is described to be implicated in memory and cognitive processes, while the ventral portion is more involved in emotion processes and regulation. Similarly, hippocampal neurogenesis also seems to mirror such functional dissociation, with the adult newborn cells of the ventral hippocampus being more preferentially susceptible to the effects of models of depression (Tanti and Belzung, 2013b). In this study, we sought to examine hippocampal neurogenesis along the dorsoventral axis of the hippocampus, upon JNK genetic and pharmacological inhibition. We found that the stages of generation and maturation of newly born neurons are generally more prominently affected in the ventral pole of the dentate gyrus upon pharmacological treatment with DJNKI-1. More precisely, in animals lacking *Jnk1* hippocampal neurogenesis resulted uniformly increased throughout the hippocampal structure, whereas, following 6-week DJNKI-1 inhibitor treatment, a remarkable increase in mature newly born granule cells co-expressing BrdU and NeuN markers was detected only in the ventral dentate gyrus (Study I, Fig.3l). Correspondingly, the pool of immature newly born neurons expressing doublecortin marker was found heightened only in the ventral portion of the dentate gyrus following DJNKI-1chronic infusion,

while the dorsal pole remained unaffected (Study I, Fig.3j and 4j). Therefore, these results point out to a ventrally biased regulation of neurogenesis following inhibitor treatment. Excitingly, in line with these data, pharmacological inhibition of JNK also prompts dendritic maturation more selectively in the ventral dentate gyrus, which altogether poses a more promising therapeutic avenue for affective disorders, in view of the fact that the ventral sub-region is linked more than the dorsal to emotional behavior (Tanti and Belzung, 2013b; Wu and Hen, 2014). As previously mentioned, from the ventral portion of the DG direct monosynaptic connections arise toward areas that regulate mood such as medial prefrontal cortex which promotes anxiety-like behavior (Padilla-Coreano *et al.*, 2016). Besides, reciprocal connections to and from amygdala have been implicated in fear processing (Richardson, Strange and Dolan, 2004), as well as connections to the nucleus accumbens have been implicated in susceptibility to depression (Bagot *et al.*, 2015). Thereby, the higher number of adult born granule cells, or their heightened synaptic activity, is likely to reduce the neural activity of glutamatergic projections from the ventral hippocampus to downstream regions that govern emotional responses (Anacker and Hen, 2017).

4.1.6 Specific inhibition of the nuclear pool of JNK in adult-born granule cells of the hippocampus improves anxiety-like and depressive-like phenotype

Next, we restricted JNK inhibition to a specific population of newly born neurons: the adult born granule cells with the aim to gain a better understanding of their exact contribution to emotional behavior. Furthermore, we wanted to test whether the sole JNK inhibition in this pool of cells is sufficient to elicit an improved behavioral outcome. To do so, we engineered replication-deficient Moloney murine leukemia virus retrovirus (MLV) (van Praag *et al.*, 2002), which carried GFP reporter gene along with the inhibitor sequence of either the nuclear (NLS-JBD) or cytosolic (NES-JBD) pool of JNK (tools already described from our lab (Björkblom *et al.*, 2005)). MLV retroviruses restrict transduction to proliferating cells; additionally, we achieved specific targeting of the adult born granule cells pool by including the CAG promoter upstream our transgene sequence, which induces exogenous DNA expression only in the cells that were dividing at the time of infection and later commit to neuronal lineage (Zhao, 2006).

First, we produced and used viruses to transduce 293FT cells in order to assess specific sub-localization of our nuclear and cytosolic JNK inhibitors (Study I, Fig.4a). Then, high titre viruses were stereotactically injected in the hilus of the DG of young adult mice, which were left expressing the virus for either 4 or 8 weeks before being tested for anxiety and depression related behaviors. Then animals were sacrificed for post-mortem analysis (Study I, Fig.4b and 4g). Fig. 4c shows *in vivo* compartment-specific labelling of adult born granule cells in the dorsal and ventral hippocampus, with insets depicting an enlarged view of cell nuclei to highlight different fluorescent

