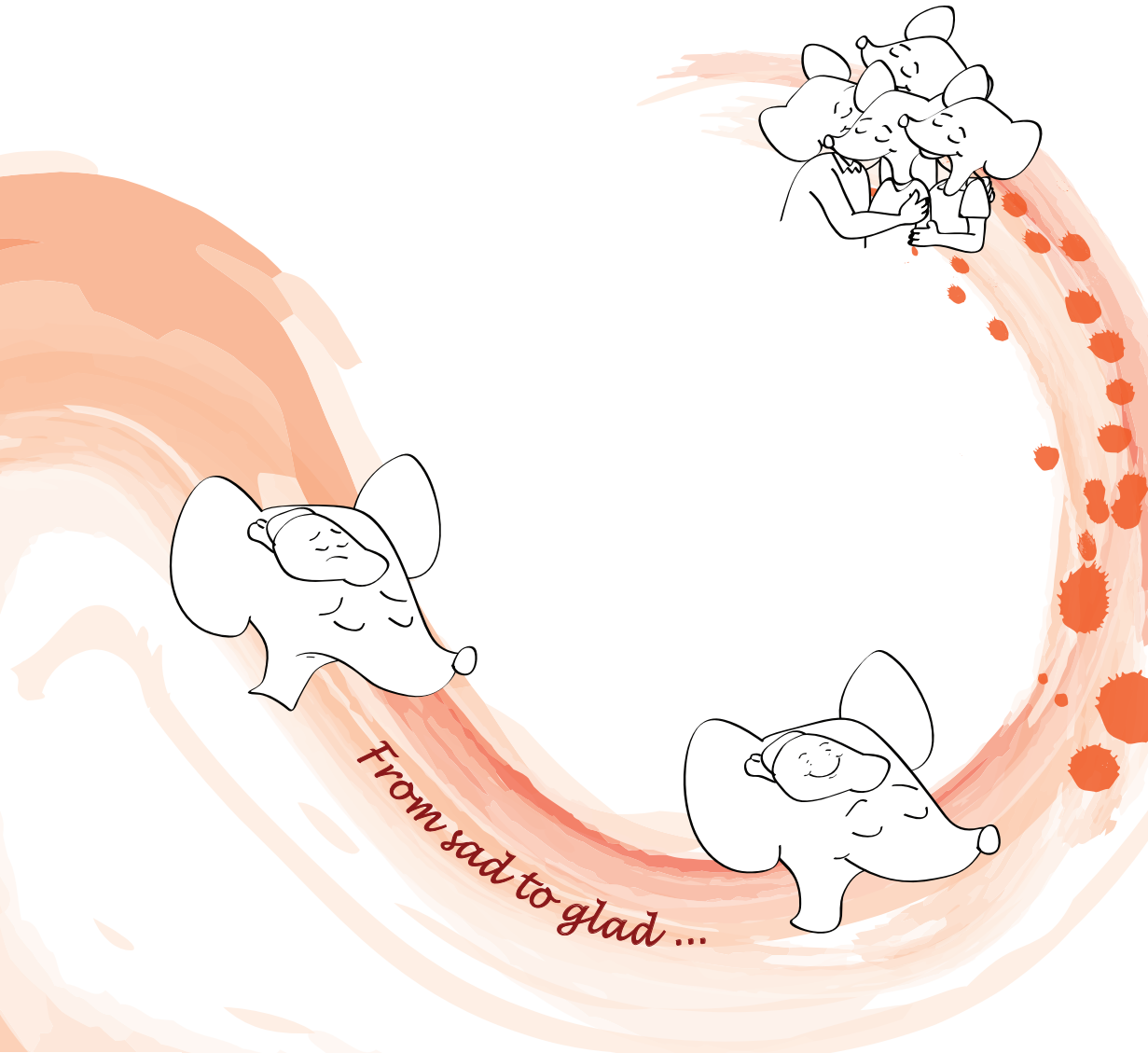
Unravelling the role of Brain-Derived Neurotrophic Factor in depression

BDNF overexpression in Serotonin Transporter Knockout Rats



DONDERS SERIES

Danielle Mendes Diniz

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BDNF overexpression in Serotonin Transporter Knockout Rats

Danielle Mendes Diniz

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General Introduction and Thesis Outline

Mood disorders

Defined as a group of psychiatric illnesses, mood disorders affect an individual's emotions, energy, and motivation (Marshall, 2012). Among the many mood disorders, one of the most outstanding is the major depressive disorder (further called depression in this chapter). Depression is characterized by depressed mood, loss of interest or pleasure in nearly all activities (anhedonia), appetite and sleep disturbances, fatigue, and loss of energy. These symptoms usually lead to significant impairment in the achievement of important tasks at home, work, or school. Additionally, depressive patients can manifest feelings of worthlessness and inappropriate guilt resulting in suicidal thoughts or actually in suicidal attempts (Morris et al., 2017). According to the World Health Organization, the total number of individuals suffering from depression worldwide is 322 million, accounting as the single largest contributor to global disability. This disorder is also the major contributor to suicide deaths, which number close to 800 000 per year (World Health Organization, 2017). Thereby, depression is among the leading causes of non-fatal health loss for nearly the last three decades (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). The diagnostic criteria for depressive disorders is established in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (American Psychiatric Association, 2013; Wenzel, 2017) and is summarized in Box 1. Despite the socio-economic impact led by this disorder, the causes leading to depression are still not fully understood. The etiology of depression is attributed to a complex interaction between environmental factors and genetic vulnerability, but specific genes have not yet been found, making it difficult to comprehend the mechanistic causes of such complex disease (Nestler et al., 2002). Although many of the treatments for depression have been shown to work effectively, these treatments do not work for all individuals, and it is common that after a successful treatment, many patients relapse (Cuijpers, 2017).

Box 1: DSM-5 Diagnostic Criteria for Major Depressive Disorder

A. Period lasting at least two weeks during which an individual experiences five or more out of nine overlapping depressive symptoms. At least one of the following two cardinal symptoms must be present during these once weeks: (1) depressed mood present most of the day nearly every day and (2) loss of interest or pleasure in nearly all activities:

1. Depressed mood most of the day, nearly every day
2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day
3. Significant weight loss when not dieting or weight gain, or decrease or increase in appetite nearly every day
4. Insomnia or hypersomnia nearly every day
5. Psychomotor agitation or retardation nearly every day
6. Fatigue or loss of energy nearly every day

7. Feelings of worthlessness or excessive or inappropriate guilt nearly every day
8. Diminished ability to think or concentrate, or indecisiveness, nearly every day
9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide

B. These depressive symptoms must be associated with clinically significant distress or impairment in important areas of functioning (e.g., social, occupational).
C. The episode is not attributable to the physiological effects of a substance or another medical condition

The hypothesis behind depression symptoms

In an attempt to understand the processes underlying the behavioral changes displayed by depressive patients, several hypotheses have been proposed. These hypotheses aim to gather clinical and preclinical data to offer guidance for the development of novel prophylactic and therapeutic approaches. One hypothesis was proposed in the early 1960s and highlighted the involvement of serotonin in the physiopathology of depression (Coppen, 1967). The serotonin hypothesis of depression was established based on the assumption that elevation of serotonin levels could relieve depressive symptoms, and the examination of this assumption led to the design of the selective serotonin reuptake inhibitors (SSRI), which target the serotonin transporter promoting an increase in the central levels of serotonin (Guan and McBride, 1988). Although deficits in serotonin levels or release are a central prediction of this hypothesis, the relation between human depression and the levels of serotonin remains unclear (Jacobsen et al., 2012). Moreover, while elevated levels of serotonin are associated with decreased depressive symptoms, it is clear that depression treatments do not work for all individuals (Cuijpers, 2017). Therefore, even though it has been demonstrated that increasing serotonin levels lead to relieve in depressive symptoms, inconclusive and inconsistent studies have shown that the serotonin hypothesis seems to be too simplistic to explain the mechanisms by which this mood disorder develops in some individuals (Thompson et al., 2015).

Taking into consideration that simply increased serotonin levels does not necessarily lead to amelioration of depressive behavioral phenotypes, further understanding of the molecular basis of depressive disorders started to be explored in light of another hypothesis for the origin of depression, namely the neurotrophic hypothesis of depression (Duman and Monteggia, 2006). Neurotrophins have a crucial role in the synaptic maturation, neuronal growth, and synaptic plasticity both during development and adulthood (Autry and Monteggia, 2012). Impaired production, release, and/or action of this class of signaling molecules is believed to have a direct association with depression (Duman and Monteggia, 2006). Among the neurotrophin family are the neurotrophins 3 and 4/5, nerve growth factor (NGF), and the brain-derived neurotrophic factor (BDNF) (Huang and Reichardt, 2001), of

which BDNF is the most abundant and one of the most investigated neurotrophins. Given that the antidepressant therapeutic action occurs in a delay of weeks, and due to BDNF's pivotal role in neuroplasticity, it has been associated with the mechanism of action of antidepressants (Duclot and Kabbaj, 2015; Duman and Monteggia, 2006; Monteggia et al., 2004). BDNF gained attention in the study of mood disorders because data from postmortem brain tissues or serum of depressive patients indicated low levels of BDNF, which were normalized following antidepressant treatment (Adachi, 2014; Sen et al., 2008). Moreover, most of the clinical studies reported a reduction in BDNF protein levels in the serum of depressive individuals. These studies showed that there is a direct correlation between antidepressant treatment and an increase in peripheral BDNF protein levels of treated patients, while untreated individuals present decreased levels of BDNF protein (Fernandes et al., 2015; Polyakova et al., 2015; Sen et al., 2008). Studies also reported abnormal mRNA BDNF or TrkB expression in the hippocampus and prefrontal cortex postmortem tissue of suicidal patients with a previous record of major depression (Dwivedi et al., 2003). Together, preclinical and clinical gene expression and imaging studies support the neurotrophic hypothesis of depression and antidepressant response. In summary, this hypothesis proposes that depression results from decreased neurotrophic support, leading to neuronal atrophy, decreased hippocampal neurogenesis and loss of glia and that antidepressant treatment blocks or reverses this neurotrophic factor deficit, and thereby reverses the atrophy and cell loss (Duman et al., 1999; Duman and Li, 2012; Duman and Monteggia, 2006).

Interestingly, there is an interaction between the two hypotheses when observing the action of SSRIs and the genetic deletion of its target receptor, the serotonin receptor (SERT). As previously discussed, SSRIs act by blocking SERT, causing increased serotonin levels in the synaptic cleft. Genetic downregulation of SERT, likewise, leads to increased central serotonin levels. However, while SSRIs are associated with increased BDNF levels, SERT deletion causes a decrease in BDNF levels (Calabrese et al., 2013; Molteni et al., 2010). Moreover, it has been well established that the genetic deletion of SERT produces an anxious and depression-like phenotype (Kalueff et al., 2010). Altogether, these data highlight that the developmental changes produced by genetic SERT deletion are in agreement with the neurotrophic hypothesis of depression, given that it induces BDNF downregulation, and consequently decreased neuronal plasticity.

Brain regions involved in depressive disorders

The underlying mechanisms by which the pathophysiology of neural circuits lead to depression remains unclear. The variability in symptoms, combined with the lack of definitive biomarkers, has presented a major challenge for modeling depression in the laboratory. However, recent approaches combining animal models of depression with

electrophysiological, optogenetics, chemogenetics and molecular analysis have begun to reveal a complex interplay between various neural circuits and cell types in encoding for depression (Han and Friedman, 2012; Lobo et al., 2014; Russo and Nestler, 2013). Commonly, brain circuitries involved in the detection, response, and interpretation of emotions have their function affected in depression. The fronto-limbic circuitry, for instance, is highly involved in mediating these stages of emotion processing (Liao et al., 2012), it includes among other brain areas the prefrontal cortex (PFC), hippocampus, ventral tegmental area (VTA), and amygdala, and evidence indicates that these regions show structural and functional alterations in depression (Yu and Chen, 2011). Indeed, different brain regions play a role in the physiopathology of depression, but since the experiments explored in this thesis focused on the PFC and hippocampus, this session will briefly discuss the involvement of such target areas in depressive disorders.

The prefrontal cortex (PFC) is a collection of interconnected neocortical areas that sends and receives projections to integrate behaviorally relevant information from limbic, cognitive, sensory, and motor regions (Miller and Cohen, 2001). The PFC is responsible for regulating cognitive, motivational, and emotional processes (Heinz et al., 2005; Pitts et al., 2016). Particularly, the prelimbic cortex (PrL), a subdivision of the medial prefrontal cortex (mPFC), primarily projects to limbic regions, including the nucleus accumbens (NAc) and the basolateral amygdala showing not only a clear connection with the reward pathway but also an involvement with the regulation of behavioral responses to stress (Choi et al., 2012; Patel et al., 2019; Vertes, 2004). In clinical studies, it has been demonstrated patients suffering from major depressive disorder presented structural and functional brain imaging changes, including reduced brain volume and activity in the PFC (Fales et al., 2009; Schulz and Arora, 2015). These structural changes in depressed patients have been confirmed in postmortem studies demonstrating a reduction in neurons and increased glial loss in the PFC, which was also accompanied by a reduction in brain-derived neurotrophic factor (BDNF) (Duman and Monteggia, 2006; Krishnan and Nestler, 2008). Interestingly, the cellular disruption in the PFC suggests that depressive disorders can be in part characterized by a loss of excitatory cortical control over limbic structures such as the NAc and amygdala, leading to maladaptive processing of reward and aversive events (Russo and Nestler, 2013).

The hippocampus is part of the limbic system and modulates emotional processing, memory, and learning. This brain area interacts with the amygdala to provide input regarding the context in which stimuli occur, and plays an inhibitory control over the glucocorticoid secretion by the hypothalamic-pituitary-adrenal axis (HPA-axis), making this area susceptible to the effects of stress (O'Leary and Cryan, 2014; Yu and Chen, 2011). Stress and other negative stimuli can change hippocampal plasticity, increasing the risk of depression (Liu et al., 2017). Depressive disorder is greatly associated with hippocampal atrophy (Taylor et al.

2014; Elbejjani et al. 2015; Santos et al. 2018; Elbejjani et al. 2014). Moreover, impaired hippocampal neurogenesis has been associated with depression (Jacobs et al., 2000). Likewise the prefrontal cortex, structural and functional changes in the hippocampus are probably associated with disruption and atrophy of neurons and glia in depression (Castrén and Rantamäki, 2010). Importantly, animal studies have shown that while impaired hippocampal neurogenesis can lead to depression (Jacobs et al., 2000), the upregulation of BDNF levels stimulated hippocampal neurogenesis (Quesseveur et al., 2013; Rossi et al., 2006).

Brain-derived neurotrophic factor (BDNF)

BDNF is one of the most extensively studied neurotrophins in the central nervous system (CNS). It was identified in the early 1950s as a peptide promoting neuronal survival and growth (Cohen et al., 1954), and later in 1982, it was first purified from pig brains (Barde et al., 1982). BDNF is considered part of the neurotrophin family, which also includes the nerve growth factor (NGF), neurotrophin 3 (NT3), and neurotrophin 4/5 (NT4/5) (Cohen, 1960; Lessmann et al., 2003). All neurotrophins mediate their biological function through binding to a common neurotrophin receptor called $p75^{NTR}$, and to their highly specific tropomyosin-related kinase receptor (NGF: TrkA, BDNF and NT4: TrkB, and NT3: TrkC) (Thoenen, 1991).

Like other neurotrophins, BDNF is synthesized as a 32 kDa pre-pro-neurotrophin precursor in the rough endoplasmatic reticulum (See figure 1). Immediately after synthesis in the endoplasmatic reticulum, the pre-signal peptide is cleaved off, and the pro-BDNF is ready to be transferred to the Golgi apparatus to be then sorted in the Trans-Golgi Network (TGN) into at least two different kinds of vesicles according to the secretory pathway. In the constitutive pathway, the secretory granules release BDNF by default when reaching the plasma membrane without the need of a triggering mechanism. The regulated pathway employs Ca^{2+}-dependent exocytosis of secretory granules. While other neurotrophins, including NGF and NT-3, prefer the constitutive pathway, BDNF is preferentially sorted into the regulated pathway in response to neuronal activity (Adachi, 2014; Benarroch, 2015; Lessmann et al., 2003; Leßmann and Brigadski, 2009; Park and Poo, 2013). Exactly where the proteolytic removal to generate the mature 13 kDa form of BDNF takes place is still controversial. Initial studies concluded that pro-neurotrophins are cleaved by proteases such as furin and pro-protein convertases in the trans-Golgi network or inside the dense core vesicles before secretion (Adachi, 2014). However, recent studies have shown that neurons can also secrete pro-BDNF, which can be subsequently processed to mature BDNF by extracellular proteases such as matrix metalloproteinases and plasmin (Pang et al., 2004). Complete understanding of the conversion from pro-BDNF to mature BDNF is still lacking, but it is important because pro-BNDF has an affinity to $p75^{NTR,}$ which initiates a cascade of

signaling pathways promoting cell apoptosis (Leßmann and Brigadski, 2009; Teng et al., 2005). Through activation of its high-affinity receptor, TrkB, mature BDNF exerts several physiological roles in the CNS including neuronal differentiation, synaptogenesis, axonal and dendritic growth of neuronal processes, formation and maturation of glutamatergic and GABAergic synapses, activity-dependent refinement of synaptic connections and synaptic plasticity (Alsina et al., 2001; Benarroch, 2015; Edelmann et al., 2014; Poo, 2001).

The human BDNF gene consists of 11 exons and nine functional promoters, and its transcription occurs through multiple 5' exons spliced to a single 3' coding exon (exon IX) (Pruunsild et al., 2007) (Figure 1). Similarly, the mouse and rat BDNF gene present a common 3' exon encoding BDNF protein and at least eight 5' noncoding exons (exons I-VIII), giving a total of nine exons and nine functional promoters (Aid et al., 2007). The majority of the mRNA BDNF in the brain are generated through promoters I, II, IV, or VI (Aid et al., 2007; Pruunsild et al., 2007). In both rodents and humans, transcription of the BDNF gene terminates at two alternative polyadenylation sites in exon IX, generating two different pools of mRNA, a short and a long 3' untranslated regions (UTRs) (Adachi, 2014). Importantly, these two mRNA groups might define the cellular localization of BDNF. For instance, An et al. (2008) demonstrated that BDNF mRNA with the long 3'-UTR is preferentially targeted to dendrites upon neuronal activation, while the short 3'-UTR may be restricted to the soma.

The functional consequences of multiple BDNF transcripts encoding the same protein together with the effects caused by distinct subcellular localization of such transcripts are still under investigation. It is hypothesized that BDNF transcripts distribution in the brain may occur to provide flexibility that allows different factors to regulate BDNF signaling in distinct cell types and circuits, thereby reinforcing specific neural networks. Baj et al. (2011) showed that in hippocampal cultures BDNF exons II and VI targets distal dendrites, whereas transcripts I and IV are localized in proximal dendrites and soma. Interestingly, the work of Maynard et al. (2016) using mutant mice with selective disruption of either BDNF promoters I, II, IV or VI demonstrated that mutant mice lacking either exon I or II displayed high levels of aggression and changes in the serotoninergic signaling, while mutant mice with disrupted exons IV or VI displayed impairments associated with GABAergic gene expression. Moreover, transcription from promoters I and II was more prominent in the adult hypothalamus, whereas transcription from promoters IV and VI was more prominent in the prefrontal cortex (PFC) and hippocampus. These results show that alternative BDNF transcripts mediate precise temporal-, spatial-, and stimulus-specific BDNF production, creating a spatial code for BDNF expression in different brain regions, cell types, and even within distinct subcellular compartments (Maynard et al., 2016).

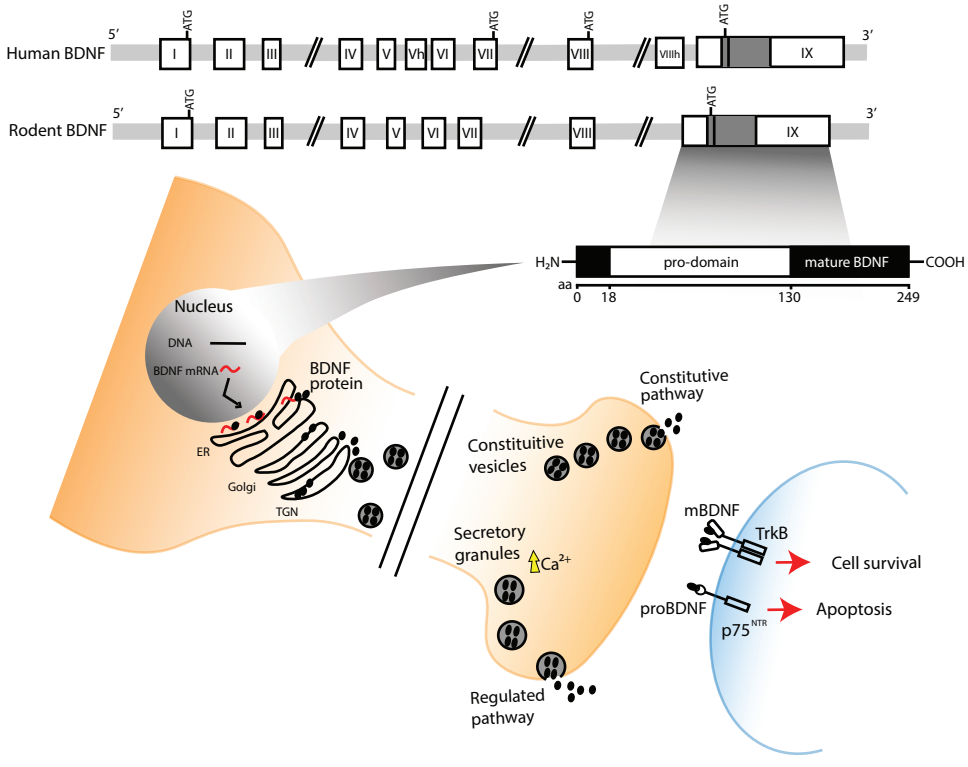

Figure 1. Schematic overview of the BDNF system. The human BDNF gene consists of 11 exons and nine functional promoters, and its transcription occurs through multiple 5' exons spliced to a single 3' coding exon (exon IX) (Pruunsild et al., 2007). Likewise, the mouse and rat BDNF gene present a common 3' exon encoding BDNF protein and at least eight 5' noncoding exons (exons I-VIII), giving a total of nine exons and nine functional promoters (Aid et al., 2007). Immediately after synthesis in the endoplasmatic reticulum (ER), the pre-signal peptide is cleaved off and the pro-BDNF is transferred to the Golgi apparatus to be then sorted in the Trans-Golgi Network (TGN). In the constitutive pathway, the secretory granules release BDNF by default when reaching the plasma membrane without the need of a triggering mechanism. The regulated pathway employs Ca^{2+}-dependent exocytosis of secretory granules. Pro-BNDF has an affinity to $p75^{NTR,}$ which promotes cell apoptosis. Mature BDNF has a high affinity for its receptor, TrkB, and through it exerts several physiological roles in the CNS (Lessmann et al., 2003; Leßmann and Brigadski, 2009)

The human serotonin transporter (SERT), BDNF, and the vulnerability to mood disorders

The serotonin transporter (SERT) is a transmembrane presynaptic protein involved in the reuptake of the released serotonin from the synaptic cleft. Therefore, it mediates rapid removal and recycling of released serotonin following neuronal stimulation, playing a critical role in the homeostatic regulation of the magnitude, duration, and spatial distribution of signals reaching serotonin receptors (Murphy et al., 2004). The human SERT is encoded by

the SLC6A4 (Solute Carrier Family 4) gene, which comprises fourteen exons and is located on chromosome 17 (17q11.2) (Lesch et al., 1994; Ramamoorthy et al., 1993). It presents a functional polymorphism in the promoter region (5-HTTLPR), generating a short and a long allele variant of the serotonin transporter. The upstream 44 base-pair insertion/deletion polymorphism is composed by the repetition of 14 (for the s - short - allele) or 16 (for the l - long -allele) repeated elements (Murphy et al., 2008). The frequency of the S allele in the population differ according to the ethnic group analyzed. A recent meta-analysis identified that among Caucasians, the S allele is present in 46% of the population, and among Asians, the frequency is 75% (Fanelli and Serretti, 2019).

The s-allelic variant is dominant and induces a decrease in the SERT expression and function in comparison to the l-allelic variant (Lesch et al., 1996). It is linked to increased risk for depression and suicidal behavior (Bleys et al., 2018; Caspi et al., 2003; Fanelli and Serretti, 2019; Homberg et al., 2014). Additionally, the short allele variant appears to be associated with insufficient response to SSRIs (Kroeze et al., 2012; Serretti et al., 2007).

Although there is still no consensus that 5-HTTLPR is associated with depression, this polymorphism has been extensively explored in gene x environment studies. Several works have highlighted that the 5-HTTLPR can modulate the influence of stressful life events on depression. The most well-established and consistent trait associated with the 5-HTTLPR s-allele is neuroticism (Lesch et al., 1996; Munafo et al., 2008; Schinka et al., 2004). Neuroticism is the trait disposition to experience negative effects, including anger, anxiety, self-consciousness, irritability, emotional instability, and depression (Widiger and Oltmanns, 2017). It has been shown that s-carriers tend to develop selective attention to negative stimuli (Pergamin-Hight et al., 2012). As such, sensitivity to negative stimuli may increase vulnerability for psychopathologies (Homberg and Lesch, 2011). Caspi et al. (2003) reported that individuals carrying the s-allelic variant of the 5-HTTLPR, as opposed to the l-variant, were at risk of developing depression when exposed to adverse psychosocial stressors in early life. More recently, a study investigating how life-stressors modulate the effect of 5-HTTLPR on depression and anxiety in a European population cohort of over 2300 subjects, demonstrated that the s-allele was a risk variant. There was also a direct association between the 5-HTTLPR s-allele and anxiety, and an interaction between 5-HTTLPR s-carriers versus recent negative life events and current depressive symptoms (Juhasz et al., 2015).

Several studies have proposed an interaction between the serotonin and the BDNF system associated with vulnerability to neuropsychological disorders. Interestingly, while BDNF has a crucial role in the synaptic maturation, neuronal growth, and synaptic plasticity both during development and adulthood (Autry and Monteggia, 2012), serotonin is implicated in many central functions, including the control of mood, sleep, cognitive functions, learning and memory, as well as ingestive- and reward-related behavior (Homberg

et al., 2014). Impaired production, release, and/or action of BDNF is believed to have a direct association with depression (Duman and Monteggia, 2006). As discussed above, the serotonin transporter plays a critical role in the homeostatic regulation of the magnitude, duration, and spatial distribution of signals in the serotonin system (Murphy et al., 2004); moreover, impaired SERT function is associated with increased risk for depression (Caspi et al., 2003). Therefore, it can be expected that these two systems interact, increasing the risk of developing mood disorders. Actually, lower serum levels of BDNF were identified in 5-HTTLPR homozygotes s-carriers (Benedetti et al., 2017; Bhang et al., 2011). Molteni et al. (2010) have also demonstrated BDNF mRNA downregulation in leukocytes of s-carriers.

Lessons from the Serotonin Transporter Knockout ($SERT^{-/-}$) Rodents

Although rodents do not carry the human serotonin transporter linked polymorphic region (5-HTTLPR), genetic deletion or partial deletion of the SERT in rodents leads to anxiety- and depression-related phenotypes (Kalueff et al., 2010; Olivier et al., 2008). There is increasing evidence that particularly heterozygous 5-HTT knockout ($SERT^{+/-}$) mice respond to prenatal, early life, and adult psychosocial stress, as seen in s-allele carriers (Bartolomucci et al., 2010; Carola et al., 2008; Jones et al., 2010). Moreover, $SERT^{-/-}$ rats seems to present increased responsiveness to stress throughout the lifetime (Schipper et al., 2011). Therefore, these data support the association between an increased risk for mood disorders and the functionality of SERT.

The $SERT^{-/-}$ rats were first generated through an N-ethyl-N-nitrosourea (ENU)-driven target-selected mutagenesis (Smits et al., 2006) by Homberg and co-workers (Homberg et al., 2007). These animals have a Wistar background and present stable phenotype across generations and laboratories (Olivier et al., 2010). The lack of SERT during development allows the investigation of potential compensatory mechanisms and the developmental effects of reduced neuronal plasticity. Indeed, it has been demonstrated that SERT deletion leads to a persistent reduction in the expression BDNF (Molteni et al., 2010) and of the immediate early gene Arc (Molteni et al., 2009). BDNF mRNA and protein are specially downregulated in the ventral hippocampus and prefrontal cortex of $SERT^{-/-}$ and $SERT^{-/+}$ rats (Calabrese et al., 2015; Guidotti et al., 2012; Molteni et al., 2010), both areas in which neuroplasticity plays an important functional role (Castrén and Rantamäki, 2010). Reductions in BDNF and Arc expressions are already present in adolescence and are sustained by epigenetic changes suggesting that they may represent developmental consequences of SERT deletion.

SERT$^{-/-}$ rats also present downregulation of transcriptional factors involved in the regulation of BDNF, such as CREB, Arnt2, CaRF, NFkB, and Npas4. Changes in Npas4 are directly correlated with decreased BDNF exons I and IV (Guidotti et al., 2012). Npas4 plays a role in the development of inhibitory synapses by regulating the expression of activity-dependent genes, which in turn control the number of GABA-releasing synapses that form on excitatory neurons (Lin et al., 2008). The GABAergic system communicates with other neurotransmitter networks, and its downregulation can lead to an anxiety-like behavioral outcome (Lydiard, 2003; Millan, 2003). It has been demonstrated that promoter IV-driven BDNF transcription has a critical role in GABAergic transmission (Sakata et al., 2009) and mice with a selective deficiency of promoter-IV-dependent expression of BDNF show depression-like behavior (Sakata et al., 2010). Importantly, SERT$^{-/-}$ rats present changes in the functioning of the GABAergic system suggesting a link between Npas4, BDNF, and GABA in contributing to the phenotypic changes observed in SERT$^{-/-}$ rats. (Calabrese et al., 2013; Luoni et al., 2013; Miceli et al., 2017; Schipper et al., 2019).

Aim and Outline of the thesis

As discussed above, there is converging evidence that inherited SERT downregulation is associated with a developmental decline in BDNF levels (Calabrese et al., 2013). The decreased developmental neurotrophic support might set the stage for decreased inhibitory control over excitatory neurons in hippocampal and prefrontal cortex areas, which hinders adaptive response to unpredictable and uncontrollable stressors due to decreased neuroplasticity, leading to increased risk for depression (Schipper et al., 2019). Indeed, the SERT$^{-/-}$ rats present anxiety- and depression-like phenotype, including anhedonia-like behavior in the sucrose preference test, increased immobility in the forced swim test, and decreased time spent in the central part of the open field (Olivier et al., 2008). Additionally, SERT$^{-/-}$ rats displayed increased levels of basal CORT under control conditions, showing altered basal hypothalamic-pituitary-adrenal axis (HPA-axis) activity (van der Doelen et al., 2014). Therefore, in concordance with the neurotrophic hypothesis of depression, this thesis aimed to investigate whether overexpressing BDNF in the SERT$^{-/-}$ rats would remediate the low BDNF mRNA and BDNF protein levels in SERT$^{-/-}$ rats, and whether BDNF upregulation would in return meliorate their depression-like symptoms.