distribution (Study I, Fig.4c). Besides, staining of virally labeled newly born neurons with Prox1, a marker of granule cells, revealed that all GFP-expressing cells had, as expected, typical granule cell layer morphology, and, additionally, they co-localized with Prox1(Study I, Suppl. Fig.1e). 4-week post injection, mice who received the nuclear inhibitor of JNK exhibited low anxiety-like behavior in the EPM, exploring the open arms significantly more than their control counterparts (Study I, Fig.4d), and increasing head dipping and rearing behavior (Study I, Fig.4e). However, no anxiolytic effect of NES-JBD was detected in these animals. In addition, this cohort of mice underwent FST, but only a slight trend towards an effect of treatment was observed on immobility scores at 4-week time point (Study I, Fig.4f), suggesting that antidepressant effect of JNK inhibition might require longer time to occur. Hence, we tested mice at 8-week time point and the NLS-JBD group of animals displayed significantly reduced immobility in the FST (Study I, Fig.4h) and, consistently, low anhedonic state in the sucrose preference test (Study I, Fig.4i). In parallel, animals receiving the cytosolic inhibitor of JNK did not differ from control group mice (though a small trend could be detected).

Lastly, we employed stereological analysis to estimate the total number of virally infected cells in the dentate gyri of these animals that were effectively able to induce the observed behavioral changes. Moreover, we sought to address whether this viral approach of JNK inhibition altered the number of adult born neurons of the hippocampus. We did not detect any effect of treatment on the total number of targeted cells, however and remarkably, we observed that about 120 infected cells per dentate gyrus were responsible for the above behavioral outcome (Study I, Fig.4j). Altogether, the data presented in this paragraph imply that the nuclear pool of JNK might have a primary role in supporting anxiety-like and depressive-like behavior from the neurogenic niche of the hippocampus. We had previously used the mitotic inhibitor AraC to ablate neurogenesis in the hippocampus and demonstrated that the beneficial effects of JNK inhibition were dependent on neurogenesis. However, AraC, as well as irradiance or other methods that ablate neurogenesis present the inability to control their spatial distribution in the brain. Therefore, one cannot rule out off-target effects on other regions or cells. Here, we delivered our inhibitory sequence strictly to newborn granule cells, and, surprisingly, inhibition solely in these cells was sufficient to lower anxiety- and depressive-like behavior, with no increase in the total number of granule cells. Hence, in line with previous reports (João M Bessa *et al.*, 2009; Marlatt, Lucassen and van Praag, 2010) we observed that this behavioural switch was independent on increased neurogenesis. In addition, we observed that the primarily site for the antidepressant and anxiolytic action of JNK inhibitors in the DG is the nucleus, which is consistent with our previously reported cytoprotective effect of inhibition of nuclear JNK (Björkblom *et al.*, 2008). On the other hand, we hypothesize that the cytosolic pool may only a play secondary role. Similarly, we also expect that

the effect on anxiety-like behavior observed upon infusion with DJNKI-1 depends on nuclear JNK inhibition, as others have shown that DJNKI-1 accumulates passively in neuronal nuclei (Repici *et al.*, 2007). The nuclear pool of JNK has been shown to target many transcription factors (Yang, Sharrocks and Whitmarsh, 2013), including the glucocorticoid receptor (Yang, Sharrocks and Whitmarsh, 2013) with an overall effect of modulation of several cellular outcomes. Furthermore, since the adult granule cells of the DG have been shown to have a robust transcriptional response to external stimuli (Jaeger *et al.*, 2018), the action of JNK in this context may be particularly significant. All in all, this finding carries the novelty of being the first to induce a specific genetic manipulation of the adult-born granule cell population aiding accurate characterization of its function in anxiolytic and antidepressant outcomes.

4.2 Hippocampal circuit regulation of mood controlled by adult born granule cells (Study II)

Previous data from Study I showed that the specific nuclear targeted inhibition of JNK activity in the adult generated granule cells of the dentate gyrus lowers anxiety-like and depressive-like behavior in mice. This prompted us to clarify more deeply JNK's role in modifying the DG activity. After assessing morphological changes of the adult born granule cell (abGC) population upon JNK inhibition, we employed a cutting-edge technique, known as fiber photometry (FP) to directly monitor *in vivo* calcium changes coming from the abGCs, while the animals were freely performing behavioral tests related to anxiety. The importance of this study relies on the fact that, for the first time, abGCs global calcium activity has been monitored *in vivo* in the presence of JNK inhibition and in the context of anxiety-related behavior. The following preliminary data bring us closer to a comprehensive understanding of adult hippocampal neurogenesis synaptic contribution to emotion regulation and JNK's potential therapeutic role.

4.2.1 Analysis of morphological changes of virally labeled adult generated granule cells, in the presence of JNK inhibition

In Fig.4 of Study I, we observed that genetic and pharmacological inhibition of JNK increase complexity of abGCs, possibly promoting their integration in the trisynaptic circuitry of the DG. In order to address the question as to whether these changes also occurred upon viral inhibition of JNK in the adult born granule cells (relatively more mature neurogenic cells), a separate batch of mice underwent surgery to stereotactically deliver a mix of MLV-CAG-mCherry and MLV-CAG-GFP-NLSJBD viruses in the hilus of the DG. 8 weeks post injection, animals were tested for anxiety-like behavior, sacrificed and histological analysis were performed to assess successful

co-expression of the viruses. We chose to inject two types of viruses because the NLSJBD sequence localizes GFP expression only in the nucleus, thereby preventing us from visualizing fluorescence in the dendrite compartment, for morphological analysis. MLV-CAG-mCherry virus, instead, allows us to overcome such issue and by visualizing co-expressing neurons, we are able to analyze changes at the level of dendrite complexity and dendritic spine. We wanted to test whether increased dendritic complexity and/or dendritic spine density and sub-type would underlie any anxious-related behavioral improvement. Indeed, we subjected our mice to EPM (Study II, Suppl. Fig.1A) test, in which we found that mice carrying JNK inhibition spent more time exploring the open arms of the maze, and they additionally displayed increased number of entries to the open arms (Study II, Suppl. Fig.1B), when compared to animals infected with a control mix of viruses (MLV-CAG-GFP + MLV-CAG-mCherry). However, when we scored the dendritic complexity of the co-expressing adult born granule cells (Study II, Fig.1A), we could not detect any difference between the two groups of mice (Study II, Fig.1B). Of note, dendritic complexity was estimated as number of labelled neurons displaying tertiary dendrites (David *et al.*, 2009) over the total number of abGCs analyzed, yielding to maturation index. Next, we manually sub-categorized dendritic spines, based on morphology, into stubby, mushroom, and thin, and we looked for changes in the percentage of each spine sub-type, along with total spine density. We did not find any significant difference between the two groups for neither of the two parameters considered in this analysis (Study II, Fig.1C, 1D and 1E), suggesting that inhibition of JNK activity does not affect the newly born granule cells potential of innervation from the perforant path, at least 8-weeks post-injection. On one hand, this is consistent with previous data published from our lab (Bjorkblom, 2005), which show that *in vitro* inhibition of nuclear JNK does not produce significant changes on dendrites in cerebellar granule neurons. However, this is in contrast with data shown above where genetic and pharmacological ablation of JNK increase dendritic field size in immature granule cells. One possible explanation of this could be that JNK may transiently regulate dendritic arborization, as morphology on virally labeled granule cells was analyzed at their latest development phase in cells expressing Prox1 marker (Study I, Suppl. Fig.1e) (Iwano *et al.*, 2012). On the other hand, for *Jnk1* knockout mice or DJNKI-1 treated animals the analysis was performed on DCX expressing cells, signifying earlier maturation stage (Brown *et al.*, 2003; Snyder *et al.*, 2009). From two to six weeks after birth, new granule cells display a lower threshold for activation by perforant-path excitatory input and this synaptic innervation supports maintenance and maturation of dendritic arborization. On a molecular level, immature granule cells excitability is sustained by high expression of the NKCC1 cotransporter, which intriguingly, has been shown to be phosphorylated by JNK *in vitro* (Klein, Lamitina and O'Neill, 1999). On the other hand, at eight weeks after birth, abGCs reach similar properties to early-

born granule cells of the DG (Ming and Song, 2011) and the "critical period" (Bergami *et al.*, 2015) characterized by enhanced excitation is no longer detectable and it may be related to the discrepancy reported here. Moreover, we observed a significant positive correlation between anxiolytic behavior in the EPM and dendrite complexity or spine density, upon nuclear JNK inhibition. This implies that increased ability of the abGCs to receive cortical inputs is predictive of decreased anxious behavior in the elevated plus maze, which prompted us to ask next whether JNK modulation of abGC firing rate could be relevant to explain anxiety-like behavior. Acquired data is discussed in the next paragraphs.