To this end, in **chapter 2** of this thesis, I present the results from the BDNF overexpression in the prefrontal cortex (PFC). In this study, we targeted a subregion within the medial prefrontal cortex, the prelimbic cortex, due to its connectivity with the reward pathway, which is affected in depression and other mood disorders (Nestler and Carlezon, 2006; Vertes, 2004). We specifically intended to upregulate BDNF exon IV because this transcript is downregulated in the mPFC of SERT-/- rats (Molteni et al., 2010). In the first

experiment, we aimed to check the gene expression following the lentivirus infusion one and two weeks after surgery. After that, we verified the overexpression of the total BDNF mRNA in the prelimbic and infralimbic cortices of the $SERT^{+/+}$ rats through RT-qPCR analysis. In a second experiment, following the viral infusions, $SERT^{-/-}$ rats and wild-type controls were submitted to behavioral testing. Behavior experiments included: (1) sucrose consumption test, in which we assessed the rats preference for sucrose over water and the total sucrose intake in grams; (2) forced swim test, where we scored for immobility, mobility and strong/high mobility behaviors; (3) novelty-induced locomotor activity, in which Phenotyper® cages were used to evaluate distance moved, velocity, and time and frequency in the center; and (4) HPA-axis reactivity test, a test where the response of the HPA-axis upon acute restraint stress was checked through measurement of the CORT levels. Next, we sacrificed the animals that underwent behavioral testing and measured the post-behavior BDNF mRNA levels of total BDNF, BDNF IV and BDNF VI in the prelimbic and infralimbic cortices.

In **chapter 3**, we investigated the same parameters, but targeting the hippocampus. We targeted the ventral area of the hippocampus because it has been demonstrated that BDNF is especially downregulated in this area of $SERT^{-/-}$ rats (Calabrese et al., 2013). Therefore, in the first experiment, we analyzed the temporal dynamics of BDNF IV overexpression one, two, and four weeks following lentivirus infusion in the ventral hippocampus (vHIP) of $SERT^{+/+}$ rats. In this experiment, we measured BDNF mRNA levels of total BDNF, BDNF IV, and BDNF VI in the prelimbic and infralimbic cortices, and in the vHIP. In a second experiment, following viral infusion, $SERT^{-/-}$ and $SERT^{+/+}$ rats were submitted to the sucrose consumption test, forced swim test, novelty-induced locomotor activity, and HPA-axis reactivity tests.

In **chapter 4**, we describe a pilot experiment performed to develop and characterize a non-viral gene carrier containing features to enable delivery through the blood-brain-barrier. We demonstrated that the synthesis of this lipid-based nanocarrier, namely liposome, was feasible. The physicochemical characterization of the liposomes are presented, including particle size and distribution (polydispersity index – PDI), and surface charge (zeta-potential) measurements. Moreover, we encapsulated pDNA and measured loading efficiency, and in vitro transfection. This chapter also discusses the perspectives for future use of the liposomal nanocarrier as a non-viral vector to the target delivery of pBDNF into the hippocampus.

Finally, **chapter 5** provides a general discussion and suggests directions for future perspectives regarding the research described in this thesis.

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BDNF Overexpression in the Prelimbic Cortex Does Not Reduce Anxiety- and Depression-like Behavior in Serotonin Knockout Rats

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Abstract

Depressive disorders are one of the leading causes of non-fatal health loss in the last decade. Adding to the burden, the available treatments not always properly work for some individuals. There is, therefore, a constant effort from clinical and preclinical studies to bring forward a better understanding of the disease and look for novel alternative therapies. Two target systems very well explored are the serotonin and the brain-derived neurotrophic factor (BDNF) systems. Selective serotonin reuptake inhibitors (SSRIs), a commonly used class of antidepressants, target the serotonin transporter (SERT) and increase serotonin levels, which in turn also leads to an increase in BDNF. A rat model lacking SERT (SERT knockout) has been a useful tool to study the interplay between serotonin and BDNF. $SERT^{-/-}$ rats present increased extracellular levels of serotonin, yet BDNF levels are decreased, especially in the prefrontal cortex (PFC) and hippocampus. The animals further display anxiety- and depression-like behavior. Therefore, BDNF might mediate the phenotype expressed by the $SERT^{-/-}$ rats. In this study, we sought to investigate whether overexpression of BDNF in the brain of $SERT^{-/-}$ rats would rescue its anxious and depressive-like behavior. Through stereotaxic surgery, $SERT^{-/-}$ and wild-type (WT) rats received BDNF or GFP lentivirus microinfusions into the prelimbic cortex subregion of the mPFC and were submitted to the sucrose consumption, open field test, and forced swim tests. Additionally, we measured hypothalamus-pituitary-adrenal (HPA)-axis reactivity. The results revealed that $SERT^{-/-}$ rats presented decreased sucrose intake, decreased locomotor activity, and increased escape-oriented behavior in the forced swim test compared to WT rats. BDNF upregulation in WT rats caused alterations in the HPA-axis function, resulting in elevated basal plasma corticosterone levels and decreased plasma corticosterone upon stress. In conclusion, BDNF overexpression in the PrL, in general, did not rescue $SERT^{-/-}$ rats from its depression- and anxiety-like behavior, and in WT animals, it caused a malfunction in the HPA-axis.

Keywords: BDNF, Serotonin knockout rats, Prefrontal cortex, Depression, Anxiety.

Introduction

All around the world, individuals are experiencing the damaging symptoms caused by depressive disorders, such as depressed mood, loss of interest or pleasure in nearly all activities (anhedonia), appetite and sleep disturbances, fatigue, and loos of energy. These symptoms usually lead to significant impairment in the achievement of essential tasks at home, work, or school. Additionally, depressive patients can manifest feelings of worthlessness and inappropriate guilt resulting in suicidal thoughts or actually in suicidal attempts (Morris et al., 2017). The rate of individuals suffering from depression is very high; by 2015, it had reached 322 million worldwide (WHO 2017). Thereby, depression is among the leading causes of non-fatal health loss for nearly the last three decades (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). Moreover, although many of the treatments for depression have been shown to work effectively, these treatments do not work for all individuals, and commonly after a successful treatment, many patients relapse (Cuijpers, 2017). Furthermore, despite the socio-economic impact led by this disorder, the causes leading to depression are still not fully understood. The etiology of depression is attributed to a complex interaction between environmental factors and genetic vulnerability, but specific genes have not yet been found, making it challenging to comprehend the mechanistic causes of such complex disease (Nestler et al., 2002).

Clinical and preclinical studies are attempting to elucidate the pathophysiological processes underlying depression. These efforts brought to light, for instance, the serotonin hypothesis of depression in the early 1960s, which is based on the observation that antidepressant drugs can increase the concentration of monoamines, especially serotonin, in the brain (Coppen, 1967). However, although it has been demonstrated that increasing serotonin levels using, for example, selective serotonin reuptake inhibitors (SSRIs), leads to relieve in depressive symptoms, inconclusive and inconsistent studies have shown that the serotonin hypothesis seems to be too simplistic to explain the mechanisms by which this mood disorder develops in some individuals (Thompson et al., 2015). One particular observation involves the role of the serotonin reuptake transporter (SERT), which is responsible for regulating extracellular serotonin levels, and it is the target of SSRIs. Genetic down-regulation of SERT leads to increased central levels of serotonin, reproducing, thereby, the effects of SSRIs. However, it is well established that SERT downregulation is also associated with anxious and depressive phenotypes. Therefore, although antidepressants increase serotonin levels, other systems might be involved in the antidepressant response. The SERT knockout (SERT$^{-/-}$) rats, for example, are characterized by a complete lack of SERT and increased extracellular serotonin levels (Homberg et al., 2007), yet show anxious and depressive-like phenotypes. This animal model presented anhedonia-like behavior in the sucrose preference test, increased immobility in the forced swim test, and decreased time

spent in the central part of the open field, indicating that anxiety levels and depressive-like behavior are increased (Olivier et al., 2008). Additionally, SERT$^{-/-}$ rats displayed increased levels of basal plasma corticosterone (CORT) levels under control conditions, showing altered basal hypothalamic-pituitary-adrenal axis (HPA-axis) activity (van der Doelen et al., 2014).

Taking into consideration that simply increased serotonin levels does not necessarily lead to amelioration of depressive behavioral phenotypes, further understanding of the molecular basis of depressive disorders started to be explored in light of another hypothesis for the origin of depression, namely the neurotrophic hypothesis of depression (Duman and Monteggia, 2006). Neurotrophins have a crucial role in the synaptic maturation, neuronal growth, and synaptic plasticity both during development and adulthood (Autry and Monteggia, 2012). Impaired production, release, and/or action of this class of signaling molecules is believed to have a direct association with depression (Duman and Monteggia, 2006). Among the neurotrophin family are the neurotrophins 3 and 4/5, nerve growth factor (NGF), and the brain-derived neurotrophic factor (BDNF) (Huang and Reichardt, 2001), of which BDNF is the most abundant and one of the most investigated neurotrophins.

Several studies support the neurotrophin hypothesis of depression and point to the involvement of BDNF in the physiopathology of this disorder. Most of the clinical studies reported a reduction in BDNF protein levels in the serum of depressive individuals. These studies showed that there is a direct correlation between antidepressant treatment and an increase in peripheral BDNF protein levels of treated patients, while untreated individuals present decreased levels of BDNF protein (Fernandes et al., 2015; Polyakova et al., 2015; Sen et al., 2008). Studies also reported abnormal mRNA BDNF or TrkB expression in the hippocampus and prefrontal cortex post-mortem tissue of suicidal patients with a previous record of major depression (Dwivedi et al., 2003).

Noteworthy is the observation that, while antidepressant treatment induces increases in BDNF levels, the genetic manipulation of the SERT in rats causes a decrease in BDNF levels. As mentioned above, although inherited SERT downregulation in SERT$^{-/-}$ rats is associated with constitutive increased levels of serotonin, these animals present anxiety- and depression-like behavior (Homberg et al., 2014). Moreover, in agreement with the neurotrophic hypothesis of depression, it was shown that SERT$^{-/-}$ rats present, under basal conditions, downregulation of BDNF mRNA and protein levels in the hippocampus and prefrontal cortex (Molteni et al. 2010; Calabrese et al. 2013). Further, total BDNF mRNA levels (exon IX) were significantly downregulated and the reduction of BDNF gene expression observed in the prefrontal cortex of SERT$^{-/-}$ rats was shown to be due, at least in part, to epigenetic changes affecting the promoter regions of exons IV and VI (Molteni et al., 2010). Therefore, while pharmacological increase of serotonin through SSRIs leads to an increase in

BDNF levels, genetic deletion of the SERT leads to likewise increased serotonin levels but decreased in BDNF levels.

BDNF presents a complex gene structure; its regulation occurs at transcriptional, translational, and post-translational levels. The human gene presents 11 different exons regulated by nine promoters (Pruunsild et al., 2007), and the rodent gene consists of nine distinct exons with eight 5' untranslated exons and one protein-coding 3' exon (Aid et al., 2007). The multiple BDNF exons generate a wide diversity of BDNF transcripts that differentially control BDNF protein expression in an activity-dependent and tissue-specific manner (Autry and Monteggia, 2012; Mercado et al., 2017; Miranda et al., 2019). The BDNF mature protein is subject to post-translational modifications. It is synthesized as its precursor preproBDNF in the endoplasmic reticulum (ER), where the pre-domain is cleaved generating proBDNF; proBDNF is then transferred to the Golgi apparatus to be sorted into secretory vesicles (Lessmann et al., 2003). Extracellular or co-released endopeptidases are responsible for removing the pro-domain, which can happen in different stages following the secretion of the pro-protein (Leßmann and Brigadski, 2009). Interestingly, not only the mature form of BDNF has a cellular function. While mature BDNF has an affinity for the tropomyosin-related kinase receptor TrkB receptor promoting synaptic plasticity, proBDNF has a preference for the p75NTR receptor, which activates the pathway for cellular apoptosis (Teng et al., 2005).

Different brain regions play a role in the physiopathology of depression, with the prefrontal cortex being one of them. It has been demonstrated that major depressive disorder, for example, is associated with structural and functional brain imaging changes, including reduced brain volume and activity in the PFC (Schulz and Arora, 2015). These structural changes in depressed patients have been confirmed in post-mortem studies demonstrating a reduction in neurons and glial loss in the PFC, which is accompanied by a reduction in BDNF in this brain area (Duman and Monteggia, 2006; Krishnan and Nestler, 2008). In fact, the prefrontal cortex (PFC) is a well-known brain region responsible for processes such as cognitive, motivational, and emotional regulation (Heinz et al., 2005; Pitts et al., 2016). Interestingly, the prelimbic cortex (PrL), a subdivision of the medial prefrontal cortex (mPFC), primarily projects to limbic regions, including the nucleus accumbens and the basolateral amygdala showing not only a clear connection with the reward pathway, but also involvement with the regulation of behavioral responses to stress (Choi et al., 2012; Patel et al., 2019; Vertes, 2004).

Given the reduced levels of BDNF in the PFC of SERT$^{-/-}$ rats (Calabrese et al., 2013; Molteni et al., 2010) and the role of BDNF in supporting neuronal plasticity that is particularly affected in depressive disorders (Miranda et al., 2019), we sought to investigate whether BDNF gene overexpression can rescue the anxiety- and depression-like behavior of these rats. We selected the PrL as a target due to its connectivity with the reward pathway, which

is affected in depression and other mood disorders (Nestler and Carlezon, 2006; Vertes, 2004). For gene overexpression, the BDNF exon IV was chosen because notably, this transcript is downregulated in the mPFC of $SERT^{-/-}$ rats (Calabrese et al., 2013).

Material and Methods

Animals

$SERT^{-/-}$ rats (Slc6a41Hubr) were generated by N-ethyl-N-nitrosourea (ENU)-induced mutagenesis on a Wistar background (Smits et al., 2006). $SERT^{-/-}$ rats were derived from crossing heterozygous 5-HT transporter knockout ($SERT^{+/-}$) rats that were outcrossed for at least 15 generations with wild-type $Wistar^{Crl:WI}$ rats obtained from Charles River Laboratories (Horst, the Netherlands). Ear punches were taken at the age of 21 days for genotyping, which was done by LGC (Hoddesdon, United Kingdom). $SERT^{+/+}$ rats were used to check BDNF virus overexpression in naïve animals. For the behavioral experiments, due to breeding difficulties, we didn't achieve the required number of $SERT^{+/+}$ rats from the nests. Therefore, we used $SERT^{-/-}$ rats and wild-type $Wistar^{Crl:WI}$ rats (WT rats) from Charles River (Horst, the Netherlands) as behavioral wild-type controls (see experimental design in figure 1). All animals were housed in temperature-controlled rooms (21 °C) with standard 12/12-h day/night-cycle (lights on at 7:00 am) and food and water available ad libitum. 5-7 days before surgery, animals were socially housed in individually ventilated (IVC) cages for habituation. After surgery, animals were separately housed in the IVC cages until the end of the sucrose preference test, thereafter the animals we socially housed again and kept under the same temperature and day/night-cycle throughout the entire experiment. All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

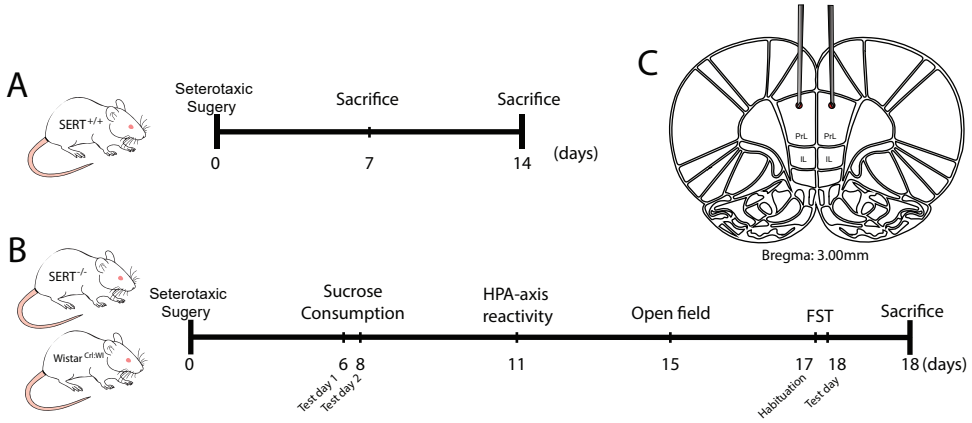

Figure 1. Schematic representation of the experimental design. A) Evaluation of BDNF overexpression in naïve SERT $^{+/+}$ rats one and two weeks following viral infusion. B) Behavioral tests: Viral infusion followed by behavioral tests including sucrose consumption test, HPA-axis reactivity, open field, and forced swim test (FST). C) Representation of the local o the infusion of either BDNF or control virus.

Stereotaxic Surgery

Rats were anesthetized using isoflurane (5% induction, 2-3% maintenance). Lidocaine (10% m/v) was used for local anesthesia. Animals were fixed in a robot stereotaxic frame (StereoDrive, Neurostar, Germany). The coordinates for the site of the injection were theoretically determined based on the Paxinos & Watson (2007) rat brain atlas and checked through histological evaluation of 30 µm brain slices from dye-infused SERT$^{+/+}$ rats. The total volume of 1µL of either BDNF lentivirus particles (transcript variant IV under CMV promoter, NM_001270633.1) or pLenti-C-mGFP control lentivirus particles, was bilaterally infused into the prelimbic cortex according to the following coordinates: AP +3.0 mm, ML ±0.6 mm, DV -3.0 mm. After surgery, animals were placed in IVC cages (Sealsafe Plus GR900 green line, Tecniplast, Italy) until sacrifice.

RNA Preparation And Gene Expression Analysis By Quantitative Real-Time PC

Total RNA was isolated from the prelimbic and infralimbic region of the mPFC by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories; Segrate, Italy), according to the manufacturer's instructions, and then quantified by spectrophotometric analysis (NanoDrop™1000, Thermo Scientific). Following total RNA extraction, an aliquot of each sample was treated with DNase to avoid DNA contamination. Then, the samples were processed for real-time PCR to assess total BDNF, BDNF isoform IV, and VI. The analyses were performed by TaqMan qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.) using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well formats in triplicates as

multiplexed reactions with a normalizing internal control (36B4). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription), and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reactions. Data were analyzed with the comparative threshold cycle (ΔΔCt) method using 36B4 as a reference gene. Primers and probe for BDNF exon IV and VI were purchased from Life technologies (BDNF exon IV: ID EF125679 and BDNF exon VI: ID EF125680). Primers and probe for total BDNF and 36B4 were purchased from Eurofins MWG-Operon. Their sequences are shown below:

- total BDNF: forward primer 5′-AAGTCTGCATTACATTCCTCGA-3′, reverse primer 5′-GTTTTCTGAAAGAGGGACAGTTTAT-3′, probe 5′-TGTGGTTTGTTGCCGTTGCCAAG-3′;
- 36B4: forward primer 5′-TTCCCACTGGCTGAAAAGGT-3′, reverse primer 5′-CGCAGCCGCAAATGC-3′, probe 5′-AAGGCCTTCCTGGCC GATCCATC-3′.

Behavioral tests:

Sucrose Consumption Test

After stereotaxic surgery, animals were housed individually and provided with two bottles of water for a 5 days habituation period in which side preference was checked. The sucrose consumption test was adapted from Olivier et al. (2008) and consisted of two days of free-choice access to 24 hours sucrose versus water bottles with a water-only bottle choice in between the two days. In detail, on test-day 1, one of the water bottles was replaced by sucrose 8% solution, and animals had free drinking access for 24 hours. Next, animals received water in both bottles for 24 hours, ending with another 24 hours of free choice between water and sucrose 8% solution on test day 2. The position of the bottles was switched from sucrose consumption test day 1 to the test day 2 to prevent spatial bias. Daily, liquid intake and bodyweight were measured. The data are presented as the preference of sucrose above water (sucrose intake in ml divided by total intake X 100%) and the intake in grams of a 100% sucrose solution per kg bodyweight (intake in ml corrected for the voluminal weight of sucrose and recalculated toward a 100% solution divided by bodyweight in kg).

HPA-axis reactivity Test

HPA-axis reactivity was assessed through the measurement of corticosterone levels in the plasma. Usually, when rodents are submitted to stress, plasma concentrations of corticosterone (CORT) peak after 15 to 30 minutes and gradually decrease 60 to 90 minutes later to the pre-stress levels (de Kloet et al., 2005). Therefore, blood samples from tail cuts were collected in capillary blood collection tubes (Microvette® CB 300 Di-Kalium-EDTA,

Sarstedt, Germany) 5 minutes before, and 15 and 60 minutes after 30 minutes of restraint stress. Rodent restrainers Broome-style were used for the restraint stress (554-BSRR, Bio-services, The Netherlands). Blood samples were centrifuged (3400 rpm for 15 min at 4 ◦C), and the plasma was stored at -80 ◦C until analysis. CORT levels were measured using a radioimmunoassay (RIA) kit according to the manufacturer protocol (ImmuChemTM Double Antibody Corticosterone 125I RIA, MP Biomedicals, USA).

Open field test

Novelty-induced locomotor activity was recorded by video recording in Phenotyper® cages (Noldus Information Technology, Wageningen, The Netherlands). The cages (45 cm × 45 cm × 45 cm) were made of transparent Perspex walls and a black floor. Each cage had a top unit containing a built-in digital infrared-sensitive video camera, infrared lighting sources, and hardware needed for video recording. To explore the novelty factor, animals were not exposed to this cage previously and the cages were cleaned with 70% alcohol solution between trials to prevent transmission of olfactory cues. Spontaneous locomotor activity was monitored for 1 hour, and the following parameters were scored using Ethovision XT 11.5 (Noldus Information Technology, Wageningen, Netherlands): distance moved, velocity, frequency and time spent in the center of the cage (Manfré et al., 2017; Schipper et al., 2011a).

Forced Swim test

The forced swimming test was performed as previously described (Porsolt et al., 1978). Briefly, rats were individually placed in cylindrical glass tanks (50 cm height, 20 cm diameter) filled to a height of 30 cm with 23±1°C water. The test consisted of two sessions. In the first session, animals were submitted to a habituation period of 15 minutes, then 24 hours later, to a second session of 5 min. The video recordings of the second session were used to automatically score the movements of the rats through a computerized system (Ethovision XT 10, Noldus, The Netherlands). Scored behaviors were 'immobility', which reflects no movement at all and/or minor movements necessary to keep the nose above the water; 'mobility', indicating movement that corresponds to swimming activity; and 'strong mobility', reflecting 'escape behavior' (e.g., climbing against the walls and diving). Settings within Ethovision were adjusted based on manually recorded sessions (immobility/mobility threshold: 12; mobility/strong mobility threshold: 16.5 (Boulle et al., 2016; Van den Hove et al., 2013).

Statistical Analysis

The data were checked for outliers and normality (using the Shapiro–Wilk statistic), and extreme outliers were windsorized. Two-way analysis of variance (ANOVA) was computed for gene expression analysis, with time, genotype, and treatment as independent factors. The outcomes of SPT, Novelty-induced locomotor activity, FST, and post-behavioral gene expression were also analyzed through Two-way ANOVA considering genotype and treatment as fixed factors. Post-hoc Fisher Protected Least Significant Difference (PLSD) or independent sample t-tests were performed where applicable to compare individual group differences. All these statistical analyses were carried out using IBM® SPSS® statistics, version 23 (IBM software, USA). Regarding the HPA-axis reactivity test, a linear mixed model was implemented to account for repeated measurements, and multiple factor analysis using the LME4 package in R (3.5.1). Time, genotype, and treatment effects were modeled as a fixed effect, together with their pairwise double interactions, and their triple interactions. Subject intercepts were modeled as random effects. A likelihood-test ratio was used to assess fixed effect significance. *Post-hoc* tests were performed with the multicomp package, which accounts for multiple hypothesis testing. Significance was accepted at a $p<0.05$ threshold. Descriptive statistics are provided as mean +/- 1 standard error of the mean (SEM).

Results

Upregulation of Total BDNF mRNA in naïve SERT$^{+/+}$ rats following prelimbic BDNF lentivirus infusion

Feasibility of BDNF expression and its temporal dynamics was separately examined in a group of naïve SERT$^{+/+}$ rats. mRNA levels were evaluated one and two weeks following BDNF or GFP lentivirus infusion in the prelimbic (PrL) cortex of naïve SERT$^{+/+}$ rats. RT-qPCR was performed to measure total BDNF mRNA overexpression in the prelimbic (PrL) and in in the neighboring mPFC area infralimbic (IL). Two-way ANOVA revealed a significant main effect for treatment in the PrL ($F_{(1, 18)} = 13.790$, $p = 0.002$). PLSD *post-hoc* analysis revealed BDNF overexpression in the site of the injection (PrL) with significant total BDNF mRNA increased in the BDNF treated animals compared to the control GFP treated rats both one week ($p = 0.038$) and two weeks ($p = 0.002$) after surgery. Interestingly, while in the PrL, no time point differences were identified among control GFP treated rats, the IL samples presented a sharp rise (72.47 %, SD = 26.41) in BDNF levels in control-treated animals ($p = 0.01$). As shown in figure 2, this increase led to significantly higher BDNF levels in the IL than in the PL ($p < 0.001$). We concluded that BDNF overexpression was stable for at least 14 days, specifically in the PrL.

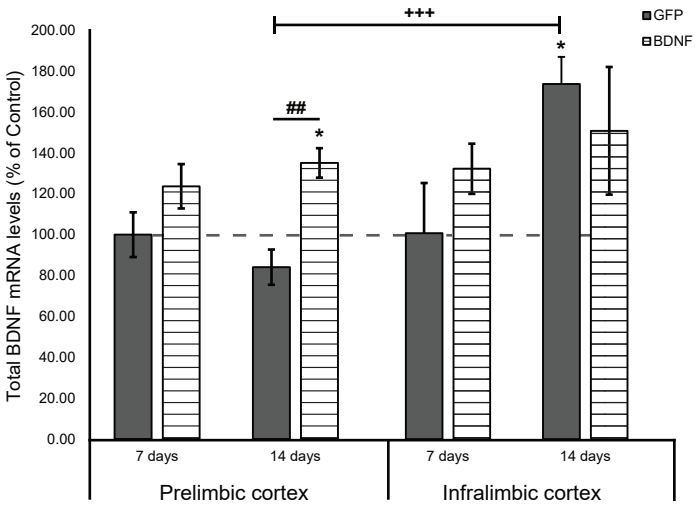

Figure 2. Modulation of total BDNF expression in SERT $^{+/+}$ animals infused with either GFP control or BDNF viral particles at 1 week and 2 weeks after stereotaxic surgery. Total BDNF mRNA levels were measured in the prelimbic cortex and infralimbic cortex. Data are expressed as fold change compared to the GFP-treated animals (set at 100%), and reflect mean ±SEM from 4-6 independent determinations. * = $p < 0.05$ *vs* GFP-7 days; ## = $p < 0.01$ *vs* GFP-14 days; +++ = $p < 0.001$ IL *vs* PrL (GFP-14 days).

Sucrose Consumption Test (SCT)

Anhedonia is marked by a reduced interest in pleasurable events, and it is present in depression. This depression-like symptom can be identified in rodents through a decrease in sucrose consumption. Animals were exposed to two days of free access to sucrose 8% solution. The results of the sucrose intake in grams and the preference for sucrose above the water are described below.

Sucrose Preference in SERT$^{-/-}$ rats is altered by BDNF overexpression

On the first day of testing, no significant main effects were observed for sucrose preference. Pairwise comparisons, however, revealed that sucrose preference was significantly reduced in the SERT$^{-/-}$ rats treated with BDNF lentivirus compared to untreated SERT$^{-/-}$ ($p = 0.05$) and control WT rats ($p = 0.018$). At the second day of testing, two-way ANOVA analysis showed a genotype as well as a genotype versus treatment interaction ($F_{(1, 40)} = 4.738$, $p = 0.035$ and $F_{(1, 40)} = 5.058$, $p = 0.030$, respectively). As seen in figure 3, the *post-hoc* analysis further demonstrated that SERT$^{-/-}$ rats treated with BDNF presented a higher preference for sucrose than control-treated SERT$^{-/-}$ animals ($p = 0.017$). Additionally, control-treated SERT$^{-/-}$ animals displayed lower sucrose preference than the control WT rats ($p = 0.002$). Therefore, in

summary, BDNF treatment in SERT$^{-/-}$ rats improved the preference for sucrose in the second day of the test.

SERT$^{-/-}$ genotype rather than BDNF overexpression modulates the rat's response to sucrose intake

As for sucrose preference, two-way ANOVA did not reveal a significant main effect for sucrose intake in SERT$^{-/-}$ versus WT rats on the first day of the test. There were also no statistically significant differences in the amount of sucrose consumed among the groups (Figure 3). On the second day of testing, however, a main genotype effect was found for sucrose consumption ($F_{(1, 42)} = 16.789$, $p < 0.001$). Moreover, *post-hoc* analysis showed that SERT$^{-/-}$ rats consumed significantly less sucrose than controls ($p < 0.05$) with no treatment differences. We concluded that as previously described (Olivier et al., 2008), the SERT$^{-/-}$ phenotype led to reduced sucrose consumption that was not rescued by the PrL BDNF transfection.

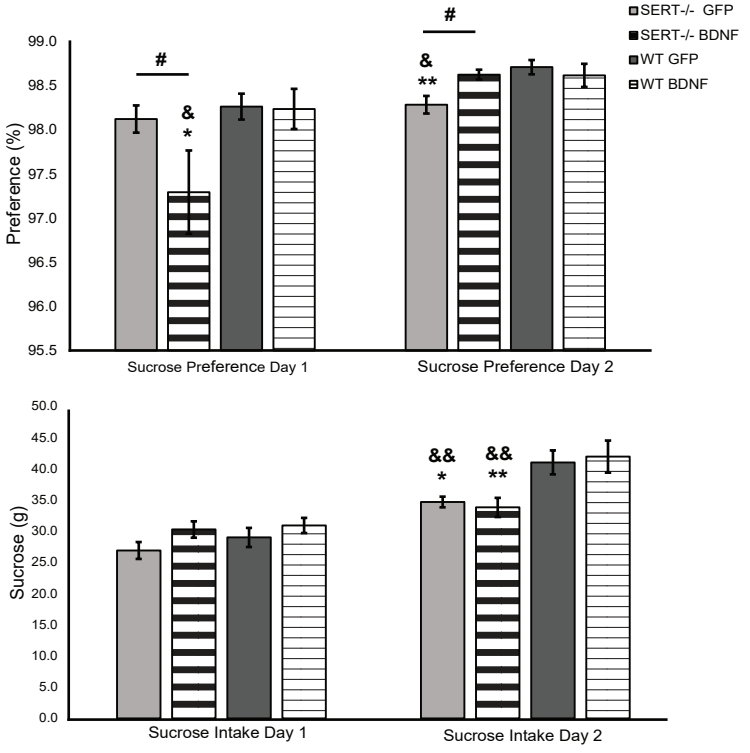

Figure 3. Sucrose consumption of 8% sucrose solution by SERT$^{-/-}$ and WT rats. Data are expressed as mean S.E.M. sucrose preference (sucrose intake/total fluid intake x 100%), and as mean S.E.M. total sucrose intake (g) per body weight (n = 10-12; * = $p < 0.05$ and ** = $p < 0.01$ vs WT-GFP; & = $p < 0.05$ and && = $p < 0.01$ vs WT-BDNF; # = $p < 0.05$ vs SERT$^{-/-}$GFP).

BDNF overexpression did not modify SERT$^{-/-}$ rats anxiety-like pattern in the Forced Swim Test

When rodents are exposed to an inescapable stressor such as in the forced swim test, their motivation to cope with stress can be quantified by the percentage of time spent on immobility (behavioral passivity) or performing a highly mobile (escape-like) behavior (Porsolt et al. 1977). We found a main genotype effect for immobility ($F_{(1,42)}$ = 7.827, p = 0.008). Unexpectedly, as figure 4 shows, immobility was decreased in both control- and BDNF-treated SERT $^{-/-}$ rats compared to WT rats (p = 0.017 and p = 0.027, respectively). Additionally, two-way ANOVA revealed a genotype main effect for high mobility ($F_{(1, 42)}$ = 8.278, p = 0.006). Particularly, *post-hoc* examination demonstrated that the time spent on high mobility swimming or escape behavior was significantly higher in SERT$^{-/-}$ rats than in WT controls (*vs.* SERT$^{-/-}$ GFP p = 0.006, *vs.* SERT$^{-/-}$ BDNF p = 0.021). In summary, surprisingly, SERT$^{-/-}$ rats presented decreased immobility and increased escape behavior compared to WT animals.