4.2.2 Experimental tools and design for in vivo recording of adult-born granule cells calcium activity

The previous paragraph illustrated the set of analyses that led us to hypothesize that the underlying mechanism of low anxiety-like behavior seen in the EPM in the JNK inhibited group of mice might lie at the synaptic transmission level. Next, in order to directly monitor in vivo intracellular calcium activity of the abGCs in response to behavioral challenges, we re-engineered replication-deficient Moloney murine leukemia virus retrovirus (MLV) (van Praag et al., 2002), carrying the genetically encoded calcium indicator GCaMP6s (Akerboom et al., 2013; Chen, Trevor J. Wardill, et al., 2013). Moreover, downstream to an IRES sequence in the same plasmid, we cloned mCherry fluorescent protein along with the nuclear-targeted JNK inhibitor sequence, so the expression of mCherry would be restricted to the nuclear compartment. Schematic representations of the constructs are depicted in Study II, Fig.2A. 293FT cells were used to validate the viruses and to assess compartment-specific localization of tagged inhibitor (Study II, Fig. 2B). Lastly, we designed our experiment so that high titre viruses were targeted to the hilus of young-adult mice, which were tested for open field (OF), elevated plus maze (EPM), social isolation (SI) and enriched environment (EE), starting at 2 weeks following injection, and then additionally at 4-, 6-, and 8-week time points (Study II, Fig. 2C). During each behavioral session, in vivo recording of the abGCs total activity was enabled by a chronically implanted fiber-optic cannula, which was clipped into longer fiber patch cords immediately before starting the trials. To confirm successful labeling of our population of interest within the adult hippocampus, we performed immunofluorescence staining following animals' sacrifice (Study II, Fig. 2D).

4.2.3 Fiber photometry recording from adult-born granule cells, upon JNK blockade

It has long been sought to correlate real-time neuronal responses to animal behavior in order to gain knowledge on synaptic circuitries underlying given effects. However,

a lack of reliable methodologies to design and execute such experiments has prevailed so far, until the advent of more advanced technologies such as optogenetics or fiber photometry (FP). The latter (Gunaydin *et al.*, 2014; Calipari *et al.*, 2017), enables sensitive recording of cell-type specific population neuronal activities in deep brain regions, while animals are freely behaving. To date, abGCs calcium activities have not been recorded in response to different behavioral settings, related to anxiety-like behavior. Hence, we sought to quantify neuronal activity changes coming from our targeted population and plotted as transient changes in fluorescence normalized to baseline, time-locked to a given behavior of interest. Moreover, we performed analysis fluorescence peaks throughout the recorded traces to determine frequency (events per seconds) and amplitude (DF/F) of peaks and assess differences in the presence of JNK inhibition. First, we exposed our virally infected mice to EE and left them free to explore the arena for 5 min, while being recorded for FP. We compared peak amplitudes of abGCs calcium events in the EE to those analyzed for OF (as a control arena), over the course of 8 weeks, for both, GCaMP6-NLSJBD mice and their control counterparts. We find that brief exposure to EE significantly increased averaged amplitudes of peaks 6 weeks post injection equally for both NLSJBD and control groups, highlighting the 6th week as a functionally relevant period for recruitment of abGCs of the DG in response to EE (Study II, Fig. 3A, 3B and 3C). Recordings from the open field maze, an anxiety-related behavioral test assessing exploratory behavior in mice, highlight once more the 6th week of age of the abGCs as the most significant in terms of heightened frequency of peaks for the GCaMP6-NLSJBD group of mice (Study II, Fig. 4A and 4B). In the social interaction test, which measures social behavior that is another feature of anxiety-like behavior, the higher peak amplitude for the GCaMP6-NLSJBD group of mice is already visible at 4 weeks, followed by a consistent tendency at 6 weeks (Study II, Fig. 4C). When we performed correlation analysis to look for matches between social behavior and neural recordings, we found positive correlations between that activity of 6- and 8-week old abGCs and SI scores in GCaMP6-NLSJBD mice (Study II, Fig. 4D and 4E). Intriguingly, however, the earlier time-point of 2 weeks post injection showed overall decreased anxiety-like behavior in the SI (Study II, Fig. 4F) and abGCs recorded activity appeared to negatively correlate with individual SI scores (Study II, Fig. 4G). Taken together these data might suggest a switch in JNK modulation of abGCs activity according to their developmental phase. Of note, we consistently observed a slight anxiolytic trend of JNK inhibition 2 weeks post injection in the EPM, with two separate cohorts of mice (Study II, Suppl. Fig. 2A and 2D). For one of these two cohorts, we employed Thy1-GCaMP6 transgenic mice infused with MLV-CAG-GFP or MLV-CAG-NLSJBD viruses. However, for this cohort of mice we did not detect any significant effect on neural activity with FP recording (Study II, Suppl. Fig. 2B and 2C).