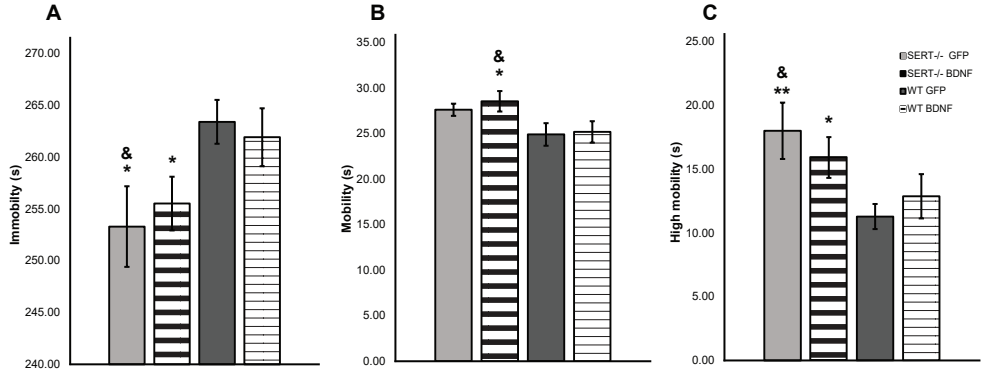

Figure 4. Mean (±SEM) measure of (A) immobility, (B) mobility, and (C) high mobility in the forced swim test. n = 10–12 rats per group. * p < 0.05 and ** p < 0.01 vs WT GFP, & p <0.001 vs WT BDNF). Two-way ANOVA, Fisher LSD post-hoc test.

Novelty-induced locomotor activity is impaired in SERT$^{-/-}$ rats

Rodents may present higher activity when they are introduced to a novel environment (Menzaghi et al., 1994). A decrease in central locomotion (frequency and time spent in the central part of the arena), together with a general decrease in the locomotion (distance moved and velocity) can be interpreted as an anxiogenic-like behavior (Prut and Belzung, 2003). In our experimental conditions, genotype played a role in the locomotor activity affecting distance moved ($F_{(1, 42)}$ = 40.708, p < 0.001), velocity ($F_{(1, 42)}$ = 41.559, p < 0.001), time spent in the center of the arena, as well as the frequency to which the animals accessed the center ($F_{(1, 42)}$ = 11.621, p = 0.001) and $F_{(1, 42)}$ = 6.743, p < 0.013, respectively). No

treatment effect was found for SERT$^{-/-}$ or WT rats exposed to the new environment. Further *post-hoc* investigation revealed that SERT$^{-/-}$ rats, independently of treatment, displayed a significant decrease in distance moved and velocity ($p < 0.0001$ for all SERT $^{-/-}$ *vs* WT rats comparisons); additionally, as it can be seen in figure 5, frequency and duration spent in the center of the arena were reduced in SERT$^{-/-}$ BNDF-treated rats (frequency: *vs* WT GFP $p = 0.04$; duration: *vs* WT GFP $p = 0.01$) and in SERT$^{-/-}$ GFP treated rats (frequency: *vs* WT GFP $p = 0.005$; duration: *vs* WT GFP $p < 0.001$, *vs* WT BDNF $p = 0.039$). In brief, exposition to a novel environment caused an anxiety-like response in SERT$^{-/-}$ rats as expressed by decreased mobility and decreased access to the center.

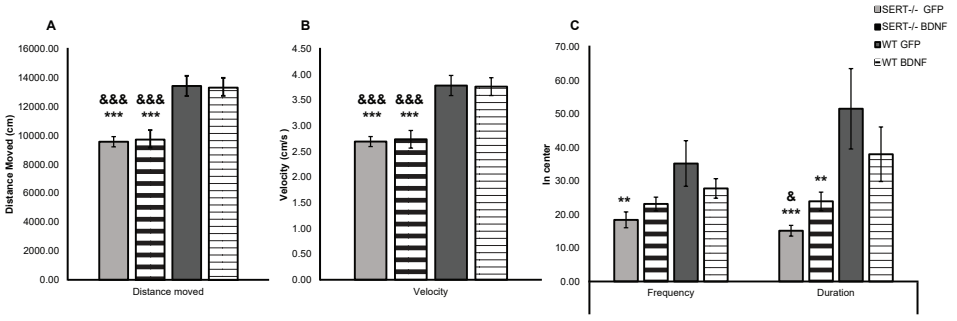

Figure 5. Novelty-induced locomotor activity expressed as mean (±SEM) measure of (A) distance moved, (B) velocity and (C) frequency and time spent in center. n = 10-12; * = p < 0.05, ** = p < 0.01, *** = p < 0.001 vs WT GFP; & = p < 0.05 and &&& = p < 0.001 vs WT BDNF.

BDNF overexpression exclusively alters HPA-axis reactivity in WT animals.

Acute restraint stress can activate the hypothalamic-pituitary-adrenal axis (HPA-axis), resulting in the release of CORT in rodents, which can be a measure of the (mal)functionality of the HPA-axis. The stress-induced increase in CORT levels relative to baseline was evaluated 15, 30, and 60 minutes after stress. A linear mixed-effect analysis indicated a triple interaction effect between treatment, genotype, and time ($F_{(15,18)} = 15.84$, $p = 0.001$). A post-hoc analysis comparing groups pairwise per time point indicated a strong difference between BDNF vs. GFP transfected WT rats at the 15 min time point ($p < 0.001$) and between WT and SERT$^{-/-}$ rats in the GFP condition ($p < 0.03$). This difference indicates that BDNF overexpression in the PrL has the potential to reduce the HPA reactivity during the first phase of the response, although only in the WT group. Interestingly, CORT baseline levels were increased in BDNF-treated WT animals (t-test *vs.* WT GFP $p = 0.003$). At the 30 min time point, a difference between BDNF-transfected WT and SERT$^{-/-}$ rats was also found ($p < 0.001$), indicative of an elevated CORT level in SERT rats relative to WT control. All but the WT BDNF group after 60 min still displayed CORT levels significantly elevated from baseline, indicative

of a strong activation of the HPA-axis, with a trend toward return near baseline after 60 min. In summary, BDNF overexpression appeared to increase basal CORT levels and decrease the HPA reactivity in the WT group, whereas the SERT$^{-/-}$ rats were unaffected.

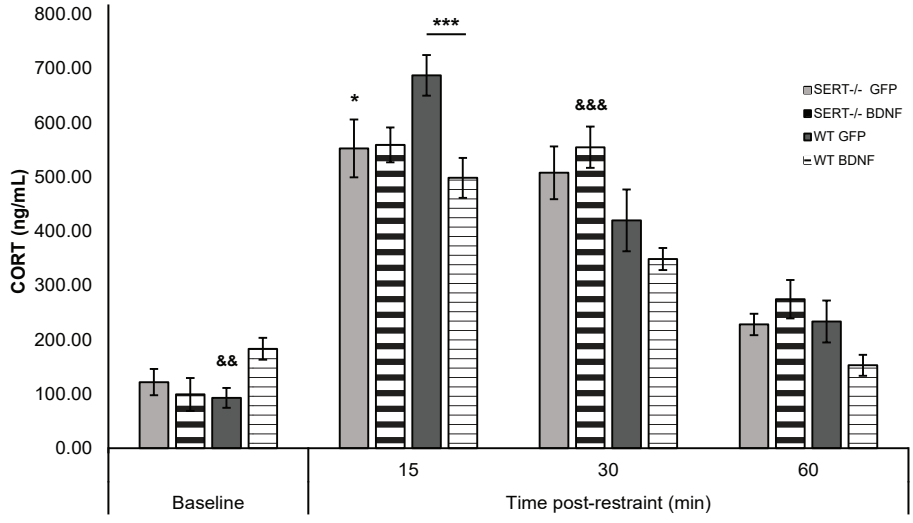

Figure 6. HPA-axis reactivity assessment. Corticosterone (CORT) levels are expressed mean ±1 standard error of the mean (SEM) of measurements from plasma samples obtained 5 minutes before restraint stress (baseline), and 15, 30, and 60 minutes post-restraint stress. n = 10-12; * = $p < 0.05$, *** = $p < 0.001$ vs WT GFP; && = $p < 0.01$ and &&& = $p < 0.001$ vs WT BDNF. Linear mixed model.

BDNF overexpression was stable in WT animals after behavioral challenge

To assess the BDNF expression induced by transfection in the behaviorally tested animals, rats were sacrificed 90 minutes after the forced swim test. Overexpression of total, exon IV, and exon VI BDNF transcripts in the PrL and IL were evaluated through RT-qPCR. No main effects were found for total BDNF or BDNF exons IV and VI in the PrL. Pairwise comparisons were computed and, as it is demonstrated in figure 7, they revealed that WT rats treated with BDNF presented higher BDNF levels than GFP-treated SERT$^{-/-}$ rats (total BDNF: $p = 0.04$, BDNF IV: $p = 0.039$, and BDNF VI: $p = 0.05$), as well as higher BDNF levels than BDNF-treated SERT$^{-/-}$ rats (BDNF IV: $p = 0.016$, and BDNF VI: $p = 0.015$).

In the IL, on the other hand, BDNF expression was decreased in both SERT$^{-/-}$ groups compared with both WT groups. As a result, a significant genotype main effect was observed for total BDNF ($F_{(1, 17)} = 22.365$, $p < 0.001$), BDNF IV ($F_{(1, 18)} = 9.707$, $p = 0.006$), and BDNF VI ($F_{(1, 17)} = 13.397$, $p = 0.002$). Post-hoc testing demonstrated no treatment differences, but did reveal a significant BDNF downregulation of total, exon IV, and exon VI BNDF transcript levels in SERT$^{-/-}$ rats compared to WT rat controls ($p < 0.05$). In conclusion, we observed that BDNF

overexpression in the PrL of WT animals remained even after the behavioral tests; moreover, in SERT$^{-/-}$ rats no changes were found in BDNF expression levels in the PrL area compared to WT rats, but we did find a decrease in BDNF expression in the IL.

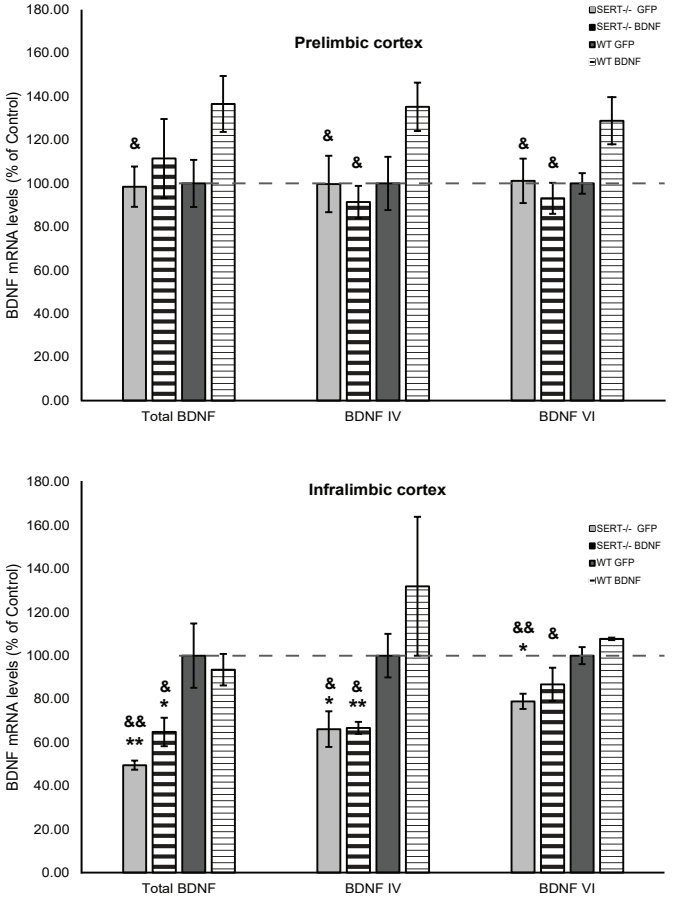

Figure 7. BDNF expression of total, exon IV, and exon VI transcripts in SERT +/+ and SERT KO animals submitted to local infusion of either GFP control or BDNF viral particles followed by sequential behavioral challenges. Total BDNF, BDNF IV, and BDNF VI mRNA levels were measured in the prelimbic cortex and infralimbic cortex. Data are expressed as percentage change compared to the WT GFP-treated animals (set at 100%), and reflect mean ±SEM from 4-6 independent determinations. * = $p < 0.05$ and ** = $p < 0.01$ vs WT GFP; & = $p < 0.05$ and && = $p < 0.01$ vs WT BDNF.

Discussion

In this study, we have demonstrated that BDNF IV lentiviral infusion into the PrL induced overexpression of total BDNF in naïve SERT$^{+/+}$ animals in a time- and brain region-dependent manner. Notably, we confirmed increased mRNA BDNF levels in the PrL of BDNF lentivirus treated SERT$^{+/+}$ rats two weeks after the surgery, as well as an unexpected BDNF upregulation

in the IL of control-treated animals. Moreover, BDNF overexpression caused different phenotypical outcomes depending on the behavioral task and the animal genotype. In WT animals, BDNF upregulation caused an alteration in the HPA-axis function following acute restraint stress, and in SERT$^{-/-}$ rats, it resulted in improvement of the anhedonia-like symptoms in the sucrose preference test. Whereas the stress coping behavior as measured in the forced swim test and locomotor activity were not affected by BDNF overexpression, rather, the SERT$^{-/-}$ genotype played a role in inducing anxiety-like phenotypes in these tests.

During the sucrose consumption test, SERT$^{-/-}$ and WT rats were submitted to two sessions of 24 hours exposition to an 8% sucrose solution. In consequence, we observed that SERT$^{-/-}$ treated with control virus did not differ in sucrose preference from WT rats on the first day of testing, but presented decreased preference in the second day. This result is in line with our previous demonstration that preference for sucrose is negatively affected in SERT$^{-/-}$ animals (Olivier et al., 2008). The differences between the first and second day of sucrose preference seen in SERT$^{-/-}$ rats have been demonstrated before in studies using a mouse animal model presenting selective disruption of BDNF IV (BDNF-KIV mice) in which BDNF-KIV mice only exhibited significantly decreased sucrose preference in the second day of sucrose test (Sakata et al., 2010). Accordingly, SERT$^{-/-}$ rats treated with BDNF IV lentivirus presented reduced sucrose preference in the first day, but increased preference in the second day when compared to control-treated SERT$^{-/-}$ and WT rats. Therefore, it is likely that BDNF IV overexpression in SERT$^{-/-}$ rats led to neophobia to a novel taste or an anxiety-like behavior upon the first exposure; while during the second exposure, when the animals were familiar with the new taste, the anhedonia-like behavior in SERT$^{-/-}$ animals was suppressed by the BDNF overexpression. Conclusively, possibly, overexpression of BDNF transcript IV in the PrL was responsible for rescuing the anhedonia-like behavior displayed by SERT$^{-/-}$ rats.

Concerning the forced swim test, no BDNF treatment differences were found, but we did notice apparent genotype behavioral differences. In contrast to previous studies employing SERT$^{-/-}$ rats and mice, SERT$^{-/-}$ rats spent less, rather than more, time on immobility than WT animals (Lira et al., 2003; Olivier et al., 2008). In agreement with our current results, studies in other animal models presenting alterations in the BDNF system have also shown that subjects with decreased BDNF levels did not display higher immobility in the forced swim test compared to controls (Duman and Monteggia, 2006; Sakata et al., 2010). In our experimental conditions, decreased immobility can be justified by the fact that SERT$^{-/-}$ rats spent proportionally more time on vigorously strong swimming (high mobility) than immobility compared to the WT animals. Several lines of research have hypothesized that this escape behavior essentially reveals an increased level of anxiety (Anyan and Amir, 2018). As mentioned before, SERT$^{-/-}$ animals have impaired BDNF expression as well as reduced expression of its transcription factors such as Npas4. Both BDNF and Npas 4, are implicated

in the establishment of the GABAergic system. The GABAergic system communicates with other neurotransmitter networks, and its downregulation can lead to an anxiety-like behavioral outcome (Lydiard, 2003; Millan, 2003). Correspondingly, $SERT^{-/-}$ rats present changes in the functioning of the GABAergic system (Calabrese et al., 2013; Luoni et al., 2013; Miceli et al., 2017; Schipper et al., 2019). Therefore, likely, reduced levels of GABA in these animals could contribute to an anxiety-like response upon behavioral challenges such as the forced swim test.

In the novelty-induced locomotor activity test, the $SERT^{-/-}$ rats presented decreased locomotor activity in a novel environment compared to the WT animals. This decrease indicates that the reduced immobility and increased high mobility observed in $SERT^{-/-}$ rats in the forced swim test was not due to increased locomotor activity. Moreover, similarly to the forced swim test, the decreased locomotor activity seen in the $SERT^{-/-}$ rats contradicts previous studies using $SERT^{-/-}$ rats, which report no genotype differences between naïve $SERT^{-/-}$ and $SERT^{+/+}$ rats in the novelty-induced locomotor activity test (Homberg et al. 2008; Schipper et al. 2011). However, in contrast to the findings in rats, studies in $SERT^{-/-}$ mice have shown that in an unfamiliar environment, $SERT^{-/-}$ mice displayed reduced locomotor activity and anxiety-like behavior similar to our results (Alexandre, 2006; Kalueff et al., 2007). Hence, the reduced distance moved, lower velocity, and especially the decreased time and frequency in the center of the arena might be a reflection of the overall increased anxiety-like behavior displayed by $SERT^{-/-}$ rats which have also been described in $SERT^{-/-}$ mice (Olivier et al., 2008).

We further evaluated the HPA-axis activity to assess the effects of BDNF overexpression in $SERT^{-/-}$ and WT rats. Our results revealed that basal levels of CORT were not altered in $SERT^{-/-}$ rats compared to control-treated WT rats. Conversely, we found that BDNF upregulation in WT rats generated increased basal levels of CORT in this group when compared to control WT animals. Moreover, after acute restraint stress, WT rats treated with BDNF presented similar HPA-axis response to the $SERT^{-/-}$ animals, namely a decreased elevation in CORT levels compared to control-treated WT rats. Therefore, these results show that BDNF upregulation altered the HPA-axis function in WT rats with no effects in $SERT^{-/-}$ animals. Concerning the HPA-axis disturbances seen in WT rats overexpressing BDNF, likely basal HPA-axis hyperactivity and decreased response to stress might have been facilitated by a discrepancy between the rate of mature BDNF (mBDNF) protein to its precursor proBDNF, favoring the later one. This imbalance in the proBDNF/mBDNF was confirmed before in a study showing that BDNF overexpression can lead to an increase in the release of uncleaved proBDNF (Leßmann and Brigadski, 2009). While mBDNF supports plasticity through its high affinity for the TrkB receptor, proBDNF has an affinity for the p75NTR receptor, which mediates apoptotic signaling leading to neuronal death (Woo et al. 2005; Leßmann and

Brigadski 2009). Although both HPA hyperactivity and increased proBDNF are present in depressive disorders (Bai et al. 2016; Zhou et al. 2013; Arborelius et al. 1999; Nestler et al. 2002; Pariante and Lightman 2008; van Bodegom, Homberg, and Henckens 2017), the direct proof that the deleterious effects of proBDNF are the underlying cause to HPA-axis malfunction is still lacking. Yet another possibility we tend to support to explain the disruption in the HPA-axis reactivity in WT rats overexpressing BDNF is that the gene upregulation led to exceeding levels of the mature form of the BDNF protein. Accordingly, the exceeding mBDNF in the PrL would preferentially inhibit CORT production at the amygdala level, shortening the HPA-axis response loop, resulting in decreased CORT release upon stress in comparison to control animals. The PFC, which is one of the key regions in the control of the HPA-axis, presents inhibitory connections with the amygdala and PVN (van Bodegom et al., 2017), and it has been demonstrated that when BDNF is overexpressed in the PFC, it can undergo anterograde transport and cause BDNF overexpression in the amygdala (McGinty et al., 2010). BDNF, especially exon IV, is known for its critical role in GABAergic transmission (Sakata et al., 2009). Brivio et al. (2020) showed that acute restraint stress increased PFC levels of total and BDNF exon IV in Sprague Dawley rats specially 1 hour following the acute stress. Total BDNF levels were also increased in the PFC following acute swim stress (Brivio et al., 2019). Therefore, acute restraint stress may have caused activity-dependent upregulation of BDNF (Leßmann and Brigadski, 2009), leading to enhancement in the GABAergic inhibitory control in the amygdala, and consequently, increased negative feedback to the HPA-axis system (Barry et al., 2017; Liu et al., 2014; Zhang et al., 2018).

In conformity, because BDNF IV, the transcript chosen to be upregulated in the present study, is activity-dependently released (Leßmann and Brigadski, 2009), we also evaluated the BDNF mRNA levels in the PrL and IL of the animals submitted to behavioral testing. Considering the intricate control over the BDNF gene, in which different isoforms are generated by distinct promoters (Aid et al., 2007), and giving that different stimuli can influence the isoforms response and cellular location, we focused not only on total mRNA but also BDNF exon IV and VI transcripts. These transcripts are involved in depressive- or anxiety-like behavior and are known to be downregulated in SERT$^{-/-}$ rats (Molteni et al., 2010; Sakata et al., 2010). As a result, in the PrL, we did observe that WT rats receiving BDNF lentivirus presented a higher BDNF expression than the WT control group and SERT$^{-/-}$ rats. However, when compared to the control WT group, this overexpression was not statistically significant. Furthermore, we did not observe any differences in the level of all analyzed BDNF transcripts when comparing both SERT$^{-/-}$ groups to control WT rats. This finding indicates that BDNF overexpression did not change levels of BDNF in SERT$^{-/-}$ rats compared to both control-treated SERT$^{-/-}$ and WT rats. However, taking into consideration that the gene expression analysis was conducted after the behavioral testing, possibly the viral transfection in the PrL

was not stable after exposing the animals to behavior challenges. Additionally, we noticed that when comparing control-treated SERT$^{-/-}$ and WT no differences in BDNF levels were found in the PrL. Conversely, in the IL, a remarkable downregulation in total BDNF, BDNF IV and BDNF VI was seen in the SERT$^{-/-}$ animals in comparison to WT controls. The observation that mRNA BDNF levels were unchanged in SERT$^{-/-}$ even after viral upregulation may help to understand the mechanisms behind the outcomes seen in the behavioral tests. For instance, we have demonstrated that SERT$^{-/-}$ rats displayed anxiety-like behavior, presenting higher activity in the forced swim test, less activity in the novelty-induced locomotor activity, and altered HPA-axis response upon restraint acute stress. A possible explanation for the overall anxiety-like behavior in SERT$^{-/-}$ rats may be based on the complex control the BDNF gene can undergo. For example, previous research showed that transcription factors that regulate the BNDF transcription, such as CREB, Arnt2, CaRF, NFkB, and Npas4, are significantly downregulated in SERT KO rats. Changes in Npas4 are directly correlated with decreased BDNF exons I and IV, bringing about the hypothesis that behavioral outcomes related to SERT knockout and BDNF downregulation in the SERT$^{-/-}$ rats might be, at least in part, also attributed to Npas4 downregulation (Guidotti et al., 2012). Therefore, it is appropriate to infer that overexpression of exogenous BDNF IV transcript in the PrL was affected by the downregulation of endogenous transcription factors in the SERT$^{-/-}$ rats. Thus, despite gene overexpression, BDNF protein levels may not have changed in the SERT$^{-/-}$ rats, explaining the lack of behavioral changes upon BDNF overexpression in these animals and its general anxiety-like behavior. On the other hand, this hypothesis does not justify the results observed in the sucrose preference test, where we revealed melioration in the anhedonia-like symptoms of SERT$^{-/-}$ rats treated with BDNF lentivirus. Therefore, because in the present study we did not investigate the levels of BDNF protein in the PrL, possibly other molecular mechanisms might be involved in the BDNF gene regulation concerning the anhedonia-like behavior.

In conclusion, we have shown that BDNF overexpression in the PrL, in general, did not rescue SERT$^{-/-}$ from its depression- and anxiety-like behavior, as demonstrated by the decreased sucrose intake, reduced locomotor activity, and increased high mobility in the forced swim test compared to controls. However, in the sucrose preference test, SERT$^{-/-}$ rats treated with BDNF IV lentivirus presented a higher preference for sucrose (that is a reduction in anhedonia-like behavior) than control SERT$^{-/-}$ animals. Furthermore, BDNF upregulation in WT rats specifically promoted alteration in the HPA-axis activity of WT rats, resulting in increased basal levels of CORT and making these rats respond similarly to the SERT$^{-/-}$ rats upon restraint stress.

This study, however, presented several limitations. For example, the overexpression of an individual transcript variant, namely BDNF IV, in the PrL may have resulted in activation

and reinforcement of particular neural networks. These networks could mainly cause HPA-axis alterations in WT animals and reduction of anhedonia-like behavior in SERT$^{-/-}$ rats, without affecting other relevant neuronal circuits involved in the behavioral challenges the animals were submitted. Moreover, although our SERT$^{+/+}$ and SERT$^{-/-}$ have a Wistar background, the outcrossing may not eliminate all additional induced mutations. Therefore, the use of commercial wild type Wistar rats as controls may pose a disadvantage when relating to our previous findings. Nevertheless, the stability of the SERT$^{-/-}$ phenotype across studies, generations, and laboratories have shown that this animal model is a useful tool for studying the effects of life-time increased extracellular serotonin and downregulated BDNF levels (Homberg et al., 2014; Olivier et al., 2010).

Despite the progress in the understanding of the biological effects of BDNF, important aspects of the BDNF gene regulation, as well as the spatiotemporal release and the precise sites of the BDNF action are still poorly understood, especially in the prefrontal cortex (Sakata et al., 2009). Different stimuli seem to differently regulate the transcription of BDNF in specific brain areas adding complexity to the study of the mechanisms behind the effects of BDNF (Adachi, 2014; Baj et al., 2011; Govindarajan et al., 2006; Maynard et al., 2016). The neurotrophic hypothesis of depression was developed based on the observation that stress, anxiety, and depression are accompanied by decreased levels of BDNF, and that several treatments used for such disorders also increase BDNF levels (Duman and Monteggia, 2006). Since vulnerability to depression can be attributed to poor neuronal plasticity (McClung and Nestler, 2008) and underlying neurobiological processes might be associated with BDNF levels, it is likely that changes in BDNF may contribute to an improvement of behavioral symptoms in depressive individuals. Furthermore, taking into account that some of the current first-line treatments for depression targeting the serotoninergic system have failed to work consistently in all patients (Cuijpers, 2017), BDNF is placed as an important candidate for therapeutic modulation in mood disorders in humans. From this perspective, since our study has shown that therapeutic approaches aiming BDNF overexpression may need to be specific to promote symptoms attenuation, it is essential to elucidate further the relevance of the BDNF downregulation found in the PFC or other brain areas of SERT$^{-/-}$ rats as regarding to the neuropathology of depression.

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3

BDNF overexpression in the ventral hippocampus promotes antidepressant- and anxiolytic-like activity in serotonin transporter knockout rats

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Abstract

Brain-derived neurotrophic factor is one of the most studied proteins playing a pivotal role in neuroplasticity events and vulnerability and resilience to stress-related disorders. Most importantly, BDNF is decreased in depressive patients, and increased after antidepressant treatment. Additionally, BDNF was found to be reduced in a genetic subset of depression susceptible patients carrying the human polymorphism in the serotonin transporter promoter region (5-HTTLPR). The serotonin knockout rat ($SERT^{-/-}$) is one of the animal models used to investigate the underlying molecular mechanisms behind the genetic susceptibility to depression in humans. $SERT^{-/-}$ rats present decreased BDNF levels, especially BDNF exon IV, in the prefrontal cortex (PFC) and ventral hippocampus (vHIP), and display anxiety- and depression-like behavior. To investigate whether upregulating BDNF in the vHIP would meliorate the phenotype of $SERT^{-/-}$ rats, we overexpressed BDNF locally into the rat brain by means of stereotaxic surgery and submitted the animals to behavioral challenges, including the sucrose consumption, the open field, and forced swim tests. Additionally, we measured hypothalamus-pituitary-adrenal (HPA)-axis reactivity. The results showed that lentivirus-induced BDNF IV overexpression in the vHIP of $SERT^{-/-}$ rats promoted higher sucrose preference and sucrose intake, on the first day of the sucrose consumption test, indicative for decreased anhedonia-like behavior. Moreover, it decreased immobility time in the forced swim test, suggesting adaptive passive coping. Additionally, BDNF upregulation increased the time spent in the center of a novel environment, implying decreased novel-induced anxiety-like behavior. Finally, it promoted a stronger decrease in plasma corticosterone levels 60 minutes after restraint stress. In conclusion, modulation of BDNF IV levels in the vHIP of $SERT^{-/-}$ rats led to a positive behavioral outcome placing BDNF upregulation in the vHIP as a potential candidate for the development new therapeutic approaches targeting the improvement of depressive symptoms.

Keywords: BDNF, Serotonin knockout rats, Hippocampus, Depression, Anxiety.

Introduction

An essential part of our daily performance has to do with our capability to adjust and respond to unforeseen events. Adaptation to unexpected environmental changes relies on our ability to develop resilience and display behavioral flexibility. This flexibility is a result of several mechanisms occurring in the brain that are usually called plasticity. Brain plasticity or neuroplasticity can be defined as the adjustments the nervous system undergoes in response to diverse stimuli to achieve reorganization at the structural, functional, or cellular connectivity level (Mateos-Aparicio and Rodríguez-Moreno, 2019). Considering this, it is expected that alterations in the brain's ability to develop neuronal plasticity can lead to maladaptation and, consequently, to the development of neuropsychiatric disorders (Duman et al., 2000). Many molecular networks might be involved in neuroplasticity, including the serotonin and the brain-derived neurotrophic factor (BDNF) systems. Serotonin is a brain-wide distributed monoamine, and its decrease has been connected to the occurrence of depression (Coppen, 1967). The levels of serotonin in the synaptic cleft are regulated by the serotonin reuptake transporter (SERT), and to restore serotonin levels in depressive subjects, drugs targeting SERT, known as selective serotonin-reuptake inhibitors (SSRI), are considered the first-line treatment for depressive disorders (Alenina and Klempin, 2015; Berger et al., 2009). While SSRIs very quickly generate a blockade of SERT in vitro, the actual clinic therapeutic effect may take several weeks (Deltheil et al., 2008). This delay suggests that the molecular mechanisms underlying depression might be more complex, requiring perhaps secondary changes in structural, functional, or cellular connectivity; thus, requiring neuronal plasticity (Duman et al., 2000, 1999).

BDNF, a neuropeptide from the neurotrophin family, plays a central role in neuroplasticity (Edelmann et al., 2014). Several studies confirmed that BDNF contributes to the brain plasticity through its positive influence in neurogenesis, cell survival, synapse formation and plasticity (Edelmann et al., 2014; Mattson et al., 2004; Park and Poo, 2013). BDNF gained attention in the study of mood disorders because data from post-mortem brain tissues or serum of depressive patients indicated low levels of BDNF, which were normalized following antidepressant treatment (Adachi, 2014; Sen et al., 2008). Given that the antidepressant therapeutic action occurs in a delay of weeks, and due to BDNF's pivotal role in neuroplasticity, it has been associated with the mechanism of action of antidepressants (Duclot and Kabbaj, 2015; Duman and Monteggia, 2006; Monteggia et al., 2004). Therefore, the relation between the serotonin system and the BDNF system in connection with the antidepressant therapeutic effect seems to overlap.

The human SERT, encoded by the SLC6A4 gene, presents a functional polymorphism in the promoter region (5-HTTLPR) generating a short and a long allele variant of the serotonin transporter (Murphy et al., 2008). The short allelic variant induces a decrease in

the SERT transcription in comparison to the long allelic variant (Lesch et al., 1996), and it is linked to increased risk for depression and suicidal behavior (Bleys et al., 2018; Caspi et al., 2003; Fanelli and Serretti, 2019; Homberg et al., 2014). Additionally, the short allele variant appears to be associated with insufficient response to SSRIs (Kroeze et al., 2012). Moreover, the short allelic variant has been related to decreased BDNF mRNA levels in white blood cells and serum from healthy 5-HTTLPR patients (Benedetti et al., 2017; Bhang et al., 2011; Molteni et al., 2010). Likewise, rodents lacking SERT ($SERT^{-/-}$ animals) display a significant BDNF mRNA and protein downregulation, especially in the hippocampus and prefrontal cortex (Calabrese et al., 2015, 2013; Molteni et al., 2010). Additionally, $SERT^{-/-}$ rats and mice present anxiety- and depression-related phenotypes (Kalueff et al., 2010; Olivier et al., 2008).

The hippocampus plays a crucial role in depression. The hippocampus modulates emotional processing, memory, learning, and controls glucocorticoid secretion by the hypothalamic-pituitary-adrenal axis (HPA-axis), making this area susceptible to the effects of stress (O'Leary et al., 2014). Stress and other negative stimuli can change hippocampal plasticity, increasing the risk of depression (Liu et al., 2017). In fact, depressive disorder is significantly associated with hippocampal atrophy (Elbejjani et al., 2015, 2014; Santos et al., 2018; Taylor et al., 2014). Furthermore, several lines of research have shown that BNDF and its high-affinity receptor (TrkB) are decreased in the hippocampus of post-mortem tissue from suicidal or depressed patients (Castrén et al., 2007; Castrén and Rantamäki, 2010; Dwivedi et al., 2003; Ray et al., 2011). Importantly, while impaired hippocampal neurogenesis can lead to depression (Jacobs et al., 2000), studies demonstrated that the upregulation of BDNF levels stimulated hippocampal neurogenesis (Quesseveur et al., 2013; Rossi et al., 2006).

In line with this, considering that BDNF levels are decreased in the ventral hippocampus of $SERT^{-/-}$ rats (Calabrese et al., 2013; Molteni et al., 2010) we sought to investigate whether BDNF gene overexpression in the ventral hippocampus was able to restore BDNF levels and anxiety- and depression-like behavior in $SERT^{-/-}$ rats. Although BDNF gene expression generates at least nine different transcripts (Aid et al., 2007), we targeted the overexpression of transcript IV because it is most downregulated in $SERT^{-/-}$ rats (Molteni et al., 2010). Moreover, BDNF IV was selected due to its activity-dependent expression, association with depressive-like phenotypes (Sakata and Duke, 2014), and due to its higher contribution to total levels of BDNF protein in the hippocampus (Maynard et al., 2016). First, we aimed to analyze the temporal dynamics of BDNF IV overexpression one, two, and four weeks following lentivirus infusion in the ventral hippocampus (vHIP) of $SERT^{+/+}$ rats. In this experiment, we measured BDNF mRNA levels of total BDNF, BDNF IV, and BDNF VI in the prelimbic and infralimbic cortices, and in the vHIP through RT-qPCR analysis. Thereafter, in a

second experiment, following the viral infusions, SERT$^{-/-}$ and SERT$^{+/+}$ rats were submitted to behavioral testing. Behavior experiments included: (1) sucrose consumption test, in which we assessed the rats preference for sucrose over water and the total sucrose intake in grams; (2) forced swim test, where we scored for immobility, mobility and strong/high mobility behaviors; (3) open field, where we measured novelty-induced locomotor activity using Phenotyper® cages to evaluate distance moved, velocity, and time and frequency in the center; and (4) HPA-axis reactivity test, a test where the response of the HPA-axis upon acute restraint stress was checked through measurement of plasma corticosterone (CORT) levels.

Material and Methods

Animals

SERT$^{-/-}$ rats (Slc6a41Hubr) were generated by N-ethyl-N-nitrosourea (ENU)-induced mutagenesis on a Wistar background (Smits et al., 2006) and outcrossed with commercially available Wistar$^{Crl:WI}$ rats obtained from Charles River Laboratories (Horst, the Netherlands) for at least 15 generations. Ear punches were taken at the age of 21 days for genotyping, which was done by LGC (Hoddesdon, United Kingdom). SERT$^{+/+}$ rats were used to check BDNF virus overexpression in naïve animals. For the behavioral experiments, male SERT$^{-/-}$ and wild-type (SERT$^{+/+}$) rats, weighing 350-400g at the beginning of the study were used (see experimental design in figure 1). All animals were housed in temperature-controlled rooms (21 °C) with standard 12/12-h day/night-cycle (lights on at 7:00 am) and food and water available ad libitum. 5-7 days before surgery, animals were socially housed in individually ventilated (IVC) cages for habituation. After surgery, animals were separately housed in the IVC cages until recovery. Animals were socially isolated during the sucrose consumption test; thereafter, the animals we socially housed again and kept under the same temperature and day/night-cycle throughout the entire experiment. All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, and all efforts were made to reduce the number of animals used and their suffering.

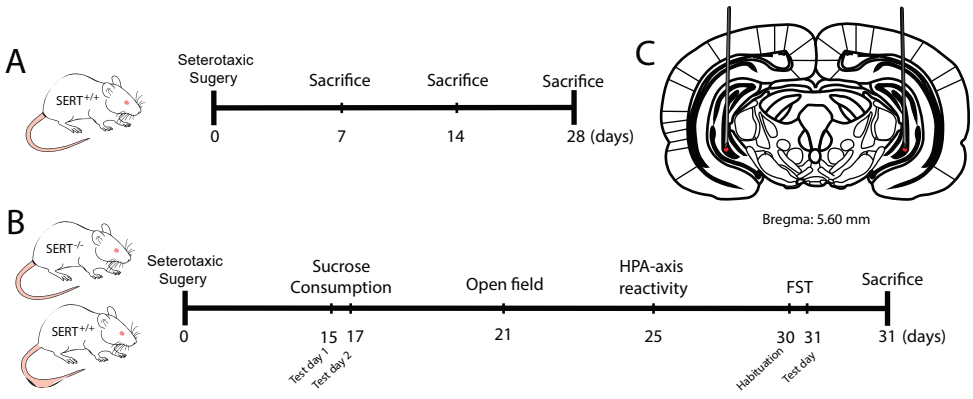

Figure 1. Schematic representation of the experimental design. A) Evaluation of BDNF overexpression in naïve $SERT^{+/+}$ rats one, two, and four weeks following viral infusion. B) Behavioral tests: Viral infusion followed by behavioral tests including sucrose consumption test, HPA-axis reactivity, open field, and forced swim test (FST). C) Representation of the local o the infusion of either BDNF or control virus.

Stereotaxic Surgery

Rats were anesthetized using isoflurane (5% induction, 2-3% maintenance). Lidocaine (10% m/v) was used for local anesthesia. Animals were fixed in a robot stereotaxic frame (StereoDrive, Neurostar, Germany). The coordinates for the site of the injection were theoretically determined based on the Paxinos & Watson (2007) rat brain atlas and checked through histological evaluation of 30 µm brain slices from dye-infused $SERT^{+/+}$ rats. The total volume of 2µL of either BDNF lentivirus particles (transcript variant IV under CMV promoter, NM_001270633.1) or pLenti-C-mGFP control lentivirus particles, was bilaterally infused into the ventral hippocampus according to the following coordinates: AP -5.60 mm, ML ±5.0 mm, DV -7.6 mm. After surgery, animals were placed in IVC cages (Sealsafe Plus GR900 green line, Tecniplast, Italy) until sacrifice.

RNA Preparation And Gene Expression Analysis By Quantitative Real-Time PC

Total RNA was isolated from the prelimbic and infralimbic region of the mPFC by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories; Segrate, Italy), according to the manufacturer's instructions, and then quantified by spectrophotometric analysis (NanoDrop™1000, Thermo Scientific). Following total RNA extraction, an aliquot of each sample was treated with DNase to avoid DNA contamination. Then, the samples were processed for real-time PCR to assess total BDNF, BDNF isoform IV, and VI. The analyses were performed by TaqMan qRT–PCR instrument (CFX384 real-time system, Bio-Rad Laboratories S.r.l.) using the iScript one-step RT–PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384-well formats in triplicates as

multiplexed reactions with a normalizing internal control (36B4). Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription), and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reactions. Data were analyzed with the comparative threshold cycle (ΔΔCt) method using 36B4 as a reference gene. Primers and probe for BDNF exon IV and VI were purchased from Life Technologies (BDNF exon IV: ID EF125679 and BDNF exon VI: ID EF125680). Primers and probe for total BDNF and 36B4 were purchased from Eurofins MWG-Operon. Their sequences are shown below:

- total BDNF: forward primer 5′-AAGTCTGCATTACATTCCTCGA-3′, reverse primer 5′-GTTTTCTGAAAGAGGGACAGTTTAT-3′, probe 5′-TGTGGTTTGTTGCCGTTGCCAAG-3′;
- 36B4: forward primer 5′-TTCCCACTGGCTGAAAAGGT-3′, reverse primer 5′-CGCAGCCGCAAATGC-3′, probe 5′-AAGGCCTTCCTGGCC GATCCATC-3′.

Behavioral tests:

Sucrose Consumption Test

After stereotaxic surgery, animals were housed individually and provided with two bottles of water for habituation. Before the test, side preference was checked for five days. The sucrose consumption test was adapted from Olivier et al. (2008) and consisted of two days of free-choice access to 24 hours sucrose versus water bottles with a water-only bottle choice in between the two days. In detail, on test day 1, one of the water bottles was replaced by sucrose 8% solution, and animals had free drinking access for a period of 24 hours. Next, animals received water in both bottles for 24 hours, ending with another 24 hours of free choice between water and sucrose 8% solution on test day 2. The position of the bottles was switched from sucrose consumption test day 1 to the test day 2 to prevent spatial bias. Daily, liquid intake and bodyweight were measured. The data are presented as the preference of sucrose above water (sucrose intake in ml divided by total intake X 100%) and the intake in grams of a 100% sucrose solution per kg bodyweight (intake in ml corrected for the voluminal weight of sucrose and recalculated toward a 100% solution divided by bodyweight in kg).

HPA-axis reactivity Test

The hypothalamic-pituitary-adrenal (HPA) axis reactivity was assessed through the measurement of corticosterone levels in the plasma. Usually, when rodents are submitted to stress, plasma concentrations of corticosterone (CORT) peak after 15 to 30 minutes and gradually decrease 60 to 90 minutes later to the pre-stress levels (de Kloet et al., 2005).

Therefore, blood samples from tail cuts were collected in capillary blood collection tubes (Microvette® CB 300 Di-Kalium-EDTA, Sarstedt, Germany) 5 minutes before, and 15 and 60 minutes after 30 minutes of restraint stress. Rodent restrainers Broome-style were used for the restraint stress (554-BSRR, Bio-services, The Netherlands). Blood samples were centrifuged (3400 rpm for 15 min at 4 ◦C), and the plasma was stored at -80 ◦C until analysis. CORT levels were measured using a liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Open field test

Novelty-induced locomotor activity was recorded by video recording in Phenotyper® cages (Noldus Information Technology, Wageningen, The Netherlands). The cages (45 cm × 45 cm × 45 cm) were made of transparent Perspex walls and a black floor. Each cage had a top unit containing a built-in digital infrared-sensitive video camera, infrared lighting sources, and hardware needed for video recording. To explore the novelty factor, animals were not exposed to this cage previously, and the cages were cleaned with 70% alcohol solution between trials to prevent transmission of olfactory cues. Spontaneous locomotor activity was monitored for 1 hour, and the following parameters were scored using Ethovision XT 11.5 (Noldus Information Technology, Wageningen, Netherlands): distance moved, velocity, frequency and time spent in the center of the cage (Manfré et al., 2017; Schipper et al., 2011).

Forced Swim test

The forced swimming test was performed as previously described (Porsolt et al., 1978). Briefly, rats were individually placed in cylindrical glass tanks (50 cm height, 20 cm diameter) filled to a height of 30 cm with 23±1°C water. The test consisted of two sessions. In the first session, animals were submitted to a habituation period of 15 minutes, then 24 hours later, to a second session of 5 min. The video recordings of the second session were used to automatically score the movements of the rats through a computerized system (Ethovision XT 10, Noldus, The Netherlands). Scored behaviors were 'immobility', which reflects no movement at all and/or minor movements necessary to keep the nose above the water; 'mobility', reflecting movement that corresponds to swimming activity; and 'strong mobility', reflecting 'escape behavior' (e.g., climbing against the walls and diving). Settings within Ethovision were adjusted based on manually recorded sessions (immobility/mobility threshold: 12; mobility/strong mobility threshold: 16.5 (Pawluski et al., 2016; Van den Hove et al., 2013).

Statistical Analysis

The data were checked for outliers and normality (using the Shapiro–Wilk statistic), and extreme outliers were windsorized. Two-way analysis of variance (ANOVA) was computed for gene expression analysis, with time, genotype, and treatment as independent factors. The outcomes of sucrose consumption, open field, and forced swim tests, and post-behavioral gene expression were also analyzed through Two-way ANOVA considering genotype and treatment as fixed factors. Post-hoc Fisher's Protected Least Significant Difference (PLSD) or independent sample t-tests were performed where applicable to compare individual group differences. All these statistical analyses were carried out using IBM® SPSS® statistics, version 23 (IBM software, USA). Regarding the HPA-axis reactivity test, a linear mixed model was implemented to account for repeated measurements, and multiple factor analysis using the LME4 package in R (3.5.1). Time, genotype, and treatment effects were modeled as a fixed effect, together with their pairwise double interactions, and their triple interactions. Subject intercepts were modeled as random effects. A likelihood-test ratio was used to assess fixed effect significance. Post-hoc tests were performed with the multicomp package, which accounts for multiple hypothesis testing. Significance was accepted at a $p<0.05$ threshold. Descriptive statistics are provided as mean +/- 1 standard error of the mean (SEM).

Results

Lentivirus transfection leads to BDNF overexpression in ventral Hippocampus of SERT$^{+/+}$ rats

Molecular analyses were performed to examine the feasibility of BDNF lentivirus overexpressing exon IV in the ventral hippocampus (vHIP) of naïve SERT$^{+/+}$ rats. To check temporal dynamics, mRNA levels were evaluated one, two, and four weeks following BDNF or GFP lentivirus infusion. Due to the complexity of the BDNF gene and the distribution of its diverse transcripts variants across the brain (Aid et al., 2007), RT-qPCR was performed to measure total BDNF, BDNF IV and BDNF VI. Additionally, mRNA levels were measured not only in the vHIP but also in areas of high connectivity with the vHIP that are likewise involved in mood disorders, namely the prelimbic (PrL) and infralimbic (IL) cortex (Duman and Monteggia, 2006; Krishnan and Nestler, 2008; Schulz and Arora, 2015). The results from each brain area are detailed below.

Local BDNF IV lentivirus infusion leads *to overall BDNF overexpression in the ventral hippocampus*

Two-way ANOVA revealed significant main effects for the diverse BDNF transcripts analyzed. For example, a treatment main effect was observed for total BDNF ($F_{(1, 27)} = 5.451$, $p = 0.027$), BDNF IV ($F_{(1, 26)} = 19.077$, $p < 0.001$), and BDNF VI ($F_{(1, 27)} = 8.389$, $p = 0.007$). Moreover, an interaction between treatment and time was found for BDNF IV ($F_{(2, 26)} = 4.533$, $p = 0.02$). PLSD *post-hoc* analysis revealed that BDNF IV lentivirus infusion led to an expected upregulation of this transcript in the vHIP. BDNF IV levels in the site of the injection (vHIP) were significantly increased in $SERT^{+/+}$ BDNF-treated animals compared to the control GFP-treated rats, one ($p < 0.001$), two ($p < 0.001$), and four weeks ($p < 0.001$) after surgery. Interestingly, two weeks after the viral infusion, we also identified a higher gene expression of transcript IV in control-treated $SERT^{+/+}$ rats (*vs.* one-week GFP-treated animals, $p < 0.001$). Furthermore, as shown in **figure A**, pairwise comparisons also indicated an increase in total BNDF in $SERT^{+/+}$ BDNF-treated rats one ($p = 0.003$) and for weeks after surgery ($p = 0.032$) in comparison to one-week GFP-treated animals. Additionally, BDNF VI levels were increased one (*vs* one-week $SERT^{+/+}$ GFP, p = 0.02) and two weeks after the surgery compared to controls (*vs* one-week $SERT^{+/+}$ GFP, $p = 0.002$; *vs* two weeks $SERT^{+/+}$ GFP, $p = 0.031$). In conclusion, BDNF lentivirus infusion in the vHIP caused local overexpression of BDNF IV mRNA, which was stable for at least 4 weeks. Additionally, it led to total BDNF and BDNF VI upregulation in the vHIP.

BDNF overexpression in the vHIP leads *to BDNF mRNA downregulation in the infralimbic cortex*

A significant reduction in the analyzed BDNF transcripts in the IL was observed following the transfection of BDNF lentivirus into the vHIP. Two-way ANOVA was computed and results demonstrated main effects for treatment ($F_{(1, 28)} = 16.756$, $p < 0.001$), time ($F_{(2, 28)} = 4.170$, $p = 0.026$), and treatment *vs* time interaction ($F_{(2, 28)} = 4.903$, $p = 0.015$) for total BDNF levels. Additionally, treatment main effects were observed for BDNF IV ($F_{(1, 27)} = 12.515$, $p = 0.001$), and BDNF VI ($F_{(1, 29)} = 12.514$, $p = 0.001$). There was also a treatment *vs* time interaction ($F_{(2, 27)} = 5.610$, $p = 0.009$) for BDNF IV. Interestingly, as it can be seen in **figure B**, pairwise comparisons revealed that one week following the infusion in the vHIP, the levels of BDNF in the IL of BDNF-treated $SERT^{+/+}$ rats dropped about 40% ($p < 0.001$) compared to control-treated animals at the same time point. Notably, both BDNF- and control-treated groups presented decreased levels of BDNF in the second week after the surgery ($p = 0.05$). This decrease was sustained for at least 4 weeks in BDNF-treated animals for BDNF IV ($p = 0.038$ *vs.* one-week $SERT^{+/+}$ GFP-treated) and BDNF VI levels ($p = 0.01$ *vs.* one-week $SERT^{+/+}$ GFP-treated), but total BDNF levels were back to normal four-weeks after the virus infusion. In

conclusion, we observed that BDNF lentivirus infusion in the vHIP altered the BDNF levels in the infralimbic cortex, especially causing a significant decrease in BDNF mRNA expression one week after surgery.

BDNF overexpression in the vHIP alters BDNF expression in the prelimbic cortex

As in the IL, the levels of BDNF were decreased in the PrL of BDNF-treated SERT$^{+/+}$ one week after the viral infusion in the vHIP. Specifically, main effects for the interaction between genotype and time point were found for total BDNF ($F_{(2, 28)} = 6.044$, $p = 0.007$) and BDNF VI ($F_{(2, 28)} = 13.802$, $p < 0.001$). *Post-hoc* analysis revealed that infusion of BDNF IV lentivirus particularly disturbed BDNF expression in a time- and transcript-dependent manner. For example, at the one-week time point, all the transcripts analyzed were decreased in the SERT$^{+/+}$ rats treated with BDNF in comparison to controls (total BDNF $p = 0.038$, BDNF IV $p = 0.02$, and BDNF VI $p = 0.014$). However, as it is seen in **figure C**, two weeks following the surgery, while control rats presented decreased levels of total BDNF and BDNF VI (respectively $p = 0.024$ and $p < 0.001$ *vs.* one-week SERT$^{+/+}$ GFP-treated), SERT$^{+/+}$ rats treated with BDNF had these transcripts levels normalized to the control levels. Moreover, four weeks following the surgery, particularly the levels of BDNF VI were decreased in the BDNF-treated animals ($p < 0.001$ *vs.* one-week SERT$^{+/+}$ GFP-treated, $p = 0.003$ *vs.* 4 weeks SERT$^{+/+}$ GFP-treated). In short, BDNF overexpression in the vHIP caused decreased BDNF levels in the PrL one week after infusion; however, two and four weeks after, it especially altered the expression of BDNF VI resulting in both increased levels two weeks following surgery and decreased levels four weeks after surgery.

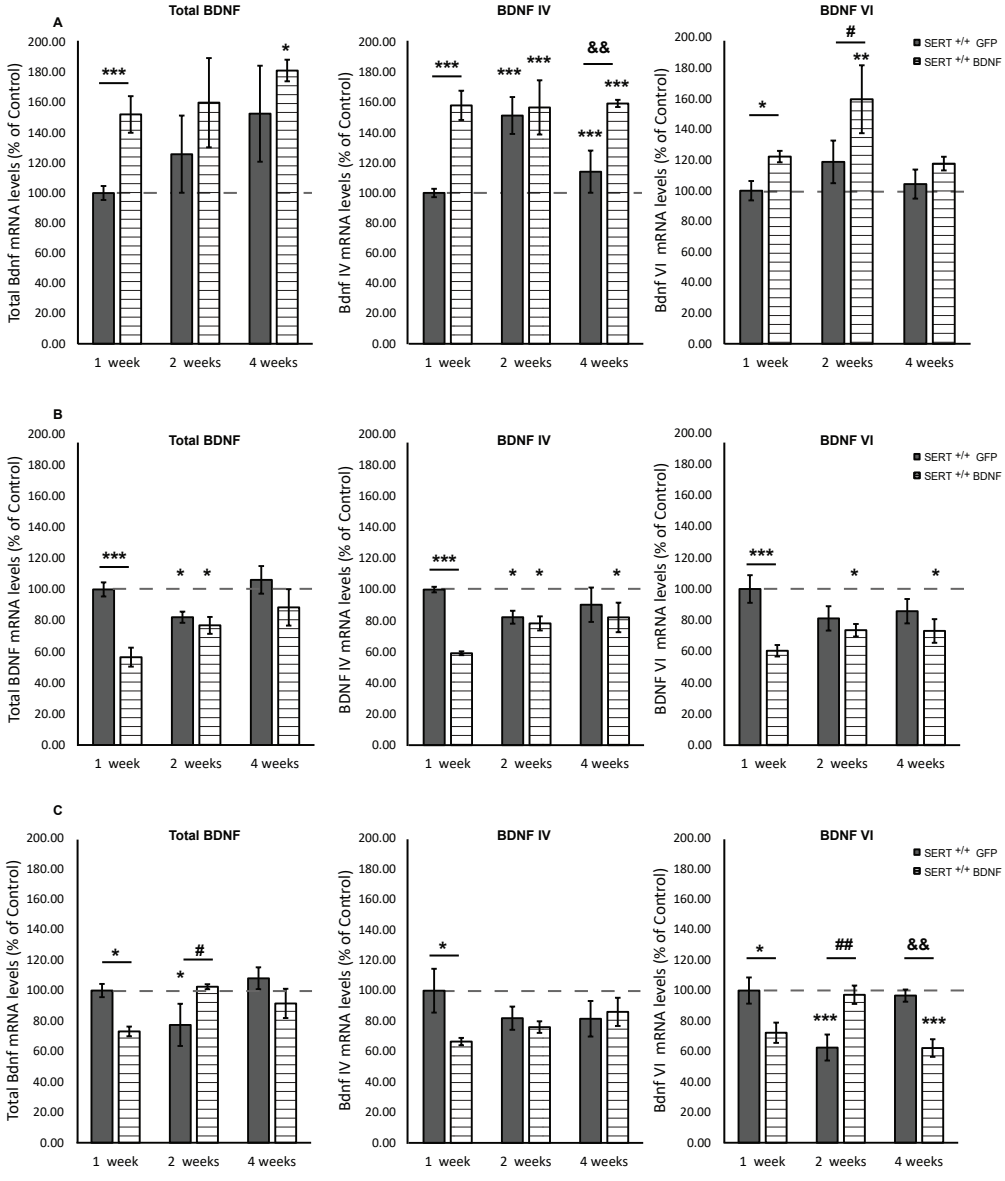

Figure 2. Modulation of total BDNF, BDNF IV, and BDNF IV transcripts expression in SERT +/+ animals infused with either GFP control or BDNF viral particles. BDNF mRNA levels were measured in the (A) ventral hippocampus, (B) infralimbic cortex, and (C) prelimbic cortex one, two, and four weeks after stereotaxic surgery. Data are expressed as fold change compared to the GFP-treated animals (set at 100%), and reflect mean ±SEM from 5-7 independent determinations. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$ vs GFP-1 week; # = $p < 0.05$, ## = $p < 0.01$, and ### = $p < 0.001$ vs GFP-2 weeks; && = $p < 0.01$ vs GFP-4 weeks.

Sucrose Consumption Test

Anhedonia is marked by a reduced interest in pleasurable events, and it is present in depression. This depression-like symptom can be identified in rodents through a decrease in sucrose consumption, which can be accessed through the sucrose consumption test. Animals were exposed to two days of free access to a sucrose 8% solution. The results of the preference for sucrose above the water and the sucrose intake in grams are described below.

Sucrose Preference: BDNF upregulation meliorates anhedonia-like behavior in SERT$^{-/-}$ rats upon first exposure to the sucrose solution

Preference for sucrose was analyzed in two sessions. In the first day of test, two-way ANOVA indicated genotype as well treatment main effects ($F_{(1, 36)}$ = 6.980, p = 0.012 and F(1, 36) = 4.854, p = 0.034, respectively). In detail, SERT$^{-/-}$ rats treated with control virus presented a significant reduction in sucrose preference compared to both controls ($p < 0.01$) and BDNF-treated SERT$^{-/-}$ rats (p = 0.006). Meanwhile, the BDNF-treated SERT$^{-/-}$ rats displayed similar sucrose preference compared to control-treated SERT$^{+/+}$ animals. On the second day, the BDNF treatment effect in SERT$^{-/-}$ was not present anymore, and only a genotype effect was found ($F_{(1, 38)}$ = 13.686, $p < 0.001$). Pairwise analysis indicated that both control- and BDNF-treated SERT$^{+/+}$ had a higher preference for sucrose than both SERT$^{-/-}$ groups ($p < 0.05$). In conclusion, as previously demonstrated, SERT$^{-/-}$ rats displayed a lower preference for sucrose than SERT$^{+/+}$ rats (Olivier et al., 2008); however, BDNF treatment improved the preference for sucrose in SERT$^{-/-}$ rats at least in the first day of the test.

Sucrose intake: BDNF overexpression exclusively modulates sucrose intake on the first day of the sucrose consumption test

As done for the sucrose preference, sucrose intake was measured in two sessions. The results reveal that, on the first day of the test, BDNF lentivirus treatment altered the behavior of SERT$^{+/+}$ and SERT$^{-/-}$ regarding the consumption of sweet solution. This effect was lost on the second day. Two-away ANOVA showed a genotype ($F_{(1, 38)}$ = 29.313, $p < 0.001$) and a treatment effect ($F_{(1, 38)}$ = 14.198, $p < 0.001$) on the first day of the test. In detail, both SERT$^{-/-}$ and SERT$^{+/+}$ rats treated with BDNF presented a significantly higher intake of sucrose than their respective GFP-treated controls (BDNF- *vs* GFP- SERT$^{-/-}$, p = 0.017; and, BDNF- *vs* GFP-SERT$^{+/+}$, p = 0.007). Moreover, compared to control SERT$^{+/+}$ rats, only the GFP-treated SERT$^{-/-}$ rats presented a lower intake of sucrose (p = 0.001). On the second day, just a genotype main effect was observed ($F_{(1, 38)}$ = 23.280, $p < 0.001$). *Post-hoc* comparisons showed that BDNF treatment did not influence the intake in the second day and that – as previously described (Olivier et al., 2008) – SERT $^{-/-}$ rats consumed less sucrose than the SERT$^{+/+}$ rats ($p < 0.01$). Taken together, the sucrose intake was influenced by the BDNF overexpression, with

a positive effect on the anhedonia-like behavior of SERT$^{-/-}$ rats and leading to an even higher sucrose intake in SERT$^{+/+}$ rats. However, this effect was only present on the first day of the test. Upon the second exposition to the sucrose bottles, the SERT$^{-/-}$ anhedonia-like phenotype returned.

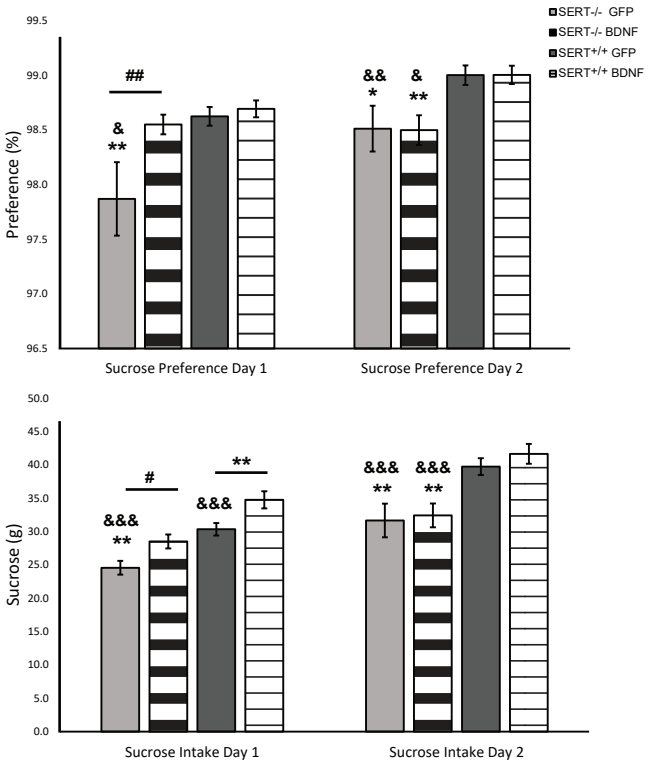

Figure 3. Sucrose consumption of 8% sucrose solution by SERT$^{-/-}$ and SERT$^{+/+}$ rats. Data are expressed as mean S.E.M. sucrose preference (sucrose intake/total fluid intake x 100%), and as mean S.E.M. total sucrose intake (g) per body weight (n = 10-12; * = $p < 0.05$ and ** = $p < 0.01$ vs SERT$^{+/+}$-GFP; & = $p < 0.05$, && = $p < 0.01$, and &&& = $p < 0.001$ vs SERT$^{+/+}$-BDNF; # = $p < 0.05$ vs SERT$^{-/-}$GFP).

BDNF overexpression decreases immobility in the forced swim test in SERT$^{-/-}$ rats

When rodents are exposed to an inescapable stressor such as in the forced swim test, their motivation to cope with stress can be quantified by the percentage of time spent on immobility (behavioral passivity) or performing a highly mobile (escape-like) behavior (Porsolt et al. 1977). Two-way ANOVA determined that for both extreme swimming modalities, immobility and high mobility, genotype main effects were found ($F_{(1,37)} = 7.589$, $p = 0.009$; and, $F_{(1,37)} = 11.237$, $p = 0.002$, respectively), while no main effects were found for

the normal swimming (mobility) behavior scoring. Moreover, pairwise comparisons demonstrated that SERT$^{-/-}$ rats were much more prompted to develop an escape-like behavior in comparison to SERT$^{+/+}$ rats ($p < 0.05$). Furthermore, as shown in **figure 3A**, we observed that BDNF upregulation in the SERT$^{-/-}$ rats resulted in a decreased immobility in comparison to both SERT$^{+/+}$ groups (*vs.* SERT$^{+/+}$ GFP, $p = 0.025$, and *vs.* SERT$^{+/+}$ BDNF, $p = 0.011$). In conclusion, BDNF overexpression did not decrease the anxiety-like behavior in SERT$^{-/-}$ rats expressed by increased time spent in the high mobility swimming modality; on the other hand, BDNF upregulation in the vHIP affected behavioral passivity, as expressed by a decrease in time spent on immobility in SERT$^{-/-}$ rats.