With these data we sought to gain a deeper understanding of JNK activity in the regulation of anxiety-like behavior from the synaptic activity of the dentate gyrus. Although these data are preliminary and repeated testing is needed to confirm results, it appears the JNK plays a major role in modulating abGCs activity during their "critical period" particularly when mice are tested for social and exploratory behaviors (two different domains that may reveal anxiety-like traits in rodents). We detected significantly increased abGCs activity in NLS-JBD mice tested for social interaction 4 weeks post-injection. Similarly, abGCs activity in NLS-JBD mice appeared heightened during the 6th week measurement in both SI and OF. Correlation analysis of 6- and 8-week old abGCs activity and interaction scores in SI revealed that higher neural activity is coupled to lower anxiety-like behavior. Therefore, JNK might be involved in modulating either the number, the synchrony, and/or the firing rate of active abGCs from around one month of their age until their 8th week of age, when instead their physiological properties are thought to overlap resident granule cells of DG. We know that the JNK pathway is involved in the of removal AMPA receptors resulting in reduced synaptic transmission in hippocampal cells (Zhu *et al.*, 2005; Myers *et al.*, 2012). In addition JNK activity has been found linked to NKCC1, a chloride co-transporter whose expression is associated to the immature granule cells hyperexcitability (Klein, Lamitina and O'Neill, 1999; Toni and Schinder, 2016). Therefore, inhibition of JNK during the critical stage of maturation in the abGCs might increase glutamatergic excitation of abGCs during anxiogenic contexts. Indeed, is also known that JNK activity plays a role in anxiety-related behaviors (Zhao *et al.*, 2017; Stefanoska *et al.*, 2018). In contrast, we found that less abGCs activity at 2-week post injection in the absence of JNK seems to be coupled to lower anxiety-like behavior. These divergent data might imply that JNK plays different roles according to the abGCs developmental age.

It has been established that exposure to novel experiences, such as enriched environments, affects DG neuronal activity and promotes abGCs survival (Kempermann, Kuhn and Gage, 1997; Cameron and Glover, 2015; Gonçalves, Schafer and Gage, 2016). We observed that our cell population of interest had maximal activity 6 weeks post-injection, which could be interpreted as a result of the previous 2 brief exposures (2nd and 4th week) in promoting the number of surviving cells, whose activity can be clearly detected at 6 weeks of age in response to EE. Interestingly, it has been recently shown that resident granule cells of the DG elevate their synaptic activity (Kirschen *et al.*, 2017) and unveil a unique transcriptional signature (Jaeger *et al.*, 2018) in response to enriched environment exposure. More generally, EE exploration induces an experience-dependent functional remodeling of the trisynaptic circuit of the hippocampus that impacts directly on the development and connectivity of the adult-generated granule neurons (Bergami *et al.*, 2015; Alvarez *et al.*, 2016). Thus, although speculative, the repeated exposure to EE might have contributed to the

significant activation of the labeled abGCs in our animals when placed in the EE and probably, the 6th week is the most relevant time point for the abGCs responsiveness to EE, since we do not detect the same activity at the following 8th week timepoint.