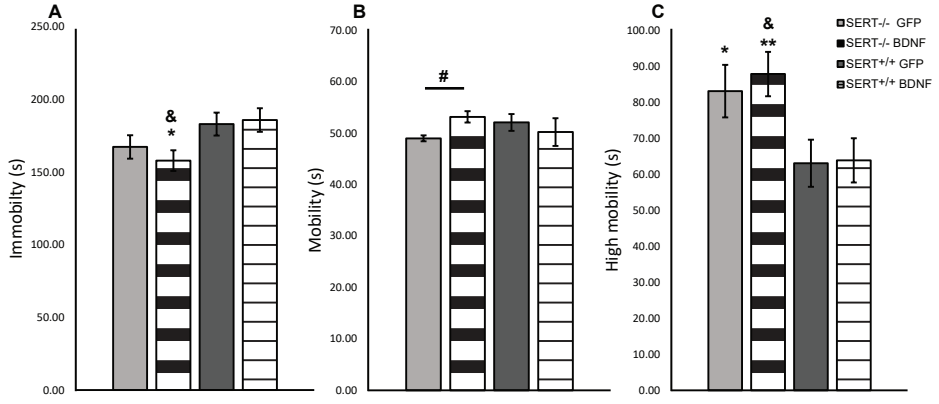

Figure 4. Mean (± SEM) measure of (A) immobility, (B) mobility, and (C) high mobility in the forced swim test (FST). n = 10–12 rats per group. * = p < 0.05, and ** = p < 0.01 vs SERT+/+ GFP; & = p < 0.05, and && = p < 0.01 vs SERT+/+ GFP; # = p < 0.05 vs SERT-/- GFP). Two-way ANOVA, Fisher LSD post-hoc test.

BDNF upregulation decreases anxiety-like behavior in SERT$^{-/-}$ rats in the open field test

Rodents may present a higher activity when they are introduced to a new environment (Menzaghi et al., 1994). A decrease in central locomotion (frequency and time spent in the central part of the arena), together with a general decrease in the locomotion (distance moved and velocity) can be interpreted as an anxiogenic-like behavior (Prut and Belzung, 2003). As previously reported, no differences between SERT$^{-/-}$ and SERT$^{+/+}$ rats were observed for the distance moved or velocity (Schipper et al., 2011). However, we did observe a genotype main effect for time spent in the center of the test-cage ($F_{(1, 37)} = 6.557$, $p = 0.015$). Further *post-hoc* analysis revealed that control-treated SERT$^{-/-}$ rats spent less time in the center of the cage than control SERT$^{+/+}$ rats ($p = 0.007$); meanwhile, BDNF injected SERT$^{-/-}$ rats spent similar time in the center compared to the SERT$^{+/+}$ rats. In conclusion, as reflected in **figure 5,** no alterations in the locomotor distance or speed were observed due to treatment

or genotype. In contrast, the duration these animals spent in the center of the novel environment highlighted the behavioral differences between SERT$^{-/-}$ and SERT$^{+/+}$ rats. BDNF upregulation in SERT$^{-/-}$ rats normalized the level of anxiety to that of SERT$^{+/+}$ rats.

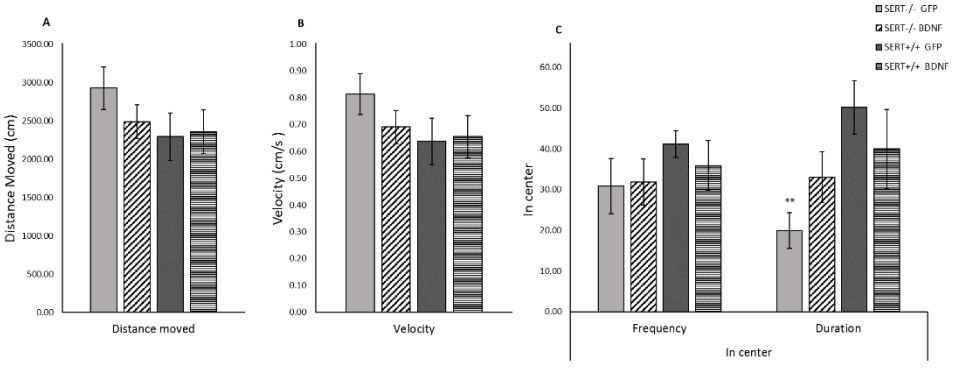

Figure 5. Novelty-induced locomotor activity expressed as mean (± SEM) (A) distance moved, (B) velocity, and (C) frequency and time spent in center. n = 10-12; ** = $p < 0.01$ vs SERT+/+ GFP). Two-way ANOVA, Fisher LSD post-hoc test.

BDNF overexpression reduces CORT levels in SERT$^{-/-}$ rats in the HPA-axis reactivity test

Acute restraint stress can activate the hypothalamic-pituitary-adrenal axis (HPA-axis), resulting in the release of CORT in rodents, which can be a measure of the (mal) functionality of the HPA-axis. The HPA-axis response to stress was evaluated 15 and 60 minutes after restraint stress. A linear mixed-effect analysis was conducted, and no interactions were found. However, pairwise comparisons indicated a significant decrease in CORT levels of BDNF-treated SERT$^{-/-}$ rats 60 minutes after acute stress (p = 0.014), indicating that BDNF overexpression in the ventral hippocampus likely reduced the HPA reactivity in the SERT$^{-/-}$ rats at this timepoint. No differences in the baseline CORT levels were found. Conclusively, while no basal or stress-induced differences in CORT levels were found between control-treated SERT$^{-/-}$ and SERT$^{+/+}$ rats, BDNF upregulation induced a decrease in the CORT levels in SERT$^{-/-}$ rats versus control-treated SERT$^{-/-}$ rats.

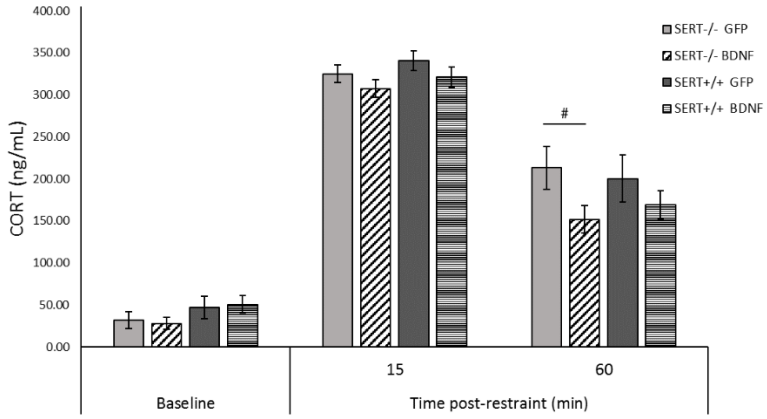

Figure 8. HPA-axis reactivity assessment. Corticosterone (CORT) levels are expressed mean ± (SEM) of plasma CORT levels 5 minutes before restraint stress (baseline), and 15 and 60 minutes post-restraint stress. n = 10-12; # = $p < 0.05$ vs SERT$^{-/-}$ GFP. Linear mixed model.

Discussion

For many years, it has been hypothesized that the intricate system regulating the BDNF gene results in the generation of BDNF transcripts that create a spatial code for BDNF gene expression throughout the brain (Baj et al., 2011). Definitive conclusions regarding the role different transcripts play in BDNF signaling are still lacking, but it is of great interest giving that BDNF mRNA variants are differently (down)regulated in individuals suffering from neuropsychiatric disorders, including mood disorders. Considering this, we aimed to investigate the effects of BDNF IV overexpression in the ventral hippocampus of animals presenting both depressive-like behavior and BDNF downregulation. Here, we report that lentivirus BDNF IV infusion into the vHIP caused a significant overexpression of BDNF in the vHIP, followed by upregulation of other BDNF transcripts, such as total and BDNF VI levels. Interestingly, we observed that the upregulation of BDNF in the vHIP disturbed the BDNF gene expression in other brain areas. Particularly, we examined the PrL and IL and noticed an accentuated decrease in BDNF expression in these areas, especially in the first week following the BDNF viral infusion. Furthermore, we demonstrated that BDNF overexpression resulted in task- and genotype-dependent modifications in the phenotypic outcome from SERT$^{-/-}$ and SERT$^{+/+}$ rats. For instance, in the sucrose consumption test, BDNF treatment improved the preference for sucrose in SERT$^{-/-}$ rats, and increased sucrose intake in SERT$^{-/-}$ and SERT$^{+/+}$ rats, but only in the first day of the test. Additionally, BDNF-infused SERT$^{-/-}$ rats presented decreased immobility in the forced swim test, and lower anxiety levels in the novelty-induced locomotor activity test compared to controls. Finally, BDNF upregulation induced a decrease in HPA-axis activity, reflected by the decreased CORT levels in SERT$^{-/-}$ rats.

Precise time-, brain region-, and stimuli-dependent BDNF production might be mediated by the expression of multiple BDNF mRNA variants (Maynard et al., 2016). At least 9 promoters are involved in the regulation of BDNF (Aid et al., 2007); among them, promoter IV is an activity-dependent gene, leading to the expression of BDNF upon neuronal activity (Sakata et al., 2010). Interestingly, molecular analysis of the BDNF IV overexpression in this study revealed exciting features regarding the BDNF IV effect over other BDNF transcripts, and regarding the vHIP connection with frontal areas of the rat brain. In short, one week after the viral infusion into the vHIP, we demonstrated that total BDNF, BDNF IV, and BDNF VI were overexpressed in the vHIP and downregulated in the PrL and IL of BDNF-infused $SERT^{+/+}$ rats. In the IL, the decreased BDNF levels of variants IV and VI were stable for at least four weeks, showing a continuous influence from the vHIP BDNF upregulation. On the other hand, in the PrL, we noticed a time-dependent control specific to total and BDNF VI. Two weeks after BDNF infusion, PrL levels of total BDNF and BDNF VI were higher in BDNF-infused animals, but in the fourth week, BDNF VI levels were again downregulated. The medial prefrontal cortex (mPFC) receives direct projections from the ventral hippocampus (Verwer et al., 1997). Studies in cultured hippocampal neurons revealed activity-induced BDNF dendritic release (Adachi, 2014; Edelmann et al., 2014; Matsuda et al., 2009). Altogether, these data indicate that, likely, BDNF upregulation in the vHIP caused negative feedback in the BDNF transcription of downstream targets such as the PrL and IL. Importantly, just as the vHIP-mPFC synchrony was shown to be modulated by negative emotions like anxiety (Adhikari et al., 2010), the vHIP to mPFC transcriptional control, might likewise induce effects on cognitive outcome. Furthermore, the different effects in PrL and IL BDNF gene expression might specifically modulate cognitive (mal)functions, considering that these two mPFC subdivisions have different projections sites (Vertes, 2004; Vidal-Gonzalez et al., 2006).

Anhedonia is a core symptom in mood disorders (Morris et al., 2017). This negative emotional state has been previously demonstrated in $SERT^{-/-}$ rats (Olivier et al., 2008), and was replicated in this study. In our experimental conditions, control-treated $SERT^{-/-}$ rats displayed anhedonia-like behavior when compared to controls $SERT^{+/+}$ rats. This anhedonia-like behavior was suppressed in the $SERT^{-/-}$ rats following BDNF upregulation. However, the enhancement in sucrose preference and sucrose intake was limited to the first day of the test, and it was lost on the second day of the test. Therefore, the upregulation of BDNF IV in the vHIP likely ameliorated the anhedonia-like symptoms in $SERT^{-/-}$ rats only when the novelty factor was present. After habituation to the novel sweet taste, the anhedonic phenotype was prevalent in $SERT^{-/-}$ animals in comparison to $SERT^{+/+}$ rats. Importantly, in a previous experiment (Diniz et al. unpublished data), we overexpressed BDNF in the PrL, and we observed exactly the opposite effect on sucrose preference. In short, $SERT^{-/-}$ rats infused with BDNF IV into the PrL preferred less sucrose than GFP-infused $SERT^{-/-}$ rats only on the first day

of the sucrose consumption test. On the second day, the BDNF-treated animals showed higher sucrose preference than GFP-treated SERT$^{-/-}$ rats. Therefore, BDNF upregulation differently modulated the response to a novel taste depending on the targeted brain region, causing both decreased sucrose preference in the PrL-infused animals, and increased sucrose over water preference in vHIP-infused animals.

Another core endophenotype in mood disorders is behavioral despair (Kalueff et al., 2010). In rodents, this symptom can be evaluated through the forced swim test (Porsolt et al., 1978). We demonstrated that, in our experimental conditions, we did not observe differences in immobility scores when comparing control-treated SERT$^{-/-}$ to SERT$^{+/+}$ rats. This result is at odds with previous studies using SERT$^{-/-}$ rats and mice, in which immobility was increased in knockout animals (Lira et al., 2003; Olivier et al., 2008). However, under the same experimental conditions, SERT$^{-/-}$ rats submitted to stereotaxic surgery targeting GFP virus infusion in the PrL also did not present increased immobility behavior in the forced swim test (Diniz et al. unpublished observations). Interestingly, BDNF upregulation in the vHIP decreased immobility time in SERT$^{-/-}$ rats in comparison to GFP-treated SERT$^{-/-}$ animals, suggesting that BDNF overexpression contributed to an adaptive learned response (Anyan and Amir, 2018). In line with this finding, Karpova et al. (2009) demonstrated that an increase in hippocampal BDNF IV caused by postnatal SSRI exposure was associated with decreased immobility in the forced swim test. Further, we demonstrated that SERT$^{-/-}$ rats, independently of the treatment, displayed higher escape-behavior than SERT$^{+/+}$ rats. Increased strong mobility in the forced swim test might indicate enhanced anxiety-like behavior displayed by SERT$^{-/-}$ rats. Anxiety-like behavior in SERT$^{-/-}$ rats might be due to changes in the functioning of the GABAergic system in SERT$^{-/-}$ rats (Calabrese et al., 2013; Guidotti et al., 2012; Luoni et al., 2013; Miceli et al., 2017; Schipper et al., 2019), causing GABA downregulation and consequently, increase anxiety-like behavior (Lydiard, 2003; Millan, 2003).

In line with this observation, we identified that in the novelty-induced locomotor activity test, SERT$^{-/-}$ rats spent less time in the center of the test-cage than SERT$^{+/+}$ rats, indicating likewise, increased anxiety-like behavior (Olivier et al., 2008). Interestingly, SERT$^{-/-}$ rats receiving BDNF virus as treatment did not differ from SERT$^{+/+}$ controls, indirectly suggesting that BDNF upregulation likely decreased the SERT$^{-/-}$ rats' anxiety-like behavior in this behavior paradigm. This BDNF anxiolytic-like action modulated by exposition to novelty is in agreement with the results from the sucrose consumption test, in which SERT$^{-/-}$ rats seems to present enhanced negative emotionality in connection to the novel taste and this behavior was reduced by BDNF IV overexpression in the vHIP. Additionally, in our experimental conditions, we replicated the previous observation that no alterations in the locomotor distance or speed were observed in SERT$^{-/-}$ rats in comparison to SERT$^{+/+}$ rats

(Homberg et al. 2008; Schipper et al. 2011). This also indicates that the decreased immobility and increased high mobility observed in SERT$^{-/-}$ rats in the forced swim test was not due to increased locomotor activity.

Adaptive responses to stressors require behavioral flexibility that depends on a plastic brain. Considering this, we also investigated the effects of hippocampal upregulation of BDNF IV on HPA-axis activity through the measurement of corticosterone (CORT) levels following acute stress. We demonstrated that 60 minutes after the acute stress, BDNF upregulation in the vHIP caused a significant decrease in CORT levels in SERT$^{-/-}$ rats compared to GFP-treated SERT$^{-/-}$ rats, indicating that BDNF overexpression affected the decreasing phase in CORT levels (de Kloet et al., 2005) by enhancing the negative feedback over the HPA-axis. Regarding the peak in CORT levels, at 15 minutes following the acute restraint stress, we observed that CORT levels were not affected by BDNF upregulation or genotype. Likewise, basal CORT levels were comparable in SERT$^{-/-}$ and SERT$^{+/+}$ rats, denoting a lack of BDNF modulation in baseline levels. In agreement with these results, studies in SERT$^{-/-}$ mice also have shown that plasma basal CORT levels (Chen et al., 2012; Li et al., 1999; Tjurmina et al., 2002) or stress-response CORT levels (Jansen et al., 2010) were not altered in comparison to SERT$^{+/+}$ mice. However, in contrast with our results, van der Doelen et al. (2014) have shown that non-stressed SERT$^{-/-}$ rats presented increased baseline levels of CORT compared to SERT$^{+/+}$ rats, whereas when submitted to early life stress, SERT$^{-/-}$ rats present lower levels of CORT than early-life-stressed SERT$^{+/+}$ rats. The data found in animals do not always match those from in human studies; however, basal HPA hyperactivity, and consequently hypercortisolemia is commonly detected in depressive patients (Nestler et al., 2002; Pariante and Lightman, 2008). In humans, the short allele generated by the single polymorphism in the serotonin transporter gene (5-HTTLPR) is associated with higher levels of waking cortisol (CORT) and increased CORT response to stress (Gotlib et al., 2008; Klein Gunnewiek et al., 2018; O'Hara et al., 2007). Therefore, the negative emotionality and neurodevelopmental changes presented by the SERT$^{-/-}$ rodents are comparable to the effects of the short allele in the human 5-HTTLPR (Homberg et al., 2014; Kalueff et al., 2010). In our experimental conditions, the SERT$^{-/-}$ rats were not stressed in their early life stage; however, they underwent surgery stress, isolation stress (in the sucrose consumption test), and were exposed to a novel environment in the locomotor activity test. Therefore, although animals were given time to recover, it is possible that the sequence of behavioral tests shaped an adaptive CORT response to stress (Belay et al., 2011) as well as determined the differential CORT basal levels (van der Doelen et al., 2014). Moreover, while BDNF overexpression in the vHIP did not alter baseline CORT levels in SERT$^{+/+}$ rats, our previous data on BDNF overexpression in the prelimbic cortex of WT rats indicated significant increased basal CORT levels in these animals. Besides, in contrast to the BDNF overexpression in the vHIP,

upregulation in the prelimbic cortex modulated the stress CORT levels 15 minutes following the acute restraint stress, causing increased CORT levels in SERT$^{-/-}$ rats and decreased CORT levels in WT animals. Altogether, these data suggest that BDNF affects the HPA-axis activity in a brain region and phase-dependent manner; also, it differently affected the animals depending on their genotype. Further studies are necessary to understand the association between the serotonin transporter gene and the HPA-axis reactivity in stress and non-stress contexts. Especially, the role of BDNF in key brain areas controlling the HPA-axis response, such as is the case of the mPFC and hippocampus, in healthy and disease will be essential to develop novel treatments to provide improved care to patients suffering from mood disorders.

Depression is a complex neuropsychiatric disorder comprising multiple neural circuit processes (Nestler et al., 2002). Animal models such as the SERT$^{-/-}$ rat comprise a useful tool to study targeted circuits that are found in both rodents and humans. Although complete function loss of the human SERT is a very rare condition, behavioral and biomolecular changes in SERT$^{-/-}$ are comparable to short allele carriers of the human 5-HTTLPR (Kalueff et al., 2010). The SERT knockout rats are characterized by a complete lack of SERT and increased extracellular serotonin levels (Homberg et al. 2007), yet show anxious and depressive-like phenotypes. In agreement with the neurotrophic hypothesis of depression, these animals present, under basal conditions, downregulation of BDNF mRNA (especially exon IV) and protein levels in the ventral hippocampus and prefrontal cortex (Calabrese et al., 2013; Molteni et al., 2010). Modulation of BDNF IV levels in the ventral hippocampus and not in the prefrontal cortex showed to have positive effect in mood disorder-related symptoms. Selective upregulation of a specific BDNF exon can lead to specific neuronal network reinforcement (Baj et al., 2011). The current study demonstrated that defining a target brain region might be critical for mediating therapeutic interventions targeting BDNF overexpression. Further characterization of the effects BDNF overexpression might exert over other brain areas targeted by the vHIP, such as the PFC and nucleus accumbens (NAc), is still needed. In addition, given that BDNF did not decrease escape behavior in the forced swim test, indicating anxiety-like behavior, it is still unclear which circuits might be involved in the forced swim test escape-behavior response. Moreover, it is uncertain whether BDNF overexpression in the vHIP restored the decreased GABAergic inhibitory regulation in SERT$^{-/-}$ rats (Calabrese et al., 2013; Luoni et al., 2013; Miceli et al., 2017; Schipper et al., 2019). Nonetheless, despite necessary further investigations, we hypothesize that BDNF IV upregulation in the vHIP might be a good candidate for modulation in the treatment of mood disorders. Performing stereotaxic surgery in humans seems not to be a feasible treatment for mood disorders in humans; however, advances in pharmaceutical technology have shown that the delivery of drugs and genes into the brain through

peripheral bloodstream route is possible (Saraiva et al., 2016). Therefore, the additional studies to understand the BDNF dynamics in the vHIP of $SERT^{-/-}$ rats might enhance the possibilities to the development of specific gene target delivery, allowing a future novel, rational treatment to bring individualized and improved care for patients with mood disorders.

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Crossing the Blood-Brain-Barrier: A bifunctional liposome for BDNF gene delivery – A Pilot Study

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Abstract

To achieve their therapeutic effect on the brain, molecules need to pass the blood-brain-barrier (BBB). Many pharmacological treatments of neuropathologies encounter the BBB as a barrier, hindering their effective use. Pharmaceutical nanotechnology based on optimal physicochemical features and taking advantage of naturally occurring permeability mechanisms, nanocarriers such as liposomes offer an attractive alternative to allow drug delivery across the BBB. Liposomes are spherical bilayer lipid-based nanocapsules that can load hydrophilic molecules in their inner compartment and on their outer surface can be functionally modified by peptides, antibodies and polyethyleneglycol (PEG). When composed of cationic lipids, liposomes can serve as gene delivery devices, encapsulating and protecting genetic material from degradation and promoting nonviral cell transfection. In this study, we aimed to develop a liposomal formulation to encapsulate a plasmid harbouring brain-derived neurotrophic factor (BDNF) and infuse these liposomes via the peripheral bloodstream into the brain. To this end, liposomes were tagged with PEG, transferrin, and arginine and characterized regarding their physical properties, such as particle size, zeta-potential and polydispersity index (PDI). Moreover, we selected liposomes preparations for plasmid DNA (pDNA) encapsulation and checked for loading efficiency, in vitro cell uptake, and transfection. The preliminary results from this pilot study revealed that we were able to replicate the liposomes synthesis described in literature, achieving compatible size, charge, PDI, and loading efficiency. However, we could not properly determine whether the conjugation of the surface ligands transferrin and arginine to PEG worked and whether they were attached to the surface of the liposomes. Additionally, we were not able to see transfection in SH-SY5Y cells after 24 or 48 hours of incubation with the pDNA loaded liposomes. In conclusion, we synthesized liposomes encapsulation pBDNF, however, further research will be necessary to address the complete physicochemical characterization of the liposomes. Furthermore, preclinical studies will be helpful to verify transfection efficiency, cytotoxicity, and in the future, safe delivery of BDNF through the BBB.

Keywords: Liposome, Nanotechnology, BBB, Gene delivery, BDNF

Introduction

The development of new treatments for curing neurological diseases has been slow due to the inability of large molecule pharmaceuticals, such as products of biotechnology, recombinant proteins or gene therapies, to cross the blood-brain barrier (BBB) (Pardridge, 2020). The central nervous system (CNS) presents a series of barriers to protect itself from invading pathogens, neurotoxic molecules, and circulating blood cells. These structures with diverse degrees of permeability include the blood-brain barrier, which is the most extensive and exclusive barrier (Saraiva et al., 2016). The blood-brain-barrier (BBB) is an anatomical and physiological barrier that is responsible for the tight regulation of the transportation of cells, molecules, and ions between the periphery and the brain. Because the selective molecular permeability of the BBB prevents most of the drugs from entering into the brain, the development of new treatments for brain diseases is complicated (Pardridge, 2012). The highly specialized endothelial cells layer has an intimate contact with brain cells, so that to reach those cells, substances such as drugs, have to present suitable lipophilicity, size, and capacity to evade active extrusion (Serlin et al., 2015). The occurrence of specific receptors on the surface of the BBB facilitates the transport of various essential molecules into the brain. Nanotechnology offers an exciting approach for improving the therapeutic management of CNS diseases. Liposomes, being functionally versatile, can be engineered for targeting these receptors, thus rendering them as promising carriers for drug and gene delivery (Sharma et al., 2012). A liposome is a kind of non-viral vector that can deliver molecules such as DNA, proteins, and drugs to the action sites, and it has been used to carry exogenous gene productions (Ganly et al., 2013). They have the great advantage of being made from a natural biodegradable lipid bilayer, which is similar to the animal cell membrane structure. Thus, considering that the morphologic appearance of liposomes resembles the natural cell membrane, they are an ideal drug-carrier system (Bozzuto, 2015).

Bangham and Horne were the pioneers in reporting the synthesis of liposomes, described by them as single or multiple concentrically organized lipid bilayers containing an inner aqueous compartment (Bangham and Horne, 1964) (Figure 1). Liposomes usually contain phospholipids as its basic constitution. This lipid molecule is made up of a hydrophilic head that interacts with aqueous solutions and two hydrophobic fatty acids chains that have an affinity with each other. In aqueous solution, due to these amphiphilic characteristics, a lipid bilayer is formed creating a lipophilic inner compartment that acts as a permeability barrier, both inward and outward (Bozzuto, 2015). Liposomes can serve as gene carriers, being then called lipoplexes. Lipoplexes have enormous potential to deliver plasmids into target cells; they contain cationic lipids that due to its positive charge, can complex with negatively charged DNA molecules. Theoretically, the electrostatic interaction promotes neutralization and enhances cell-membrane-DNA communication and transfection efficiency (Parker et al., 2003). However, the positive charges on the surface can lead to nonspecific interaction with plasma and other extracellular proteins, decreasing the transfection efficiency (Urtti et al., 2000). Thus, to increase transfection, neutral or helper lipids, such as

dioleoylphosphatidylcholine (DOPE), are used allow endosomal escape and plasmid dissociation from liposomes before degradation in lysosomes. (Parker et al., 2003).

To specifically target brain delivery, liposomes can undergo surface modifications to increase transfection efficiency. For instance, adding polyethylene glycol (PEG), or PEGylating the liposome, increases transfection efficiency, reduces toxicity, and imparts "stealth" properties (Rip et al., 2014). A widely explored liposome surface modification for brain delivery relies on the use of ligands of receptors on the brain endothelium. The cell-penetrating peptides (CPPs) are short cationic or amphipathic peptides that have the ability to transport the associated molecular cargo (e.g., peptides, proteins, oligonucleotides, liposomes, nanoparticles, bacteriophages, etc.) inside the cells (Sharma et al., 2012). Arginine, a CPP, can enhance the cellular uptake and delivery across the BBB (Morris and Labhasetwar, 2015). Moreover, the use of transferrin (Tf) is a classic method for enhancing BBB crossing via receptor-mediated endocytosis (Chen et al., 2016; dos Santos Rodrigues et al., 2020; Sharma et al., 2013, 2012; Sonali et al., 2016). In a recent study, dos Santos Rodrigues et al. (2020) successfully designed a dual-modified liposome containing CPP and transferrin ligands that promoted efficient delivery of a plasmid DNA in vitro and, into the mice brain.

Taking into consideration the large treatment inefficacy burdening individuals suffering from neuropsychiatric diseases, such as is the case of depression (Fornaro et al., 2014), the use of nanotechnology can widen up the possibilities for novel treatments. One attractive candidate is the brain-derived neurotrophic factor (BDNF), which is widely expressed in the CNS, especially in the hippocampus (Lessmann et al., 2003; Leßmann and Brigadski, 2009), and plays an important role in the regulation of several biological processes, including neuronal survival, differentiation, growth and plasticity (Alsina et al., 2001; Benarroch, 2015; Edelmann et al., 2014; Poo, 2001). It has been shown that BDNF is decreased in depressive patients (Dwivedi et al., 2003) and that antidepressant treatment increases BDNF levels (Fernandes et al., 2015; Polyakova et al., 2015; Sen et al., 2008). Local lentivirus BDNF infusion in the hippocampus alleviated depression-like behaviors in a rat model of post-stroke depression (Chen et al., 2015). Our studies in the $SERT^{-/-}$ rat model, which presents both decreased BDNF levels in the HIP and depression- and anxiety-like behaviors (Calabrese et al., 2015; Guidotti et al., 2012; Molteni et al., 2010), showed that BDNF lentivirus infusion meliorated their anxiety levels in the novelty-induced locomotor activity. Moreover, BDNF overexpression in the hippocampus of the $SERT^{-/-}$ rats, increased sucrose preference and intake in the sucrose consumption test, showing effects in anhedonia (Diniz et al. unpublished data). Despite promising results, BDNF local infusion into the human brain to treat depression seems to be not feasible. Moreover, neurotrophins, including BDNF do not readily cross the BBB (Pardridge, 2015). Therefore, the development of a delivery system to increase BDNF in the brain following a less invasive administration route might provide the opportunity to explore the enormous therapeutic potential of BDNF.

The present study sought to assess the feasibility to design a cell penetrating peptide tethered bi-ligand liposome for brain delivery of BDNF plasmid (Kim et al., 2014; Sharma et al., 2013, 2012). In this pilot experiment liposomes without any tag (plain

liposomes), liposomes tagged with transferrin (Tf-liposomes), tagged with arginine (PR-liposomes), and bifunctional liposomes (Tf-PR-liposomes) were developed and characterized for the main physical properties, such as particle size, zeta-potential, polydispersity index (PDI) was done (see schematic representation in figure 1). Moreover, selected liposomes preparations were used for pDNA encapsulation and checked for loading efficiency, in vitro cell uptake and transfection. Pilot synthesis and characterization of the liposomal delivery system aimed to assess in first instance the efficiency of BDNF plasmid encapsulation, pursuing to the further development of a non-viral system to mediate delivery across the BBB following peripheral blood stream infusion route. mRNA BDNF overexpression in the CNS is a promising approach to remediate the decreased BDNF protein levels encountered in neurological diseases such as mood disorders.

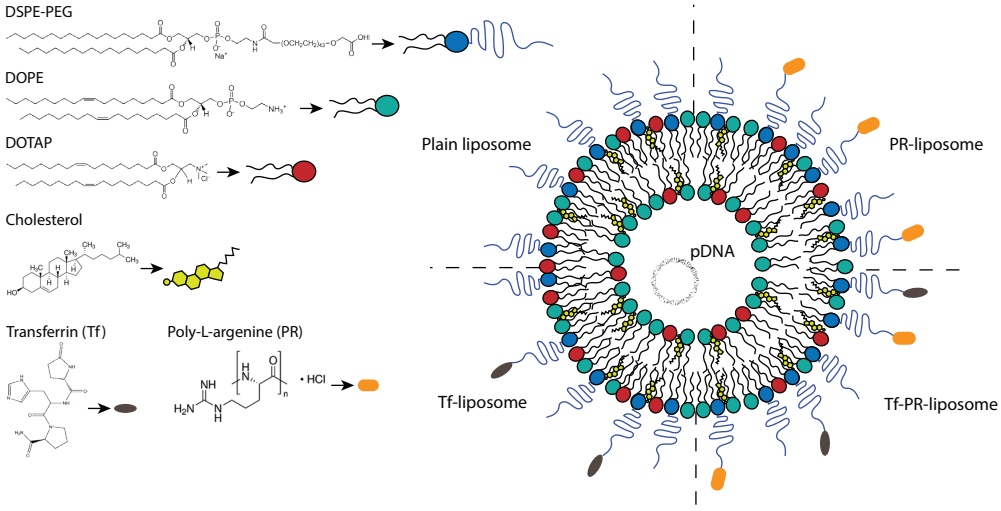

Figure 1. Schematic representation of the bifunctional liposomes. The structure of the lipids composing the liposome bilayer, the theoretical organization of the functional targeting with transferrin and/or arginine, and the encapsulation of the plasmid DNA (pDNA) are considered. According to Sharma et al. (2012), the following formulations were developed: Plain liposome (DOTAP/DOPE/DSPE-PEG/Cholesterol 45:45:8:2 mol %), PR-liposome (DOTAP/DOPE/DSPE-PEG/PR–PEG /Cholesterol 45:45:4:4:2 mol %), Tf-liposome (DOTAP/DOPE/DSPE-PEG/Tf–PEG /Cholesterol 45:45:4:4:2 mol %), Tf-PR-liposomes (DOTAP/DOPE/PR-PEG/Tf–PEG /Cholesterol 45:45:4:4:2 mol %).

Material and Methods

Materials

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol) 2000] (DSPE-PEG2000-COOH), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2 Dioleoyl-3-trimethylammonium-propane chloride (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Poly-L-arginine hydrochloride (molecular weight = 13,300 Da), holo-Transferrin human (Transferrin), 3β-Hydroxy-5-cholestene (Cholesterol), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), and N-Hydroxysuccinimide (NHS) were procured from Sigma–Aldrich Company (Darmstadt, Germany). The fluorescent dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine iodide (DiR) was obtained from Invitrogen (Landsmeer, Netherlands). BDNF (NM_012513) Rat Untagged plasmid was purchased from Origene (OriGene Technologies GmbH, Germany). All other chemicals used in this study were of analytical reagent grade.

Methods:

Coupling poly-L-arginine (PR) to DSPE-PEG

To obtain PR-liposomes, first, poly-L-arginine (PR) had to be coupled to DSPE-PEG. The synthesis procedure of PR–PEG was based on the previously described method (Huang et al., 2002). Briefly, PR was dissolved in 25 ml of 50mM sodium tetraborate buffer (STBB, pH 8.5) per gram of PR. The resulting solution was stirred vigorously for approximately 30 min and subsequently filtered through a 0.22µm Durapore® membrane (Sterile Millex GV, Sigma–Aldrich, Buchs, Switzerland) into a sterile culture tube. The appropriate stoichiometric amount of DSPE-PEG powder was then slowly added to the solution while it was continuously stirred. After another 6h of vigorous stirring at room temperature, the solution was transferred to a dialysis tube (Spectr/Por dialysis tubing, M.W.C.O. of 6–8 kDa, Spectrum Laboratories, Inc., Rancho Dominguez, CA). The synthesized product was dialyzed out for 24h in 10mM phosphate-buffered saline (PBS, pH 7.0), followed by an additional 24 h of dialysis in deionized water. The product was then freeze-dried for 48h at −70 ◦C with a pressure of 0.2 mbar.

Verification of PR-PEG coupling

Coupling efficiency was analyzed through size-exclusion high-performance liquid chromatography performed using Agilent 1100 series HPLC system (Agilent Technologies Inc., California, USA). Two tandem Shodex Protein KW803 (8.0mm×300mm, 5µm) and KW804 (8.0mm×300mm, 7µm) columns were used at ambient temperature. The mobile phase is an aqueous solution containing 20mMHEPES buffer at pH 6.5 (prepared by dissolving 4.42 g of HEPES and 0.38 g of HEPES sodium salt in 1000mL of water and filtered through a

0.45µm filter before use), and the flow rate was 0.5 mL/min. Stock solutions of 1mg/mL PR-PEG were injected. The HPLC trace was monitored with a UV/vis detector at a wavelength of 214nm and with a RI detector at an attenuation of 7.8×10^3.

Preparation of plain and PR-liposomes

Liposomes were prepared by lipid film hydration method as previously reported (Kim et al., 2010; Sharma et al., 2012). In short, stock solutions of each lipid in an organic solvent mixture ($CHCl_3$:MeOH= 2:1, v/v) were mixed in the following molar ratio: DOTAP/DOPE/DSPE-PEG/Cholesterol 45:45:8:2 and DOTAP/DOPE/DSPE-PEG/PR–PEG/Cholesterol 45:45:4:4:2 mol/mol % for plain and PR-liposomes, respectively. The organic mixture was removed on a rotary evaporator under reduced pressure with the temperature of water bath adjusted to 40 ◦C. Lipid film was hydrated with PBS buffer (pH 7.4) by vigorous vortexing. The resulting liposome dispersion was extruded 10 times (Avanti® Mini-Extruder, Avanti Polar Lipids, Inc.) using 0.2 µm polycarbonate membranes.

Development of Tf-PR liposomes

Bifunctional liposomes were prepared using the post-insertion technique (Sharma et al., 2012; Visser et al., 2005). Transferrin (Tf) was coupled to the phospholipid DSPE–PEG–COOH as reported previously (Li et al., 2009). Briefly, DSPE–PEG–COOH (remaining 4mol % of the total phospholipid content) was suspended in HEPES-buffered saline (pH 5.0) to form micelles. The micellar suspension was then treated with 360 µL of both EDC (0.5 M in H_2O) and NHS (0.5 M in H_2O) per 10 µmol of the phospholipid. Excess EDC was removed by dialysis, and the pH of the micellar suspension was adjusted to 7.3 with 0.1 N sodium hydroxide. Tf (125 µg/µmol of the lipid) was added to the resulting suspension and stirred at 25◦C for about 8h. The resulting Tf micelles were stirred overnight with PR liposomes at room temperature, and the final liposomal dispersion was passed through a Sepharose® CL-4B column to remove unbound protein.

Protein assay and Tf-binding efficacy

The average amount of transferrin conjugated to the liposome was quantified as described by (Anabousi et al., 2005). Shortly, one hundred microlitres of liposome dispersion were added to 400 µl of methanol. The mixture was vortexed and centrifuged (10s at 9000 x g). Then, 200 µl of chloroform were added, followed by vortexing and centrifugation (10s at 9000 x g). For phase separation, 300 µl of water were added, and the sample was vortexed again and centrifuged for 1 min at 9000 x g. The upper phase was carefully removed and discarded. Three hundred microliters of methanol were added to the chloroform phase and the interphase with the precipitated protein. The sample was mixed and centrifuged to pellet

the protein (2 min at 9000 x g). Then, the supernatant was removed, and the protein pellet was dried under a stream of air. The pellet was then dissolved in 20 µl of PBS (pH 7.4), and the concentration was determined with a bicinchoninic acid (BCA) protein assay using pure holo-transferrin as standard. The coupling efficiency was calculated as mg Tf/mmol PL.

BDNF plasmid transformation and purification

In order to have enough pBDNF for the liposomal formulations, the pBDNF amount was increased through bacterial transformation into E. coli (DH10β), which was performed using the heat shock method (Froger and Hall, 2007). In short, after a 10 minutes incubation in ice, the mixture of bacteria and pDNA was placed at 42°C for 90 seconds (heat shock) and then placed back in ice. LB media was added, and the transformed cells were incubated at 37°C for 45-60 min with agitation. To check that isolating colonies are irrespective of transformation efficiency, two quantities of transformed bacteria were plated. The number of pBDNF copies were quantified by OD_{600} using an UV-visible spectrophotometer (NanoDrop™, USA).

After achieving the desired amplification, pBDNF was purified using the endotoxin-free plasmid purification NucleoBond®Xtra system (Macherey-Nagel, Germany). The final concentration was measured by OD_{260} using an UV-visible spectrophotometer (NanoDrop™, USA), and diagnostic restriction digestion was used to confirm the rough structure of the plasmid on agarose gel electrophoresis 1% TEB buffer.

pDNA encapsulation

Plasmid DNA was incorporated at N/P ratio of 5 into the formulation using the encapsulation method or the complexation method as previously described (Sharma et al., 2012). Shortly, in the encapsulation method, plasmid DNA was added to the hydration buffer, which was added to the lipid film and vigorously vortexed at room temperature for about 20 minutes, followed by extrusion and size exclusion column (SEC) purification (Sharma et al., 2012). In the complexation method, pDNA was added after liposomes were extruded, gently mixed and incubated for about 30 minutes to allow the lipoplex formation. Thereafter complexed liposomes were purified through SEC (Kim et al., 2010).

Characterization of liposomes:

Particle size and zeta potential measurements

Dynamic light scattering (DLS) is a technique in physics that can be used to determine the size distribution profile of small particles in suspension or polymers in solution (Pecora, 2000). DLS measures the time-dependent fluctuations in the intensity of scattered light, which occurs because particles (liposomes) in a suspension undergo random Brownian

motion due to collisions between suspended particles and solvent molecules (Bozzuto, 2015). Therefore, the DLS allowed us to analyze the particle size and polydispersity index (PDI).

The ζ-potential is the overall charge of a particle acquires in a particular medium. A Laser Doppler Micro-electrophoresis was used to measure zeta potential. An electric field is applied to a solution of molecules or a dispersion of particles, which then move with a velocity related to their zeta potential. This velocity is measured using a patented laser interferometric technique called M3-PALS (Phase analysis Light Scattering), enabling the calculation of electrophoretic mobility, and from this, the zeta potential and zeta-potential distribution.

The size distribution, the average hydrodynamic particle size, and the zeta potential of the liposomes were evaluated using both systems described above, which are incorporated in the Zetasizer Nano (Malvern Instruments, UK). The samples were diluted in PBS-buffered saline (pH 7.4), and transferred into a disposable cuvette for particle size analysis and a capillary cell for ζ-potential measurement and inserted into the Zetasizer (Nano-ZS, Malvern Instrument, UK) at 25°C.

Measurement of plasmid BDNF Encapsulation Efficiency

The pBDNF encapsulation efficiency of the liposomes was calculated based on the previously reported method (Fillion et al., 2001; Gonçalves et al., 2004). DNA content of the samples was analyzed through a spectrophotometer. Shortly, DNA concentration was estimated by measuring the absorbance at 260nm, adjusting the A_{260} measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an A_{260} of 10 = 50 µg/mL pure dsDNA.

Concentration (µg/mL) = (A_{260} reading - A_{320} reading) x dilution factor x 50 µg/mL

Cell cultures

SH-SY5Y cells were cultured in Dulbecco's modified Eagle's Medium (DMEM, Life Technologies), with 7.5% Fetal calf serum (FCS), 400U/ml penicillin and 100µg/ml streptomycin (Gibco), at 37°C in a 5% CO_2 incubator. The cells were passed once a week, in a 1:5 dilution.

Evaluation of In-vitro Transfection Efficiency

Cells were cultured for 24 hours in a 12 wells plate, 10^3 cells/well. After these 24 hours, 1000nM of liposomes containing GFP plasmid was added. The medium was refreshed after 1 hour. The cells were kept in an Evos FL Auto 2 cell imaging system (Invitrogen). Hourly images were automatically captured to visualize the GFP expression. The cells were kept inside the Evos at 37°C in 5% CO_2.

Results and discussion

Resolving free DSPE-PEG from PEG-PR conjugate

The synthesis of the conjugate PEG-PR was particularly challenging. Because of the anticipated difficulty in revolving free PEG and PEG-conjugate, our initial efforts were focused on the identification of conditions separating these two species. We had to use different kinds of dialysis membranes to purify not only from free PEG, but also from free poly-L-arginine. During the freeze-drying procedure, we observed that the PEG-PR sample did not present loss of weight, indicating that the water molecules were not removed from the sample, and resulting in precipitation/ flocculation when we attempted to dilute PEG-PR in an organic solvent for the liposome preparation. According to Radaev and Sun (2002), during freezing, PEG tends to crystallize. This could explain increased water retention by the system (Tattini et al., 2005), and therefore solubility problems. Following the protocol proposed by Kim et al. (2010), we freeze-dried the PEG-PR solution for 48h at -70 ◦C with a pressure of 0.2 mbar. We also tried to decrease the temperature to -86°C and the pressure to 0.006 mbar; however, we encountered the same solubility problem.

Moreover, when measuring PEG-PR coupling efficiency through HPLC, we injected samples of 1mg/mL solutions of PR, PEG, and PEG-PR. We were not able to see a clear peak of the PR, PEG, or the PR-PEG conjugate. Altogether, we conclude that further studies are necessary to characterize the PEG-PR conjugation better. For example, through differential scanning calorimetry (DSC), the effects of the freeze-drying method over the structural and phasic changes in the conjugation of PEG with PR can be determined (Tattini et al., 2005). Additionally, Kim et al. (2010) confirmed the synthesis of PEG-PR through ^{1}H NMR in D_2O and gel permeation chromatography (GPC). Therefore, we will have to repeat the experiments and perhaps the use of other techniques could help in the identification of the coupling efficiency of the PEG-PR. The samples produced that achieved organic solvent solubility were used to develop the liposome formulations.

Tf-binding efficacy

We planned to access the amounts of transferrin using a bicinchoninic acid (BCA) assay following Anabousi et al. (2005). We did not conduct the entire experiment, but following the specifications of the manufacturer (BCA™, Scientific, USA), we built a calibration curve using human transferrin, which would be used as the standard to analyze the transferrin content in the liposomes. The BCA assay offers the advantage of producing a linear response curve. This response curve allows accurate determination of unknown protein concentrations in the liposome surface. Given that the PEG-Tf micelles contained transferrin concentration of about 58µg/mL, which are post-inserted to the already formed and extruded liposomes, and giving the possibility that not all chains of PEG are incorporated to

the final Tf-liposome, we built the curve with concentrations starting from 5µg/mL. The linear response curve (R^2 > 0.95) obtained is showed in **figure 2** and it will help us to further determine the amount of transferrin in the liposomes.

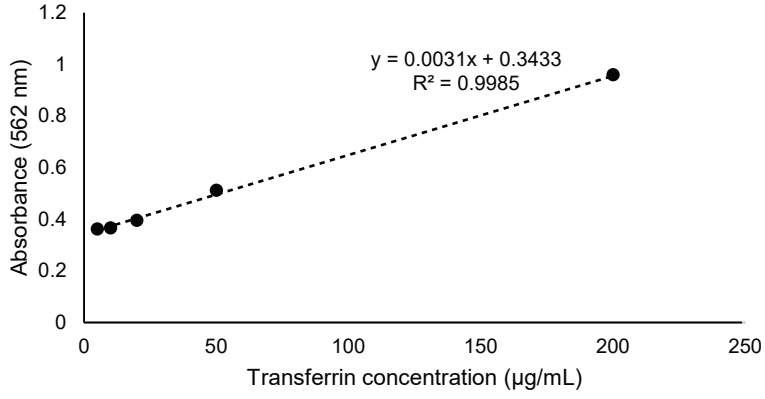

Figure 2. Standard curve for transferrin quantitation. Holo transferrin human in 0.9% saline (5–200 µg/mL) was used to generate standard curves for the BCA Protein Assay. The assay was conducted according to the manufacturer's protocols in a test-tube format.

pBDNF purification

Plasmid BDNF purification was checked by UV spectroscopy using the ratio of A_{260}/A_{280} and A_{260}/A_{230}. We verified a ratio of A_{260}/A_{280} = 1.84 ± 0.01 and A_{260}/A_{230} = 2.3, indicating that the obtained samples contained a pure plasmid DNA (NucleoBond®Xtra, Macherey-Nagel, Germany). **Figure 3** demonstrates the result of the diagnostic restriction digestion. According to the manufacturer, the pCMV6-Entry plasmid backbone plus the BDNF insert (NM_012513) has about 5.6 kb (OriGene Technologies GmbH, Germany). We notice that digestion with the restriction enzyme Hind III should have linearized both the supercoiled and nicked forms of the plasmid, but there has probably been an incomplete digestion. Therefore, although spectroscopy analysis indicates that we obtained copies of the pBDNF, to check the size and the characteristics of the plasmid correctly, we will probably need to increase the enzyme and/or increase the incubation time to ensure complete digestion.

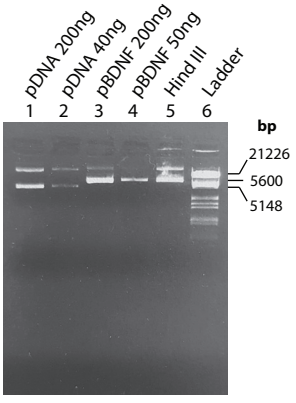

Figure 3. Evaluation of the pBDNF amplification and purification in agarose gel 1% TEB. Standard plasmids of known concentration were used (lanes 1 and 2), and two different concentrations were used to check uncut pBNDF (lanes 3 and 4). Digestion of 250ng of pBDNF by Hind III is expressed in lane 5, and marker III (Roche) was used as DNA molecular weight marker.

Particle size, zeta potential and PDI characterization of the liposomes

Determining the physicochemical properties of the liposomes is essential because it can assess its passage mechanism across the BBB (Saraiva et al., 2016). There is evidence for a inverse correlation between BBB penetration and the size of the liposome (Etame et al., 2011; Hanada et al., 2014; Sonavane et al., 2008). The preferred size for brain delivery is 100nm or smaller, but studies have shown that liposomes from 100 to 140 nm have certain advantages, such as a longer half-life in blood circulation and avoidance of plasma proteins (Ross et al., 2018). Moreover, because the BBB is negatively charged, cationic liposomes can trigger cell internalization through electrostatic interactions (Harilal et al., 2020). The physico-chemical characterization of the main properties of prepared liposomes are reported in table 1.

Table 1. Particle Size, polydispersity index (PDI), and zeta (ζ)-potential of liposomes before and after coupling of transferrin (Tf) and poly-L-arginine (PR) at pH 7.4. Data are shown as means and standard deviation (n=3). nd: not determined.

	Physical properties		
Liposome composition	Particle Size (nm)	PDI	ζ-potential (mV)
Plain (empty)	157.6 ± 1.1	0.102 ± 0.013	7.16 ± 0.72
Plain (encapsulating pBDNF)	138.9 ± 0.8	0.1065 ± 0.013	5.915 ± 0.71
Plain (complexated with pBDNF)	178.4 ± 0.8	0.082 ± 0.033	-4.18 ± 2.94
Tf-liposomes (encapsulating pBDNF)	125.1 ± 0.6	0.152 ± 0.014	-8.73 ± 1.13
PR-liposomes (encapsulating pBDNF)	124.7 ± 0.5	0.106 ± 0.014	7.83 ± 0.68
Tf-PR-liposomes (empty)	177.24 ± 75.5	nd	nd

As expressed in table 1, the different liposome preparations present diverse physical properties. As expected, the results showed that the incorporation of Tf decreased the zeta potential to a negative value. Anabousi et al. (2005) have demonstrated that using DSPE-PEG_{2000}-COOH as linker lipid led to the highest amount of bound Tf, and the negative charge of the ζ-potential indicated that Tf was attached to the liposome surface. On the other hand, usually, the coupling of PR to the liposome surface yields a higher positive charge. Sharma et al. (2012) demonstrated that PR-liposomes presented a ζ-potential of 20.25 ± 3.6 mV, which were stable after 30 days storage presenting ζ-potential of 21.30 ± 3.5 mV (Sharma et al., 2013). Kim et al. (2010) have also shown that PR-PEGylated liposomes displayed ζ-potential of 32.2 ± 3.7 mV. Therefore, considering that the ζ-potential of our PR-liposomes was very similar to that of the plain liposomes, we can suggest that PR-PEG coupling efficiency was either low or did not occur.

Furthermore, we also noticed that the different ways of loading the liposomes with pDNA generated different phisical characteristics in the plain liposomes. The addition of pDNA during liposome formation resulted in posively charged liposomes (Parker et al., 2003). Lipoplexes formulations, in which pDNA was added to the solution containing the already formed liposome (Kim et al., 2010), instead showed a slight negative ζ-potential, suggesting the presence of a higher amount of pDNA on the surface of liposomes. Sakurai et al., (2000) showed that cationic liposomes can aquire a negative charge depending on the amount of plasmid added to the formulation. Thus, it is likely that the pDNA post-added to the liposome formulation modulated the ζ-potential conferring negative surface charge to the plain liposomes.

Plasmid encapsulation efficiency

The pDNA loading efficiencies for plain, Tf, and PR-liposomes were 44.1%, 27.6%, and 57.5%, respectively. Our results are in agreement with those of Sharma et al. (2012), in which plain liposomes presented an efficiency loading of 35±4.3%, and Tf- and PR-liposomes, encapsulation efficiencies of 33±5.2% and 40±4.1%. However, we observed that several factors interfered with the absorbance measurements. For example, we initially used 1% Triton-x in order to break the liposomes and measure the freed pDNA concentration. However, we observed that the Triton-x interfered with the DNA measurement showing absorbance at 260nm. We replace the Triton-x by methanol (Podesta and Kostarelos, 2009; Zhang et al., 2010), and then, we were able to achieve more reliable results. Still, for further studies, we would like to conduct agarose gel electrophoresis to better access the pDNA content of the liposomes (Kim et al., 2010).

Gene expression

Plain liposomes and PR-liposomes encapsulating pGFP were used to access the transfection efficiency in neuroblastoma cells (SH-SY5Y) using a live-observing fluorescent microscopy. Figure 4 shows the bright field representation of the images taken during the experiment. We were not able to observe any fluorescence emission 0, 24, 48 or even 72 hours after adding the liposomes to the cells, indicating that these liposomes did not transfect the cells. The neuroblastoma cells line used in this study are often used to understand neuronal signaling. These cells are typically transfected via the calcium phosphate method or electroporation (Carrì et al., 1997; Hasegawa et al., 2004), but also different reports demonstrated successful transfection with liposomes, indicating that these cells should be sensitive to liposomal transfection (Betz et al., 2003; Obata et al., 2010). Therefore, other factors might have contributed to the failure of the transfection. It is likely, for example, that the cells did not uptake the liposomes or that the purification of the liposomes was not sufficient leading to cytotoxic effect over the cells. In conclusion, further improvement of the liposome synthesis will be necessary to achieve optimal transfection.

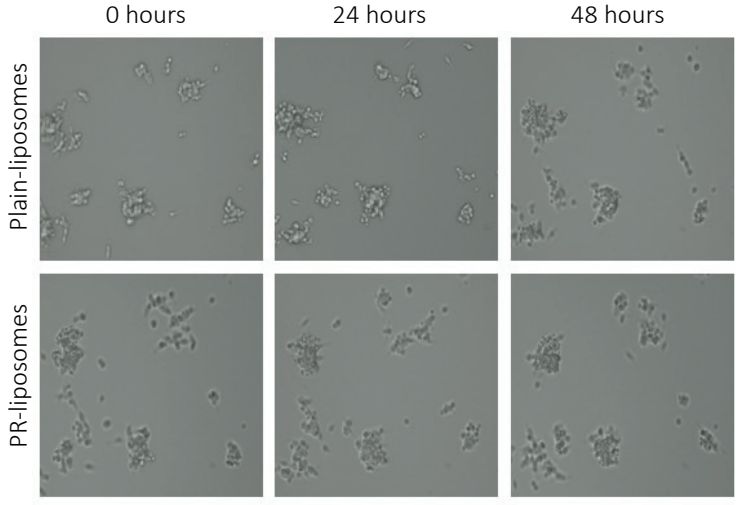

Figure 4. Live-cell imaging of SH-SY5Y cells transfected by plain and PR-liposomes using Invitrogen EVOS M7000 Imaging System. Bright-field and fluorescence pictures were taken hourly by the automated microscope. Selected bright-field images at 0, 24, and 48 hours were chosen to represent the study, but fluorescence images did not display any emission.

Conclusion and perspectives

In this study, we described the preparation of liposomal carriers to promote gene delivery through the blood-brain-barrier. We synthesized plain liposomes containing cationic lipids and PEGylated surface, PR-liposomes containing the cell-penetrating peptide arginine, and Tf-liposomes containing transferrin which promotes receptor-mediated endocytosis. Moreover, we used both conjugates to develop the bifunctional dual-targeted Tf-PR-liposomes. This pilot study revealed the challenges of tagging the liposomes with the ligands Tf and PR. Notably, we encountered several limitations when verifying the coupling efficiency between the DSPE-PEG and the conjugates PR and Tf. Purification of the free peptides from the final liposomal product is a critical step to effectively validate liposome formulations for their clinical use to ensure that the observed therapeutic effects are due to the liposome conjugate and not from non-conjugated contaminants (Manjappa et al., 2011; Nobs et al., 2004). This step in the liposome production will therefore need further analysis.

Once the pBDNF-loaded liposome carrier is well characterized, subsequent studies should evaluate the delivery of specific BDNF gene transcripts to target desired brain regions (Aid et al., 2007; Baj et al., 2011; Pruunsild et al., 2007). Besides vector modifications, gene delivery efficiency can also be dramatically enhanced through strategic modification of DNA composition and conformation, thereby improving bioavailability, biocompatibility, durability, and safety (Foldvari et al., 2016). The use of cell-type-specific promoters will confine the transgene expression to a specific cell type. For example, decreased BDNF levels in the hippocampus characterize the pathology of depression (Castrén et al., 2007; Castrén and Rantamäki, 2010; Dwivedi et al., 2003; Ray et al., 2011). Identification of the subcellular localization of the hippocampal BDNF decrease will help in the desing of a plasmid vector that contains a promoter enhancing BDNF gene expression in that specific cellular subpopulation. In principle, specifically targeting a population of neurons or glial cells might allow to achieve the therapeutic goal without off-target effects (Ingusci et al., 2019). For example, the phosphate-activated glutaminase (PAG) or the vesicular glutamate transporter (vGLUT) promoter ensures ~90% glutamatergic neuron-specific expression, whereas the glutamic acid decarboxylase (GAD) promoter ensures ~90% GABAergic neuron-specific expression (Rasmussen et al. (2007). Furthermore, the specific cellular location of the astrocyte-specific glial fibrillary acidic protein (GFAP) in the CNS has encouraged its extensive use to target transgene expression to cells of glial origin (Lee et al., 2008). Astrocytes as a target is desirable because this cell is in immediate contact with the BBB; additionally, a study designed by Quesseveur et al. (2013) indicated that BDNF released from astrocytes acts on post-synaptic cells in the hippocampus to stimulate neurogenesis, and mediate related anxiolytic-and antidepressant-like activities.

Combining several physico-chemical and genetic modifications into multicomponent non-viral vectors, such as liposomes, is an ongoing challenge but should prove possible. Optimizing both vector and genetic load provides a powerful tactic for development of effective non-viral gene therapy systems that might lead to a new therapeutic approach to deliver BDNF to the brain. Although further research is necessary to determine the safety of gene therapy and to improve drug targeting, liposome-mediated gene transfer is a promising avenue of research into the treatment of central nervous system diseases.

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5

General Discussion and Future Perspectives

Thesis objective and main findings

Depressive disorders account for the largest contributor to global disability. Impaired production, release, and/or action in the BDNF system is associated with depression, and drugs used for the treatment of depression often cause an increase in BDNF levels. How the BDNF is involved in the pathophysiology of depression remains unclear. A better understanding of the pathways affected by BDNF will provide new insights into depressive disorders and might lead to potential new therapeutic targets. This thesis aimed to unravel the behavioral and molecular consequences of BDNF overexpression in two key brain areas involved in the neuropathology of depression, the prefrontal cortex (PFC), and the hippocampus, in a rat model presenting both decreased BDNF levels and depression-like phenotype (Figure 1).

In **chapter 2,** we showed that although naïve $SERT^{+/+}$ rats present normal levels of BDNF in the PFC, these animals are still able to have the endogenous BDNF mRNA upregulated through external factors such as the virus-mediated gene overexpression. The BDNF gene upregulation was best achieved two weeks after the local infusion in the prelimbic cortex (PrL) when levels of total BDNF in the PrL were higher in BDNF-treated animals than in control GFP-treated $SERT^{+/+}$ rats. Next, we set out to investigate how BDNF overexpression in the PFC would modulate the response of $SERT^{-/-}$ rats in behavioral challenges in which such animals are known to display anxious- and/or depression-like phenotypes. We observed that in general, the BDNF upregulation did not alter the outcome from the behavioral tests in $SERT^{-/-}$ rats, nor did it modulate the response of the WT animals. Few exceptions applied. For example, in the sucrose preference test, $SERT^{-/-}$ rats receiving BDNF lentivirus presented distinct behavior from $SERT^{-/-}$ rats receiving control virus. BDNF overexpression seemed to induce neophobia, leading to a decrease in the sucrose preference, which was followed by anti-anhedonic behavior, as reflected by an increase in sucrose preference. Another exception involved the CORT levels as measured in the HPA-axis reactivity test. We found that BDNF overexpression in WT animals mediated an impairment in the HPA-axis activity, as reflected by increased CORT baseline levels and decreased CORT levels in response to stress. Further, in $SERT^{-/-}$ rats, BDNF overexpression normalized the CORT stress levels, suggesting that different mechanisms underlie the HPA-axis reactivity modulation by BDNF in $SERT^{-/-}$ and WT rats.

Considering that BDNF overexpression in the PFC failed to rescue most of the anxiety- and depression-like behaviors in the $SERT^{-/-}$ rats, we decided to target another brain area in which BDNF levels are also decreased in this animal model, the ventral hippocampus (vHIP). Therefore, in **chapter 3,** we conducted the same battery of experiments, as mentioned in **chapter 2**, to assess whether BDNF overexpression in the vHIP of $SERT^{-/-}$ rats would remediate their impaired phenotype. Several interesting outcomes were revealed in this

study. In the first experiment, we evaluated the molecular effects of BDNF lentivirus containing exon IV over other brain areas and transcripts. Especially, in the first week following the lentivirus infusion, BDNF was in $SERT^{+/+}$ overexpressed in the vHIP, but downregulated in the PrL and IL. These findings indicate not only that these areas are strongly connected, but also BDNF transcripts provide negative feedback to distal areas from the vHIP. Behaviorally, we found that BDNF overexpression in the vHIP of $SERT^{-/-}$ rats promoted a higher preference for sucrose and higher intake of sucrose solution, motivating especially the rats when they were faced with this novel taste for the first time. Likewise, in the novelty-induced locomotor test, $SERT^{-/-}$ rats infused with BDNF lentivirus spent more time in the center of the novel test cage than control-infused $SERT^{-/-}$ rats, suggesting that BDNF decreased the anxiety-like behavior expressed by $SERT^{-/-}$ rats only when BDNF was overexpressed in the vHIP.

Noteworthy, although in the experiments performed in chapter 2, we used commercially available Wistar rats as WT controls and in chapter 3 $SERT^{+/+}$ rats from our own breeding, we did not observe major differences when comparing $SERT^{-/-}$ to these two different animals. One exception applied to the novelty-induced locomotor test, in which $SERT^{-/-}$ rats presented decreased distance moved and velocity compared to commercial Wistar rat, but not compared to $SERT^{+/+}$ rats. Moreover, behaviorally speaking, Wistar and $SERT^{+/+}$ rats were both in few instances affected differently by BDNF overexpression in comparison to their respective controls; however, it seems that these differences were more related with the site of infusion than with intrinsic behavioral differences. For example, $SERT^{+/+}$ rats treated with BDNF presented increased sucrose consumption compared to control-treated $SERT^{+/+}$ on the first test day, but the Wistar rats treated with BDNF did not differ from control-treated Wistar rats. However, on the second test day, both groups of rats presented sucrose consumption comparable to GFP-infused controls. As another example, Wistar rats treated with BDNF presented increased CORT levels at baseline and decreased CORT levels after restraint stress, which was not observed in BDNF-treated $SERT^{+/+}$ rats. However, 60 minutes after stress, levels of CORT were normalized in both $SERT^{+/+}$ and Wistar rats. More importantly, we demonstrated that both animals had their BDNF levels modulated by the viral infusion, showing significantly higher BDNF gene expression than controls.