Altogether, this work confirms that the JNK pathway is a promising novel therapeutic avenue worthy of further investigation since it appears to represent a novel signaling to those previously targeted for the treatment of anxiety and depression. Moreover, gross qualitative observation of the animals did not reveal any obvious sign of sickness such as piloerection or hunched posture upon chronic treatment with JNK inhibitor, indicating that no apparent side effects seem to be associated to JNK inhibition at least in mice. This reinforces our opinion that targeting JNK signaling will have clinical benefits for the treatment of anxiety and depressive disorders in humans.

5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

This thesis was dedicated to investigating JNK's role in orchestrating structural, functional and synaptic changes in the neurogenic niche of the hippocampus, leading to anxiety-like and depressive-like behavior.

We provided evidence that JNK is a negative regulator of neurogenesis and its genetic and pharmacological inhibition elicits an increase in the number of neurogenic cells of the hippocampus, as well as, heightened complexity of their dendritic trees. Furthermore, all our approaches aimed to block JNK activity in the brain, with particular focus on the hippocampus, producing both anxiolytic and antidepressant effect in mice, thus establishing a new therapeutic target for mood disorders.

Adult hippocampal neurogenesis has been studied for more than half a century now, since the first time it was proven to be present in the adult brain (Altman and Das, 1965), and alterations in neurogenesis have been linked to clinical conditions, such as anxiety and depression (Jason S Snyder *et al.*, 2011; Toda *et al.*, 2018). Boosting the number of neurogenic cells is required for the action of antidepressants (Malberg *et al.*, 2000; Santarelli *et al.*, 2003b). Though, currently described antidepressants require at least 4 weeks to exert their therapeutic effect (Farach *et al.*, 2012). In Study I we show that inhibition of JNK1 activity in the hippocampus boosts the number of neurogenic cells throughout the whole axis of the DG (i.e., dorsal and ventral) and induces improvement of a number of behaviors in anxiety and depression related contexts. Of most interest, we demonstrated that the sole inhibition of JNK in a couple hundreds of neurogenic cells of the dentate gyrus is sufficient to elicit mood improvement of mice, thereby enabling us to believe that JNK is a valid therapeutic candidate for developing novel therapeutic strategies against mood disorders.

In Study II, we show preliminary data investigating more mechanistically how specific inhibition of nuclear JNK in the abGCs modifies synaptic activity of this population of interest, leading to a positive behavioral outcome, in the context of anxiety. It has been shown that exposure to enriched environments (EE) increases the likelihood of survival of hippocampal newly born neurons, ultimately increasing adult neurogenesis. Nevertheless, our preliminary data show, for the first time, abGCs real-time calcium activity during EE, which we find peaks at 6 weeks of age and it is not dependent on JNK inhibition. Furthermore, we show that adult-generated granule cells activity starts to be recruited at the 4th week of age during the social isolation test in the presence of JNK blockade, and we still observe it at the 6th week of age, in the open field test, suggesting that JNK inhibition plays a role in modulating abGCs activity in anxiety-related behavioral contexts. More importantly, JNK's implication

in abGCs activity seems more prominent during what is known to be the "critical period" of development of the abGCs, during which they exhibit unique electrophysiological properties rendering them distinctively contributing to the information processing within the hippocampus.

In summary, the work of this thesis starts to shed light on JNK's involvement in regulation of adult hippocampal neurogenesis and the specific contribution of abGCs synaptic activity on anxiety-like behavior. Our investigation requires additional experiments to more deeply dissect the exact intracellular consequences of JNK inhibition and how these affect neuronal firing. Moreover, subsequent experiments will also need to clarify how the overall changing DG synaptic activity, in the presence of JNK inhibition, reverberates on the global activity of the trisynaptic circuitry of the hippocampus, and how this consequently influences emotional behavior.

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