In **chapter 4**, we demonstrated a pilot study aiming for the development and characterization of a gene delivery device to carry plasmid BDNF. We reported the synthesis of a nanocarrier ranging 120-200nm in size, composed by cationic lipids, and termed liposome. To enhance the transfection capability, reduce toxicity, and increase the circulating time, we labeled the liposomes with polyethylene glycol (PEG). Furthermore, we functionalized the liposomes, attaching to the PEG chains peptides to promote blood-brain-barrier (BBB) targeting. We used Tf and arginine to enhance BBB crossing via receptor-

mediated endocytosis. Finally, the liposomes were tested for their plasmid DNA loading capacity and in vitro transfection efficiency. In conclusion, in this pilot study, we reported that, although there are still features that need to be refined in the liposomes, such as the PEG to peptides conjugation reactions, the development of a gene carrier was feasible. Thus, further improvement of the liposomal gene delivery system could enable BDNF overexpression in targeted brain areas to promote novel treatments for depressive patients.

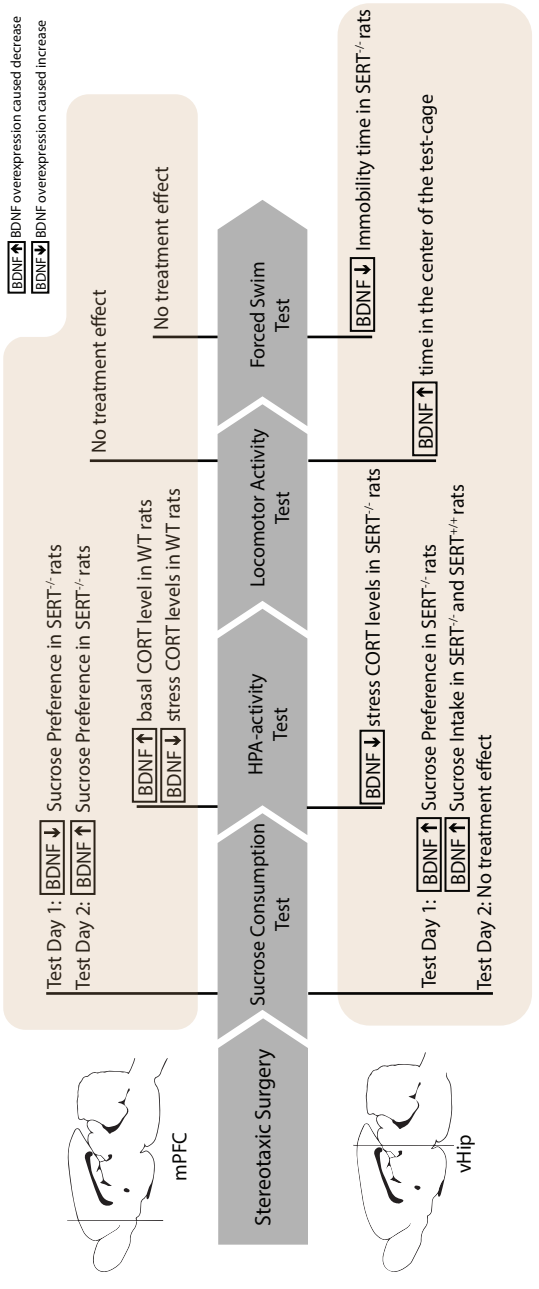

Figure 1. Schematic representacion of the main behavioral findings.

Sucrose consumption test, anhedonia, BDNF, and depression – translating the findings

In humans, one of the core symptoms of depressive disorders is anhedonia. In the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)(American Psychiatric Association, 2013), anhedonia is described as a loss of interest or pleasure in nearly all activities. Therefore, generally speaking, the definition of anhedonia in humans by the DMS conceptualizes anhedonia as a steady-state, mood-like phenomenon, in which individuals exhibit a general tendency to feel unmotivated or fail to experience pleasure. The assessment of anhedonic symptom has primarily been achieved through self-report instruments. These reports consist of a questionnaire in which the answers given by the patient are rated and reveal the severity of anhedonia (Andreasen, 1989). Some classically used self-report instruments are the Chapman Anhedonia Scale (Chapman et al., 1976), the Scale of Negative Symptoms (SANS; Nancy C. Andreasen 1982), the Fawcett–Clark Pleasure Scale (FCPS; Fawcett et al., 1983) and the Snaith–Hamilton Pleasure Scale (SHAPS; Snaith et al., 1995). Unfortunately, anhedonia clinical diagnosis through these self-report tools does not discriminate between a decrease in motivation and a reduction in experienced pleasure (Treadway and Zald, 2011).

With the deepening research of anhedonia, studies both in animal and in humans (Berridge and Robinson, 2003; Kringelbach and Berridge, 2009) have indicated that pleasure experience is not a unitary construct, but can be parsed into two distinct subcomponents, namely anticipatory (or wanting) and consummatory (or liking) pleasure (Berridge and Kringelbach, 2008). The anticipatory pleasure factor captures the future-oriented pleasure, while the consummatory pleasure factor captures in-the-moment pleasure. To capture specifically these two distinguishing components, Gard et al. (2006) developed the Temporal Experience of Pleasure Scale (TEPS) based on theoretical models of anticipatory and consummatory pleasure. This scale is a promising advance towards identifying in clinical populations the two aspects of anhedonia in the face of enjoyable activities: the lack of motivation (“wanting”) and the deficits in hedonic capacity (“liking”) (Cooper et al., 2018). The dissociation between the different components of anhedonia consists of an important step towards the comprehension of the neurobiological substrates underlying the expression of this symptom, especially because anhedonia is a particularly difficult symptom to treat. In fact, substantial evidence suggests that current first-line pharmacotherapies (e.g., SSRIs) do not adequately address anhedonia in depression (Di Giannantonio and Martinotti, 2012; Grassi et al., 2010; Treadway and Zald, 2011; Yee et al., 2015), and the presence of anhedonic symptoms is a predictor of poor treatment response (McMakin et al., 2012; Price et al., 2009; Spijker et al., 2001).

Ineffective treatment outcome using highly selective SSRI medication is not uncommon, indicating that other neurotransmitters than serotonin, might play a role in anhedonia. For instance, s-allele carriers of the human 5-HTTLPR, which present decreased expression of the serotonin transporter, and therefore increased extracellular levels of serotonin (Homberg et al., 2014), displayed reduced responsiveness to reward when exposed to stress, indicating anhedonic behavior (Nikolova et al., 2011). Additionally, studies have confirmed increased negative attentional bias in short allele carriers (Fox et al., 2009) what can contribute to the development of anhedonia. Under these circumstances, further characterization of anhedonia in animal models is very useful to study the underlying mechanisms behind the abnormal reward-based decision-making seen in depressed patients (Treadway and Zald, 2011).

The most frequently used test to measure sensitivity to reward in rodents is the consumption of, or the preference for, palatable sweet solutions, sucrose or saccharin. In the two-bottles paradigm, animals can choose between a palatable sweet solution and plain water. A decreased consumption of, or preference for, palatable solutions is considered to reflect a condition of "hedonic deficit," or anhedonia. Because rodents can show a side preference in drinking behavior that can affect the test outcome, the position of the water and sucrose solution bottles is switched during the test (Strekalova et al., 2004).

As discussed in **chapter 1**, rodents do not carry the human serotonin transporter linked polymorphic region (5-HTTLPR), but genetic deletion or partial deletion of the SERT in rodents leads to anxiety- and depression-related phenotypes (Kalueff et al., 2010; J. D.A. Olivier et al., 2008). $SERT^{-/-}$ rats present, for example, hyposensitivity to natural rewards displayed by its decreased sucrose consumption (J. D.A. Olivier et al., 2008). Interestingly, in the study presented in this thesis, we have demonstrated that BDNF overexpression modulated the sucrose preference and sucrose intake in $SERT^{-/-}$ rats depending on the targeted brain region, causing both decreased sucrose preference in the PrL-infused animals, and increased sucrose over water preference in the vHIP-infused animals. This different response to sucrose solution was especially evident on the first day of the test, when the animals were presented to a novel and unexpected reward. BDNF upregulation in the hippocampus favored the positive response to the reward, and in the PFC, it caused a neophobia in animals naïve to sucrose, therefore showing that the difference was not strictly related to the hedonic state, but a deficit in reward processing. Accordingly, when the previously unexpected reward becomes predicted, BDNF in the vHIP was not involved in the sucrose associative learning and reinforcement anymore. However, the preference for sucrose was increased in PFC-infused animals, showing that reward motivation was enhanced when the reward became predictable. These results highlight the fact that BDNF

might modulate specific neuronal circuitries in different brain regions, and emphasizes that pleasure experience is not a unitary construct.

Although distinct neural regions code for separate reward processes, the circuits connecting these regions allow an individual to (i) sense a pleasant stimulus; (ii) compute reward value and associated costs; (iii) determine effort requirements to obtain that stimulus; (iv) decide to obtain that stimulus; and (v) anticipate and increase motivation to obtain that stimulus. Der-Avakian and Markou (2012) proposed neural bases of the construct of anhedonia, which reflects deficits in hedonic capacity and is also closely linked to the constructs of reward valuation, decision-making, anticipation, and motivation. According to their review based in several clinical and preclinical studies, the hedonic perception of rewards is mediated primarily by endogenous opioid, GABA, and endocannabinoid systems in the nucleus accumbens (NAc), ventral pallidum, and orbitofrontal cortex (OFC). The OFC and ventral striatum receive inputs from sensory cortices and calculate the reward values. The OFC then projects reward value information to the anterior cingulate cortex (ACC) to incorporate costs, benefits, and reinforcement history to determine the effort required for different possible actions. The ACC sends projections to the anterior ventromedial prefrontal cortex (vmPFC) and dorsolateral prefrontal cortex (dlPFC), which are involved in decision-making based on reward value, effort and reinforcement history regarding future actions. Glutamatergic efferents relay this information to the NAc, which receives dopaminergic and glutamatergic inputs from the ventral tegmental area (VTA) and amygdala, respectively; these provide incentive salience properties and increase motivation to carry out the goal-directed action planned in the PFC (see review Der-Avakian and Markou, 2012). This proposed circuit might be an incomplete view of the neurobiology mediating different aspects of anhedonia and other reward-related deficits. Indeed, the multiple reciprocal connections between different PFC subregions, as well as reciprocal connections with the NAc, VTA, amygdala, and hippocampus, probably play an important role in regulating the behavioral response to rewards (Der-Avakian and Markou, 2012)

Interestingly, Verheij et al., (2016) demonstrated that brain-wide inhibition of TrkB signaling did not affect operant responding for a highly palatable sweet solution in Wistar rats; however, it did alter cocaine intake, suggesting that BDNF signaling in the mesolimbic dopamine pathway, which is involved in reward, is not recruited by sweet reinforcers. Applying this information to our $SERT^{-/-}$ rat model and considering that $SERT^{-/-}$ rats do not present compensatory alterations in the dopaminergic system (Homberg et al., 2007), we could agree that BDNF downregulation in the $SERT^{-/-}$ rats does not disturb the dopaminergic pathway. Nevertheless, although general inhibition of BDNF signaling might not alter sweet intake, our study does show that reinforcement of BDNF signaling by overexpression in the ventral hippocampus and mPFC resulted in altered sucrose consumption in $SERT^{-/-}$ rats,

suggesting that cocaine vs. natural rewards in SERT$^{-/-}$ rats are either differently mediated by BDNF or neural circuits and mediators of the construct of anhedonia are differently affected by BDNF. Indeed, deficit in consummatory anhedonia, assessed in animals as a decrease in sucrose intake or preference, is particularly mediated by the nucleus accumbens (NAc) and ventromedial prefrontal cortex (vmPFC) and involves stimulation via opioid and $GABA_A$ receptors (Der-Avakian and Markou, 2012; Treadway and Zald, 2011). Cocaine promotes dopamine (DA) signaling in the NAc, primarily by acting on DA terminals in this region to increase extracellular DA levels (Koo et al., 2012). In the context of drug addiction, BDNF is best characterized for its role in promoting the neural and behavioral plasticity induced by cocaine or other stimulants via actions on the mesolimbic dopamine (DA) system, where the BDNF pathway is engaged in a feed-forward loop that promotes further actions of stimulant drugs (Graham et al., 2009; Lobo et al., 2010; Lu et al., 2004; Verheij et al., 2016). Interestingly, opiates also act on the ventral tegmental area (VTA), and nucleus accumbens (NAc) to produce reward acutely and addiction chronically (Koo et al., 2012), but differently from cocaine, opiates promote DA signaling to the NAc by inhibiting local g-aminobutyric acid (GABA) interneurons in the VTA, which then disinhibit (activate) VTA DA neurons (Hyman et al., 2006). Opioids and cocaine have opposite effects on BDNF levels in the ventral tegmental area (VTA). While cocaine and other stimulants increase BDNF levels in the VTA, opiates downregulate intracellular BDNF signaling in this area (Berhow et al., 1995; Lu et al., 2004; Vargas-Perez et al., 2009).

Altogether, these data show that distinct substances producing pleasure in individuals are diversely regulated by the different brain circuitries involving reward. Moreover, BDNF shows to be differently modulated in these circuitries, thus proving the likelihood that BDNF modulates anhedonia differently in the PFC and the hippocampus. We propose that in the SERT$^{-/-}$ rats, BDNF upregulation in the PFC might enhance top-down control over the NAc through glutamatergic efferents (Der-Avakian and Markou, 2012) increasing GABA activity and decreasing DA release, consequently decreasing sucrose preference. BDNF in the hippocampus might have enhanced the GABAergic inhibitory regulation over the amygdala which is decreased in SERT$^{-/-}$ rats (Calabrese et al., 2013; Luoni et al., 2013; Miceli et al., 2017; Schipper et al., 2019) causing an increased response to sucrose. On the second day of the test, predictability of the reward caused a switch in the SERT$^{-/-}$ rats response to sucrose, indicating different roles of BDNF in the PFC and Hippocampus regarding the consummatory, anticipatory or motivational circuits involved in anhedonia (Der-Avakian and Markou, 2012). Therefore, an examination of the implications of such discrete differences in BDNF action over the anhedonic-like behavior in SERT$^{-/-}$ rats might provide further clarification of the pathways involved in the expression of the

anhedonic behavior, contributing to the development of a therapeutic strategy to address this core symptom of the human depressive disorder.

Forced Swim test and Novelty-induced locomotor test, anxiety, despair, and depression – translating the findings

Another cardinal symptom in depressive disorder is depressed mood. According to the DMS-5 depressed mood is defined as a feeling of sadness, emptiness, or hopelessness (American Psychiatric Association, 2013). Hopelessness is associated with negative perspectives concerning the future and plays a key role in the trajectory of depression (Assari and Lankarani, 2016). Several studies have documented hopelessness as a strong predictor of suicidal behavior (Britton et al., 2008; David Klonsky et al., 2012) showing that such symptom requires attention when dealing with depressed patients. The depressed mood can also be accompanied by fatigue or loss of energy, psychomotor agitation or retardation, and further typical symptoms are anxiety, agitation or restlessness. Theoretically, 681 combinations of symptoms can exist that meet the DSM-5 diagnosis of depression (Akil et al., 2018), and symptoms of depression occur in other disorders. Altogether, the complexity of depressive symptoms contributes to difficulties in providing behavioral tests that completely exposes some of these symptoms. For example, assessing "feelings of worthlessness or excessive or inappropriate guilt" is impossible in animals, as well as accessing hopelessness or suicidal thoughts.

In the 1970s, Roger Porsolt and colleagues introduced the forced swimming test for mice (Porsolt et al., 1977) and rats (Porsolt et al., 1978), and this work became a landmark for depression modeling in many respects. Initially, the FST was developed to predict the clinical efficacy of antidepressant drugs (Bogdanova et al., 2017). In his earlier observations, Porsolt proposed that immobility in the FST reflected a state of lowered mood in the rat and showed that antidepressants reduced this immobility (PORSOLT et al., 1977). Later, the forced swimming test was presented as a depression animal model under the concept of behavioral despair (Bogdanova et al., 2017). However, although the FST is considered the gold standard for studying depression-like behaviors, there are strong reasons to question the interpretation that immobility represents 'despair' and escape-directed behaviors such as climbing represent the absence of a depression-like phenotype (Anyan and Amir, 2018). It has been proposed that the forced swim test is useful to investigate coping strategies in the face of a stressor, providing valuable information in the process of achieving stress adaptation, which is impaired in depression (de Kloet and Molendijk, 2016; Herman, 2013). Moreover, while immobility behavior can be seen as a learned response, escape-directed behaviors might be driven by an anxiety-like response (Anyan and Amir, 2018), what can even

be an indication that these two responses in the FST are differently activated. Yet, both can have implications in the physiopathology of depression.

Anxiety-like phenotypes in rodents are often based on activity levels, such as reduced exploratory activity in the open field. Since its development by Hall (1934), the open field test (OFT) has become a very widely used tool in behavioral research. The procedure consists of subjecting an animal, usually a rodent, to an unknown environment from which escape is prevented by surrounding walls (Walsh and Cummins, 1976). An increase in central locomotion or in time spent in the central part of the device without modification of total locomotion and vertical exploration can be interpreted as an anxiolytic-like effect. At the same time, the contrary, that is, a decrease in these variables, is associated with anxiogenic effects (Prut and Belzung, 2003). In fact, anxiety behavior in the open field is triggered by two factors: social isolation (the animal is separated from its social group) and neophobia (as the arena is different relative to the animal's breeding or natural environment). In rodents, forced confrontation with novelty is stressful (Misslin and Cigrang, 1986). Generally, the novelty-induced locomotor activity test will reveal the animal response to a stressful event, assessing the changes in emotionality induced by exposure to a novel environment (Choleris et al., 2001). Therefore, anxiolytic treatments do not themselves increase exploration in the open field, but they decrease the stress-induced inhibition of exploration behavior. Exploring the neuronal basis of altered locomotor activity can help in the treatment of the psychomotor agitation or retardation symptoms seen in depression (American Psychiatric Association, 2013).

Depression rarely strikes alone. In clinical populations, depression is highly comorbid with anxiety disorders, with estimates as high as 80% (Lamers et al., 2011). Despite the high degree of comorbidity between depression and anxiety in clinical populations, only a few studies are examining individual differences in comorbidity between depression- and anxiety-like behaviors in rodents. Comorbid anxiety and depression are often more resistant to pharmacologic treatment, and patients with coexisting disorders have a poorer medical prognosis than do patients with either disorder alone (Ballenger, 2000). In light of the high complexity of depression and of its high comorbidity with anxiety disorders, the chance of succeeding in developing comprehensive animal models that reflect the relative influences of contributing factors accurately in humans is probably quite poor. However, a specific symptom or subset of symptoms can be modeled (Czéh et al., 2016). Gene knockout animals are one of the methodologies that can be employed to model comorbidity of clinical populations. The $SERT^{-/-}$ animal model, for example, presents alteration in the serotonin system, which is implicated in both depression and anxiety disorders in clinical populations; moreover, most commonly used drugs to treat these disorders target the 5-HT system (Anyan and Amir, 2018). Behavioral characterization of the $SERT^{-/-}$ rat model revealed that

the animals display anxiety- and depression-related behavior in the elevated plus-maze, open field, forced swim test, and sucrose consumption tests (Olivier et al., 2008). Although animals models of depression cannot replicate all the symptomology of depressed humans, genetically modified animals such as the $SERT^{-/-}$ rat provides a model based on environmental or social stressors, which is highly relevant in research aiming to understand the underlying pathophysiology of major depression (Czéh et al., 2016).

The HPA-axis: stress, BDNF, and depression – translating the findings

The most significant susceptibility factor for depression is acute traumatic or chronic stress (Duman et al., 2016). Approximately 60% of cases of depressive episodes are preceded by exposure to stressors, especially psychosocial stressors (Juruena, 2014). Clinical studies show that stressful life events can precipitate depressive episodes in vulnerable individuals (McLaughlin et al., 2010), and childhood stress in the form of abuse or neglect increases the risk of depression later in life (McLaughlin et al., 2020; Pechtel and Pizzagalli, 2011). Genetic factors can also increase the risk of developing depression upon stress. For instance, the effects of stressful life events on the individual risk to develop depression have been shown to be associated with the serotonin transporter (5-HTT) promoter-linked polymorphic region (5-HTTLPR) genotype (Caspi et al., 2003). Although some meta-analyses could not confirm this gene × environment (G × E) interaction (Munafò et al., 2009; Risch et al., 2009), others have shown that the interaction is especially significant after a history of early life stress (ELS) (Karg et al., 2011). Specifically, individuals with the short (S) allele of the 5-HTTLPR polymorphism were found to be more sensitive to the depressogenic effects of stress (Clarke et al., 2010; Karg et al., 2011; Kiyohara and Yoshimasu, 2010).

A hallmark feature of the stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased levels of circulating glucocorticoids, which is designed to provide maximum physiological support in the acute phase of the 'fight-or-flight' response. However, repeated exposure to stress and sustained elevation of glucocorticoids has deleterious effects on multiple organ systems, including the brain (Duman et al., 2016). Abnormal activation of the HPA axis, as well as increased circulating levels of cortisol, is one potential explanation for many of the features of depression, and many previous studies have described an impaired HPA negative feedback, leading to hypercortisolemia, in the more severe forms of depression (De Kloet et al., 1998; Gold et al., 1988). Elevated concentrations of glucocorticoids function at multiple levels to influence neuronal function and behavior. Notably, chronic exposure of rodents to adrenal glucocorticoids decreases synaptic number and function and causes atrophy of neurons in the PFC and hippocampus, regions undergoing

atrophy and disruption of connectivity in individuals with depression (Liu and Aghajanian, 2008; Magarin˜os and McEwen, 1995; Martin and Wellman, 2011).

Evidence for the clinical relevance of the aberrant HPA-axis function has accumulated over the years. Elevated basal cortisol has, for example, been shown predictive of the risk for depressive episodes (Goodyer et al., 2001). In contrast, successful antidepressant treatment is associated with the resolution of the impaired HPA-axis negative feedback (Pariante, 2006) by restoring corticosteroid receptor expression in the brain (Pariante and Lightman, 2008) that also predicts the patient's long-term clinical outcome (Pariante, 2006). In s-allele carriers of the 5-HTTLPR polymorphism, it has been reported increased basal activity of the HPA-axis (Chen et al., 2009; Goodyer et al., 2010, 2009; O'Hara et al., 2007; Wankerl et al., 2010; Wüst et al., 2009). Moreover, S/S homozygotes show increased CORT stress reactivity compared with individuals carrying a long (L) allele of the 5-HTTLPR (Gotlib et al., 2008; Miller et al., 2013; Way and Taylor, 2010). In mice, 5-HTT knockout ($SERT^{-/-}$) leads to increased adrenomedullary but not CORT responses to stress, and basal plasma CORT levels have been reported to be unaltered or lower in $SERT^{-/-}$ mice (Bartolomucci et al., 2010; Hohoff et al., 2013; Jansen et al., 2010; Lanfumey et al., 2000; Li et al., 1999; Spinelli et al., 2013; van den Hove et al., 2011). Contrary to the $SERT^{-/-}$ mice, in $SERT^{-/-}$ rats, it has been shown that in control conditions, basal CORT levels were increased, and exposure to maternal stress decreased the basal levels at adulthood (van der Doelen et al., 2014). However, the results reported in this thesis are in agreement with the mice data, for we did not find alterations in the basal levels of CORT compared to $SERT^{+/+}$ and Wistar rats.

An integration between the HPA-axis and BDNF has been explored. Cortisol (or corticosterone in rodents) mediates its action, including feedback regulation of the HPA axis, through two distinct intracellular corticosteroid receptor subtypes referred to as mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (Juruena, 2014). It has been demonstrated that inhibition of glucocorticoid receptors (GR) negatively affects BDNF-induced TrkB phosphorylation and its downstream signaling pathways, whereas a short activation of GR is associated with the long-lasting BDNF-delivered mechanisms required for memory consolidation (Begni et al., 2016). The activity of the HPA-axis in animal models can be checked using restraint stress. Restraint stress is one of the most commonly used means to induce stress in rodent models. Unlike social stressors, it is highly controllable and can be employed to assess acute stress or repeated stress events (Bath et al., 2013). Acute and chronic restraint stress seems to modulate BDNF levels in preclinical studies differently. Acute restraint in rats led to a rapid increase in BDNF mRNA expression in the hippocampus, which was then downregulated 180 min after to levels below that of non-stressed controls (Marmigère et al., 2003). Also, 2 hours of acute immobilization stress significantly decreased

BDNF mRNA in the rat hippocampus (Lee et al., 2008). Nair et al. (2007) demonstrated that different BDNF mRNA transcripts could be involved in the response to stress. Rats that underwent 2 hours immobilization stress administered once (acute) decreased all BDNF splice variants but had differing effects on BDNF I/II (increase) and III/IV (decrease) when administered chronically (Nair et al., 2007). Moreover, Chiba et al. (2012) have shown that chronic restraint stress (CRS) did not promote changes in the BDNF protein levels in the PFC, others have also reported that CRS had no significant effect on the expression of BDNF in the hippocampus (Kuroda and McEwen, 1998; Reagan et al., 2007). These inconsistencies might be related to methodological differences such as type of stressor, handling, the detection methods, or even species/strain differences.

Likewise, in the study presented in this thesis, we have demonstrated that BDNF overexpression in the ventral hippocampus or the prefrontal cortex did not alter basal levels of CORT in $SERT^{-/-}$ and $SERT^{+/+}$ rats. Moreover, after acute stress, CORT levels were also comparable in control-treated and BDNF-treated $SERT^{-/-}$ and $SERT^{+/+}$ rats. Therefore, it seems that BDNF had not significantly influenced the HPA-axis activity in $SERT^{-/-}$ and $SERT^{+/+}$ rats. Contrary to the responses in $SERT^{-/-}$ and $SERT^{+/+}$ rats, BDNF overexpression in the PFC of Wistar rats increased basal CORT levels in these animals and decreased response to acute restraint stress. Acute stress seems to have the general effect of increasing glutamatergic neurotransmission in the PFC (Popoli et al., 2012), attenuating projections from the amygdala to the PFC (Maroun and Richter-Levin, 2003), resulting in HPA-axis activation (Herman et al., 2004). Therefore, in the Wistar rats, it is possible that BDNF overexpression in the PFC might have caused an increase in glutamate release leading to impaired synaptic plasticity and altered HPA-axis functioning (Duman et al., 2016).

BDNF is known to promote neuronal differentiation and growth, synapse formation and plasticity (Park and Poo, 2013). The functional significance of altered BDNF expression is highlighted by studies demonstrating that stress and depression can lead to neuronal atrophy and cell loss in key limbic brain regions implicated in depression, including the amygdala, prefrontal cortex, and hippocampus, and that antidepressant treatment can block or reverse these effects (Duman and Monteggia, 2006). However, in the framework of depression, it has been shown that the rate of individuals presenting poor response or unresponsiveness to antidepressant treatment is very high (Berlim and Turecki, 2007; Collo and Merlo Pich, 2020). Therefore, further understanding of BDNF modulation in the context of stress using genetic and molecular tools, such as the $SERT^{-/-}$ rat model, will be determinant to more clearly delineate the relationship between gene expression and stress-associated outcomes and provide potential novel targets for the treatment of stress-related mood disorders, including depression.

Hippocampal synaptic plasticity, BDNF, and implications in depression

The hippocampus is the most commonly studied brain region in depression research. Several meta-analyses of volumetric magnetic resonance imaging (MRI) studies in patients with depression have shown volume reductions in the hippocampus (Arnone et al., 2012; Campbell et al., 2004; McKinnon et al., 2009). Compared with healthy controls, depressive patients present about 4 to 5% reduction in hippocampal volume (Videbech and Ravnkilde, 2004). Depressive patients also display an increased rate of apoptosis in the dentate gyrus, CA1, and CA3 areas of the hippocampus. These studies suggest that both apoptosis and atrophy may occur in depression (Lucassen et al., 2001; Stockmeier et al., 2004). Depressive patients not only present morphometric changes in the hippocampus but also functional deficits have been observed. Functional magnetic resonance imaging (fMRI) studies revealed abnormal hippocampal activation during a recollection memory task in depressive patients (Milne et al., 2012) and abnormal response to aversive events (Johnston et al., 2015). The 5-HTTLPR polymorphism has also been associated with disturbances in hippocampal function and morphology. Depressed patients presenting homozygosity for the 5-HTTLPR short (S) allele displayed smaller hippocampal volume (Eker et al., 2011; Phillips et al., 2015). Several hypotheses have been proposed to explain the smaller hippocampal volumetric volume in depressed individuals. One of these hypotheses is that hippocampal reduction in depression might be explained by cellular loss, including negative changes in neuronal and glial content (Rajkowska, 2000). For instance, analysis of postmortem tissue from depressive patients revealed lowered levels of neuron and glial extensions and reduced pyramidal soma size (Stockmeier et al., 2004).

Animal studies have been crucial in determining the molecular processes underlying the clinical cognitive, morphological, and functional impairment seen in the hippocampus of depressed subjects. These studies have focused on neurotrophic and neurogenic mechanisms underlying neuronal plasticity in the hippocampus (Duman, 2004). A role for neurotrophic factors in cell atrophy and loss is supported by evidence that stress or depression decreases the expression of certain factors, such as BDNF, in limbic brain regions, including the hippocampus and prefrontal cortex (Duman and Li, 2012). Considering that BDNF plays an important role in neuronal survival (Yu and Chen, 2011), it is not at odds that depressive patients present a reduction in BDNF levels. Several clinical studies have confirmed decreased BDNF levels in the serum of depressive patients (Sen et al., 2008) and postmortem studies demonstrated decreased BDNF in the hippocampus of depressed individuals (Dwivedi et al., 2003) and the PFC and hippocampus of suicidal patients (Pandey et al., 2008). A large body of evidence demonstrates that a reduction in BDNF levels is associated with deficits or impairment of neuronal plasticity, which can have a role in anxiety

and major depression (Calabrese et al., 2009; Krishnan and Nestler, 2008; Martinowich et al., 2007; Pittenger and Duman, 2008). For instance, in $SERT^{-/-}$ rats, it has been demonstrated altered neuronal plasticity, as indicated by the reduction of activity-regulated cytoskeleton-associated protein (*Arc*) and BDNF expression levels in the hippocampus and prefrontal cortex (Molteni et al., 2010, 2009). $SERT^{-/-}$ rats also present impairment of the GABAergic system in the hippocampus (Guidotti et al., 2012), which is implicated in anxiety and depression (Möhler, 2012). Moreover, it has recently been demonstrated that promoter IV-driven BDNF transcription has a critical role in GABAergic transmission (Sakata et al., 2009), and mice with a selective deficiency of promoter-IV-dependent expression of BDNF show depression-like behavior (Sakata et al., 2010).

BDNF has also been implicated in hippocampal synaptic plasticity. Synaptic plasticity describes the process by which connections between two neurons, or synapses, change in strength. Plasticity is often accompanied by structural alterations of the synapses (Lu et al., 2008). These modifications include either the strengthening or weakening of synaptic connections, termed long-term potentiation (LTP) and long-term depression (LTD), respectively (Woo et al., 2005). LTP is an activity-induced long-lasting increase in the excitatory synaptic strength, and it has been most studied in the hippocampus (Leal et al., 2015). Hippocampal LTP typically presents three sequential phases: short-term potentiation, early LTP (E-LTP), and late LTP (L-LTP). Short-term and E-LTP requires modification of existing proteins and their trafficking at synapses but not de novo protein synthesis (Malenka and Bear, 2004). L-LTP requires changes in gene expression and de novo protein synthesis and lasts for hours or even days (Abraham, 2003; Muller et al., 2005). Changes in hippocampal plasticity can result from stress and other negative stimuli such as depression, which can downregulate synaptic proteins and growth factors required for hippocampal LTP in animal models (Liu et al., 2017). BDNF has emerged as a key regulatory protein in synaptic plasticity, and the available experimental evidence supports a model in which increases in endogenous BDNF following L-LTP stimuli may be crucial for L-LTP maintenance (Aarse et al., 2016; Lu et al., 2008). Application of exogenous BDNF facilitates LTP induction in young (postnatal day 12-13) hippocampal slices (Figurov et al., 1996), and blocking the dendritic localization of BDNF mRNA in vivo reduced BDNF protein levels in dendrites, resulting in a selective impairment of LTP in dendrites (An et al., 2008). Lin et al. (2018) demonstrated that the deletion of BDNF in CA3 or CA1 areas of the hippocampus revealed that presynaptic BDNF is involved in LTP induction, while postsynaptic BDNF contributes to LTP maintenance.

Together, these results suggest a critical role for dendritically synthesized BDNF in synaptic plasticity. This is important considering that BDNF gene transcription generates multiple mRNA splice variants (Aid et al., 2007), which seems to segregate differently within the neuronal compartments. For example, the two BDNF transcript variants explored in this

thesis for its greater abundance in the brain and possible involvement in the physiopathology of depression have slightly different patterns of segregation (Lieb et al., 2018; Sakata et al., 2010; Zheleznyakova et al., 2016). BDNF variant 4 segregates to the proximal dendrites and variant 6 to the distal dendrites. Moreover, in more mature neurons, exon 4, was able to regulate also the number of secondary dendrites similarly to the "dendritic" isoforms 6 (G. Baj et al., 2011; Baj et al., 2013, 2016; Singer et al., 2018).

Recently, LeGates et al. (2018) demonstrated that, for example, the long-term potentiation (LTP) that occurs at the hippocampus to nucleus accumbens (NAc) synapses modulate response to natural rewards. Conversely, chronic stress, which induces anhedonia, decreased the strength of this synapse and impaired LTP, whereas antidepressant treatment was accompanied by a reversal of these stress-induced changes (LeGates et al., 2018). These data imply that stress may induce anhedonia by impairing the hippocampus to NAc synaptic function and LTP. Indeed, we have determined in chapter 2 that BDNF overexpression in the hippocampus increased sucrose preference in $SERT^{-/-}$ rats, thus it is likely that BDNF facilitation of LTP in the hippocampus increased preference for this natural reward (Figurov et al., 1996).

Furthermore, Conrad et al. (2011) have shown that anxiety-like behavior in the novel open-field and the elevated zero maze tests were associated blunted LTP in the bed nucleus of the stria terminalis (BNST). The BNST is a subregion of the extended amygdala, which serves as a relay of corticolimbic (including the amygdala, ventral hippocampus, and medial prefrontal cortex) information to the paraventricular nucleus of the hypothalamus (PVN) to directly influence the stress response (Goode and Maren, 2017). In our experimental conditions, generally, BDNF did not reduce the anxiety-like behavior of $SERT^{-/-}$ rats, as demonstrated by increased escape-behavior in the forced swim test, and decreased time spent in the center of a novel open field. However, BDNF overexpression in the hippocampus of $SERT^{-/-}$ rats normalized to the $SERT^{+/+}$ controls, the time spent in the center of the novel open field in the novelty-induced locomotor activity test, suggesting that BDNF might have enhanced LTP in the BNST through the inputs received from the ventral hippocampus.

Collectively, these data support the potential therapeutic of restoring BDNF levels in the hippocampus aiming enhancement of the neuronal plasticity in this brain region, given that the hippocampus is involved in emotional processing, which integrity might be compromised in depression (Castrén et al., 2007). Moreover, it shows that investigation of the spatial segregation of BDNF transcripts are critical for mediating the therapeutic effects of treatments and may provide key information about the neuronal circuits involved in the therapeutic response.

Treatment-resistant Depression - therapeutic options and BDNF involvement

Treatment-resistant depression (TRD) is a lack of symptomatic response to adequate first-line pharmacological therapy (Trivedi et al., 2006). Approximately 15-30% of the patients suffering from depression are classified as resistant (Berlim and Turecki, 2007; Collo and Merlo Pich, 2020), and because antidepressant response seems to cluster in families, it is suggested that genetic factors might play a role in it (Franchini et al., 1998; O'Reilly et al., 1994). An example of such a genetic contribution to resistance is the human serotonin transporter functional polymorphism. Individuals carrying the short allele of the 5-HTTLPR polymorphism showed an increased risk for non-response to SSRIs (Bonvicini et al., 2010) or have shown to require a longer time to respond to the treatment with difficulties to reach remission (Serretti et al., 2007).

Interestingly, TRD patients have also shown decreased levels of mRNA BDNF. Studies revealed that patients suffering from depression and presenting treatment resistance displayed lower leukocytes mRNA BDNF levels (Hong et al., 2014). Serum BDNF (sBDNF) has been pointed as a potential biomarker for TRD. sBDNF has been consistently identified as decreased in depressed patients, including TRD patients (Allen et al., 2015; Molendijk et al., 2014, 2011; Pisoni et al., 2018). In fact, two of the most used treatments used for attempting to meliorate the depressive symptoms in TRD, namely electroconvulsive therapy and ketamine, have, ultimately, effects on the BDNF levels. The current gold standard for TRD is electroconvulsive therapy (ECT) (Geddes et al., 2003) and sub-anesthetic doses of the NMDA receptor antagonist ketamine has also shown to produce rapid antidepressant effects in TRD patients (Allen et al., 2015; Ghasemi et al., 2014; Murrough et al., 2013). Although some studies concluded that ECT does not have effects in the levels of BDNF, a recent systematic review and meta-analysis study analyzing reports from the last 30 years, found a correlation between ECT and increases in BDNF levels (Luan et al., 2020). The exact mechanisms underlying response to ECT remain unclear; however, successful ECT treatment is linked to increases in BDNF (Altar et al., 2004; Rocha et al., 2016). The principal function of the electrical stimulus delivered with ECT is to induce a generalized tonic-clonic seizure (Farzan et al., 2014). According to the neurobiochemical hypothesis of the ECT mechanism of action, ECT modulates the process of neurotransmission and influences the expression as well as the release of a great variety of neurochemicals in the brain, including transcription factors, neurotransmitters, neurotrophic factors, and hormones (Singh and Kar, 2017). In rodents, it has been demonstrated that a single ECT treatment results in 10—20-fold BDNF gene overexpression after ECT in rat hippocampal dentate gyrus. Moreover, long-term ECT treatment (once daily for 7—10d) also induces BDNF mRNA expression (Nibuya et al., 1995). Similar to ECT, the precise mechanisms underlying ketamine's antidepressant

effects are currently not understood. It is suggested that ketamine causes a burst of glutamate that is thought to occur via disinhibition of GABA interneurons; NMDA receptors drive the tonic firing of these GABA interneurons, and the active, open channel state allows ketamine to enter and block channel activity. The resulting glutamate burst stimulates AMPA receptors causing depolarization and activation of voltage-dependent Ca^{2+} channels, leading to the release of BDNF (Duman et al., 2016; Duman and Li, 2012). In clinical studies, plasma or serum BDNF levels were shown to be increased a few hours after ketamine administration (Duncan et al., 2013; Haile et al., 2014; Woelfer et al., 2019).

Despite the promising effects of ECT and ketamine in the treatment of TRD, still both present significant side-effects and unknown long-term effects. ECT, for example, induces a series of common mild adverse effects, but it also can potentially induce serious adverse events, such as long-term retrograde amnesia (Andrade et al., 2016). Ketamine first received FDA approval in 1970 as a rapid-acting general anesthetic. Regardless of 50 years of clinical experience with ketamine as an anesthetic agent, there are no postmarketing surveillance data on the use of ketamine for any psychiatric indication to provide information on its safety and effectiveness (Sanacora et al., 2017). Recently, the s-enantiomer of ketamine (esketamine) was approved by the FDA in the form of nasal spray for the treatment of TRD in conjunction with an oral antidepressant, but only to be used under the supervision of a health care provider. Moreover, patients must be monitored by a health care provider for at least two hours after receiving the medication (U.S. Food and Drug Administration, 2019). Additionally, esketamine has boxed warnings for risk of sedation and dissociation, potential for abuse and misuse, and increased risk for suicidal thoughts and behaviors in pediatric and young adult populations (Bahr et al., 2019).

Future directions - Potential new therapeutic options targeting BDNF: Gene therapy (viral and non-viral vectors)

The dimension of the clinical burden caused by depression reflects, in part, the limited effectiveness of current therapies. As discussed above, present-day antidepressant medications are related to elevated rates of partial responsiveness or lack of responsiveness, delayed response onset of weeks to months, and decreased duration of efficacy (Gaynes et al., 2009). The lack of understanding regarding the underlying mechanisms behind the pathophysiology of depression hinders the development of novel drugs to address the faults in the current medications. Moreover, depression is clinically very heterogenic, and it is diagnosed through very broad subjective criteria, for which there are no reliable biomarkers. Despite these complexities, progress is being made. For example, several studies have

identified that susceptibility to depression is influenced by a variety of genetic, epigenetic, endocrine and environmental risk factors. Additionally, studies showed that altered brain structure and function are present in depression, being the most consistent finding the reduced volume of the prefrontal cortex (PFC) and hippocampus; moreover, as largely explored in this thesis, there is clinical and preclinical evidence that depression is marked by a decrease in the expression and function of BDNF (Björkholm and Monteggia, 2016).

Nevertheless, while BDNF is involved in almost all antidepressant therapies, is it sufficient to cause symptoms' remission? As previously discussed in this thesis, although BDNF has great potential to be a therapeutic targeted in depression, several critical molecular processes have to be taken into consideration. For instance, BDNF signaling in the ventral tegmental area (VTA) to the nucleus accumbens pathway seems to produce effects that are in many ways opposite to the effects of BDNF in the hippocampus (Nestler and Carlezon, 2006). BDNF injected directly into the VTA induces depression-like behavior and, conversely, blocking BDNF signaling in nucleus accumbens, a brain region, which receives afferents from the VTA, induces an antidepressant-like effect (Eisch et al., 2003). Bilateral BDNF-infusions into the hippocampus or the ventricles induces antidepressant-like effects in preclinical animal models that can persist for several days (Shirayama et al., 2002; Siuciak et al., 1996). Additionally, as we have shown in the **chapter 2 and 3** of this thesis, BDNF injection in the prelimbic cortex promoted different behavioral response in $SERT^{-/-}$ rats when compared to the animals injected in the ventral hippocampus.

Taking BDNF properties into consideration, much research has been done focusing on BDNF activity, and since BDNF protein does not readily cross the blood-brain barrier, there is growing interest in targeting the TrkB signaling. However, despite some success in preclinical studies with molecules targeting TrkB (Liu et al., 2016), BDNF-TrkB signaling is under precise control, thus administering exogenous BDNF or TrkB agonists may not result in the expected outcomes. Moreover, prolonged use or improper localization of the stimulation can lead to, due to BDNF's trophic effects, deleterious side effects (Price et al., 2007). Given that BDNF-TrkB signaling may contribute differently to the symptomatology of depression depending on the brain region where it acts, it is not certain that a global increase in BDNF expression or TrkB activation induces an overall antidepressant effect, although some experiments have supported this hypothesis (Duman and Voleti, 2012; Schmidt and Duman, 2010).

Due to the potential disadvantages of global BDNF protein upregulation and drawbacks related to exogenous protein administration, one promising therapeutic option is gene therapy, which could offer endogenously expressed protein and possibly a lifelong treatment. Gene therapy is a comprehensive term that includes many forms of nucleic acid delivery, which can be mediated by recombinant laboratory-designed viral vectors that infect

a wide variety of cells and may integrate into the genome or be retained episomally (Kaufmann et al., 2013). Viral vectors transduce target cells that can then produce therapeutic protein. One early gene therapy vector employed in human clinical studies was the non-integrating adenovirus. Adenoviral vectors can carry a large 'genetic payload', have a wide tropism, and can be produced at high titer. However, they are also highly immunogenic and demonstrate relatively short-lived transgene expression. In comparison to adenoviral vectors, adeno-associated virus (AAV) carries considerably less genetic material. AAV vectors have natural and engineered serotypes that convey wide tropism, are less immunogenic than adenovirus, and can display long-term expression in non-dividing cells (Nagree et al., 2019). In the mid-2000s, the use of lentivirus vectors derived from HIV-1 gained traction (Schröder et al., 2002). The introduction of third-generation lentivirus constructs with self- inactivating elements in the 3′-LTR and a modified U3 promoter region in the 5′-LTR minimizes the chance of viral replication, greatly reduces the potential for transactivation of adjacent patient genes, and consequently increases the safety of lentivirus. Lentivirus can infect non-dividing cells, including neurons, and promote long-term gene expression (Ingusci et al., 2019). While lentivirus pseudotypes can be modulated to improve the transduction of specific cell types, vesicular stomatitis virus-G protein (VSV-G) is most commonly used as it confers a stable envelope that facilitates broad tropism. Compared to the tissue-restricted expression promoters, direct CNS-injected vectors being used clinically have employed ubiquitous promoters – most using a combination of a β-actin promoter and a CMV enhancer (Nagree et al., 2019). Over time, lentiviruses have become the preferred integrating vector for clinical development, especially because therapeutic gene(s) and/or the regulation sequence to be delivered very often exceed the payload capacity of AAV viral vectors, which is approximately 4.8 kb. Thus, for example, encapsulation of the BDNF sequence used in our experiments, measuring about 5.6 kb, was only possible in a lentivirus which supports a payload of up to 9 kb (Ingusci et al., 2019). Therefore, in our present study, we have used a lentivirus with both features – VSV-G packaging system and CMV promoter – to transfect prefrontal and hippocampal cells, and drive the BDNF protein upregulation through a cDNA of BDNF exon IV.

Evidently, although possible, local intracerebral infusion of either the BDNF gene or protein is not a feasible treatment for depression in humans, especially not on a large scale. Moreover, despite the ability of lentiviruses to cross the blood-brain-barrier (BBB) upon peripheral infusion (Patrick et al., 2002), the costs involved in producing enough lentivirus for direct injection, and serum-inactivation of the VSV-G pseudotype, have thus far prevented much clinical investigation hindering further confirmation of the safety of lentivirus (Nagree et al., 2019).

Alternatively, non-viral vectors have emerged for its many advantages over viral approaches, displaying low cost, easier production, increased biosafety, and reduced pathogenicity (Ramamoorth and Narvekar, 2015). In the last decade, the number of non-viral gene therapy clinical trials has dramatically increased with at least 40 nanoparticle-based gene therapies entering various stages of clinical trials. Moreover, in 2018 the Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved for the first time an RNAi therapy-delivering nanoparticle administered intravenously – Patisiran/ONPATTRO, marketed by Alnylam – for the silencing of a specific gene responsible for the expression of transthyretin, which can cause hereditary transthyretin amyloidosis (Anselmo and Mitragotri, 2019). Non-viral gene delivery systems are engineered complexes or nanoparticles (NPs) composed of the required nucleic acid (plasmid, RNA, oligonucleotides) and a single or a combination of materials such as cationic lipids, surfactants, peptides, polysaccharides, metals (gold, magnetic iron) and synthetic polymers (poly(DL-lactide-co-glycolide acid) (PLGA), polyethyleneimine (PEI), polyamidoamine dendrimer (PAMAM)) (Foldvari et al., 2016). Such gene delivery systems have the advantage of offering flexibility in selecting complexing agents and targeting moieties to provide localized gene expression.

Modifications of the outer surface of nanoparticles can improve brain delivery. For instance, adding polyethylene glycol (PEG), or PEGylation, increased transfection efficiency, reduced toxicity, and imparted “stealth” properties (Rip et al., 2014). Arginine addition enhanced the cellular uptake and delivery across the BBB (Morris and Labhasetwar, 2015), and tagging liposome NPs with transferrin receptor-ligand facilitated transcytosis across the BBB and endocytosis into CNS cells (Chen et al., 2016). Using both the PEGylation and transferrin strategy, Xing et al. (2016) demonstrated that a non-viral liposome-mediated the transfer of BDNF across the BBB. Besides vector modifications, gene delivery efficiency can also be dramatically enhanced through strategic modification of DNA composition and conformation, thereby improving bioavailability, biocompatibility, durability, and safety (Foldvari et al., 2016). The use of cell-type-specific promoters, for example, might be useful for confining the transgene expression to a specific cell type. In principle, specifically labeling a population of neurons or glial cells might allow researchers and clinicians to achieve the therapeutic goal without incurring in off-target effects (Ingusci et al., 2019). Rasmussen et al. (2007) demonstrated that the phosphate-activated glutaminase (PAG) or the vesicular glutamate transporter (vGLUT) promoter ensures ~90% glutamatergic neuron-specific expression, whereas the glutamic acid decarboxylase (GAD) promoter ensures ~90% GABAergic neuron-specific expression. Therefore, optimizing both vector and genetic load provides a powerful tactic for the development of effective non-viral gene therapy systems that might lead to a new therapeutic approach targeting BDNF.

There is certainly no doubt that BDNF is one of the central mediators and modulators of synaptic plasticity in the CNS. Numerous excellent reviews covered this topic and summarized the convincing evidence that BDNF promotes neuronal differentiation of stem cells, axonal and dendritic growth of neuronal processes, formation and maturation of glutamatergic and GABAergic synapses, and activity-dependent refinement of synaptic connections, like long-term potentiation (LTP), underlying learning and memory formation (Bramham and Messaoudi, 2005; Edelmann et al., 2014; Gottmann et al., 2009; Park and Poo, 2013). These features make BDNF such an attractive candidate for regulation. However, the promising results in BDNF targeting will enormously depend on better characterization of the BDNF system in normal physiological conditions and in abnormal functioning, such as is the case for depression. Much still unknown regarding the gene control, spatial segregation, and function of the diverse BDNF split variants. Moreover, little is known regarding the correlation of different BDNF release sites and release modes (i.e., constitutive vs. regulated release) with distinct functions of BDNF in synaptic plasticity.

The use of animal models offer the possibility of neurobiological analysis at a higher resolution, allowing experiments on selected components in the brain circuits that may underlie psychopathology as well as the possibility of screening novel drugs with clinical potential. Analyses at this higher neurobiological level of resolution and preclinical drug screening are necessary to understand depression and enhance our capabilities to treat the disorder. The resultant increased understanding of brain function can potentially guide the enhancement of human mental health as a preventive measure (Harro, 2019). In the context of depression, serotonin (5-HT) and brain-derived neurotrophic factor (BDNF) are known to modulate behavioral responses to stress and to mediate the therapeutic efficacy of antidepressant agents through neuroplastic and epigenetic mechanisms (Homberg et al., 2014). The $SERT^{-/-}$ rat model brings these two systems together and provides the opportunity to understand the developmental interaction of the serotoninergic and BDNF systems. Given that early life stress significantly influence maladaptation at adulthood (Boulle et al., 2016; van Bodegom et al., 2017), it will be interesting to investigate BDNF overexpression in earlier stages of the $SERT^{-/-}$ rat life span, to check whether manipulations at developmental phases could better rescue depression and anxiety-like behaviors at adulthood. Also, further studies applying other behavioral paradigms, such as elevated plus maze, ambiguous cue interpretation (ACI) test, sucrose self-administration, and social interaction, might further clarify some of our findings. These tests could give us further information regarding BDNF effects on aspects of the anxious state of the $SERT^{-/-}$ rats, its pessimistic bias, and facets of motivational and consummatory anhedonia.

Furthermore, it will be interesting to explore further the effects of BDNF overexpression in the ventral hippocampus and investigate, for example, whether BDNF

promoted neuroplasticity, or restored GABAergic transmission. Also, this thesis was limited to the study of the BDNF effects in the hippocampus and prefrontal cortex, but it will be important to investigate the effects over other brain areas to which vHIP and PFC share connectivity, such as the amygdala and nucleus accumbens (Der-Avakian and Markou, 2012; Treadway and Zald, 2011). Especially considering that, as we have shown in chapter 2, local viral infusion in the ventral hippocampus had effects in BDNF gene expression in the prefrontal cortex, and affected other BDNF transcripts, such as total BDNF and BDNF exon VI. Therefore, it will be very valuable to evaluate overall secondary changes caused by BDNF in distal areas from the targeted brain area. We believe that by applying the above discussed pharmaceutical nanotechnologies to reach cell-specificity and promoter specificity within specific neuronal circuits might provide key information in the development of novel therapeutics to alleviate depressive symptoms.

Conclusion

Based on the findings presented in this thesis, we proposed several interesting directions for future research, as summarized above. We presented for the first time the molecular and behavioral effects of BDNF overexpression in the $SERT^{-/-}$ rat model and highlighted the importance of preclinical studies to increase our understanding of the neurobiological mechanisms underlying depressive disorders for improving therapeutic methods. The molecular investigation of BDNF overexpression using a lentivirus vector demonstrated that BDNF is modulated temporally and spatially. Moreover, the upregulation of BDNF IV affected the total BDNF and the expression of BDNF VI, confirming previous studies showing spatial segregation of BDNF transcripts and/or possible BDNF effects in distal areas after overexpression in specific sites (Gabriele Baj et al., 2011; Edelmann et al., 2014). Behavioral examination using classical paradigms such as the sucrose consumption test, the forced swim test, the acute restraint stress, and the novelty-induced locomotor activity test provided some characterization of the depression- and anxiety-like behavior displayed by the $SERT^{-/-}$ rat model. BDNF overexpression in the selected areas – the ventral hippocampus and prelimbic area of the medial prefrontal cortex – did not modulate the $SERT^{-/-}$ deficits in all the analyzed behavioral tests. However, the few modifications promoted by BDNF overexpression, together with further studies, should unravel the mechanisms underpinning the BDNF role in the vHIP and PFC of the $SERT^{-/-}$ rats.

This thesis, therefore, opens the way to new molecular and behavioral studies that can be employed to investigate further the effects of BDNF overexpression in a rat model presenting both decreased BDNF expression and anxiety- and depression-like phenotype features commonly found in clinical studies of patients suffering from mood-related

disorders. We hope that the collective efforts of the field will ultimately achieve the goal of promoting alternative therapeutic options for those struggling with resistance to current antidepressant options, thus contributing to the improvement of the human mental health.

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6

English summary

Nederlandse samenvatting /*Dutch summary*

Resumo em Português / *Portuguese summary*

English summary

Defined as a group of psychiatric illness, mood disorders affect individual's emotions, energy and motivation. Among the many mood disorders, one of the most outstanding is major depressive disorder. Depression is characterized by depressed mood, loss of interest or pleasure in nearly all activities (anhedonia), appetite and sleep disturbances, and loss of energy. These symptoms usually lead to significant impairment in the achievement of important tasks at home, work, or school. Additionally, depressive patients can manifest feelings of worthlessness and inappropriate guilt resulting in suicidal thoughts, or even attempts. The etiology of depression is attributed to a complex interaction between environmental factors and genetic vulnerability, but specific genes have not yet been found, making it difficult to comprehend the mechanistic causes of such complex disease.

Clinical and preclinical studies are attempting to elucidate the processes underlying depression. These efforts brought to light the serotonin hypothesis of depression in the early 1960s. Increasing serotonin levels by means of selective serotonin reuptake inhibitors (SSRIs) were shown to relieve depressive symptoms. However, inconclusive and inconsistent studies have indicated that the serotonin hypothesis seems to be too simplistic to explain the mechanisms by which this mood disorder develops in some individuals. Actually, current antidepressant treatments do not work for all individuals, and it is common that after a successful treatment, many patients relapse. Additionally, about 15-30% of the patients are classified as resistant, and because antidepressant response seems to cluster in families, it is suggested that genetic factors might play a role in it. An example of such genetic contribution to resistance is the human serotonin transporter functional polymorphism. Individuals carrying the short (s) allele of the 5-HTTLPR polymorphism showed increased risk for non-responsivity to SSRIs, or have shown to require more time to respond to the treatment with difficulties to reach remission.

Several works have highlighted that the 5-HTTLPR can modulate the influence of stressful life events on depression. The most well-established and consistent trait associated with the 5-HTTLPR s-allele is neuroticism, which is the trait disposition to experience negative effects, including anger, anxiety, self-consciousness, irritability, emotional instability, and depression. It has been shown that 5-HTTLPR s-carriers tend to develop selective attention to negative stimuli. As such, sensitivity to negative stimuli may increase vulnerability for psychopathologies. Clinical studies revealed that individuals carrying the s-allelic variant of the 5-HTTLPR, as opposed to the l-variant, were at risk to develop depression when exposed to adverse psychosocial stressors in early life.

While rodents do not carry the human serotonin transporter linked polymorphic region (5-HTTLPR), genetic deletion or partial deletion of the SERT in rodents leads to anxiety- and depression-related phenotypes. There is increasing evidence that particularly

heterozygous 5-HTT knockout ($SERT^{+/-}$) mice respond to prenatal, early life and adult psychosocial stress, as seen in s-allele carriers. $SERT^{-/-}$ rats presented anhedonia-like behavior in the sucrose preference test, increased immobility in the forced-swim test, and decreased time spent in the central part of the open field, indicating that anxiety levels and depressive-like behaviour are increased. Additionally, $SERT^{-/-}$ rats displayed increased levels of basal corticosterone (CORT) under control conditions, showing altered basal hypothalamic-pituitary-adrenal axis (HPA-axis) activity.

Taking into consideration that simply increased serotonin levels does not necessarily leads to amelioration of depressive behavioral phenotypes, the neurotrophic hypothesis of depression was introduced. Neurotrophins are involved in synaptic maturation, neuronal growth, and synaptic plasticity both during development and adulthood. Impaired production, release, and/or action of neurotrophins is believed to have a direct association with depression. The brain-derived neurotrophic factor (BDNF) is the most abundant and one of the most investigated neurotrophins. Several clinical studies reported a reduction in BDNF protein levels in the serum of depressive individuals, showing a direct correlation between antidepressant treatment and an increase in peripheral BDNF protein levels of treated patients, while untreated individuals present decreased levels of BDNF protein. Studies also reported abnormal mRNA BDNF or TrkB expression in the hippocampus and prefrontal cortex postmortem tissue of suicidal patients with previous record of major depression.

Interestingly, although antidepressant treatment induces increases in BDNF levels, the genetic manipulation of the SERT in rats causes a decrease in BDNF levels. Therefore, in agreement with the neurotrophic hypothesis of depression, $SERT^{-/-}$ rats present, under basal conditions, downregulation of BDNF mRNA and protein levels in the hippocampus and prefrontal cortex. Further, total BDNF mRNA levels (exon IX) were significantly downregulated and the reduction of BDNF gene expression observed in the prefrontal cortex of $SERT^{-/-}$ rats was shown to be due, at least in part, to epigenetic changes affecting the promoter regions of exons IV and VI.

Major depressive disorder is associated with structural and functional brain imaging changes, including reduced brain volume and activity in the PFC. These structural changes in depressed patients have been confirmed in post-mortem studies demonstrating a reduction in neurons and glial loss in the PFC, which is accompanied by a reduction in BDNF in this brain area. Given the association between depression and PFC alterations in human studies, together with the reduced levels of BDNF in the prefrontal cortex (PFC) of $SERT^{-/-}$ rats and the role of BDNF in supporting neuronal plasticity, which is particularly affected in depressive disorders, **chapter 2** aimed to investigate the effects of BDNF overexpression in the PFC of $SERT^{-/-}$ rats. We demonstrated that BDNF gene upregulation was best achieved two weeks after the local infusion in the prelimbic cortex (PrL), a subregion of the PFC, when levels of

total BDNF in the PrL were higher in BDNF-treated animals than in the control GFP-treated $SERT^{+/+}$ rats. Unexpectedly, we observed that in general, the BDNF upregulation did not alter the outcome from the behavioral tests in $SERT^{-/-}$ rats, nor did it modulate the response of the wild-type (WT) animals. Few exceptions applied. For example, in the sucrose preference test, BDNF overexpression in $SERT^{-/-}$ rats seemed to induce neophobia, leading to a decrease in the sucrose preference, which was followed by anti-anhedonic behavior as reflected by an increase in sucrose preference. Another exception involved the CORT levels as measured in the hypothalamus-pituitary-adrenal (HPA)-axis reactivity test. We found that BDNF overexpression in WT animals mediated an impairment in HPA-axis activity, increasing CORT baseline levels and decreasing CORT levels in response to stress. Further, in $SERT^{-/-}$ rats BDNF overexpression normalized the CORT stress levels, suggesting that different mechanisms underlie the HPA-axis reactivity modulation by BDNF in $SERT^{-/-}$ and WT rats.

Since BDNF overexpression in the PFC failed to rescue the anxiety- and depression-like behaviors in the $SERT^{-/-}$ rats, our next target was the ventral hippocampus (vHIP). The hippocampus also plays a key role in depression; it modulates emotional processing, memory and learning, and controls glucocorticoid secretion by the HPA-axis, making this axis susceptible to the effects of stress. Stress and other negative stimuli can change hippocampal plasticity, increasing the risk for depression(Liu et al., 2017). Further, depressive disorder is associated with hippocampal atrophy, and BNDF and its high affinity receptor (TrkB) are decreased in the hippocampus of post-mortem tissue from suicidal or depressed patients. In line with this, and considering that BDNF levels are decreased in the hippocampus of $SERT^{-/-}$ rats, we studied in **chapter 3** the effect of BDNF overexpression in the vHIP on behaviour using the same battery of experiments as mentioned in **chapter 2**. Interestingly, one week following the lentivirus infusion in $SERT^{+/+}$, BDNF was overexpressed in the vHIP, but downregulated in the prelimbic cortex and infralimbic cortex, main subregions of the PFC. These findings indicate not only that these areas are strongly connected, but also that BDNF transcripts provided negative feedback to distal areas from the vHIP. Behaviorally, we found that BDNF overexpression in the vHIP of $SERT^{-/-}$ rats promoted increased sucrose intake and preference, motivating the rats especially when they were faced with this novel taste for the first time. Likewise, in the novelty-induced locomotor test, $SERT^{-/-}$ rats infused with BDNF lentivirus spent more time in the center of the novel test cage than control-infused $SERT^{-/-}$ rats, suggesting that BDNF decreased the anxiety-like behavior expressed by $SERT^{-/-}$ rats only when BDNF was overexpressed in the vHIP.

Therefore, as shown in the **chapter 2 and 3** of this thesis, BDNF injection in the prelimbic cortex promoted different behavioral response in $SERT^{-/-}$ rats when compared to the animals injected in the ventral hippocampus. Likewise, other studies have shown distinct BDNF effects depending on the targeted brain region. Therefore, it seems that global BDNF

protein upregulation might have potential disadvantages. Rather, brain specificity is necessary to achieve the desired effects of BDNF. However, although possible, local intracerebral infusion of either the BDNF gene or protein is not a feasible treatment for depression in humans, especially not at a large scale. Additionally, despite the ability of lentiviruses to cross the blood-brain-barrier (BBB) upon peripheral infusion(Patrick et al., 2002), the costs involved in producing enough lentivirus for direct injection, have thus far prevented much clinical investigation, hindering further confirmation of the safety of lentivirus. Alternatively, non-viral vectors have emerged for its many advantages over viral approaches, displaying low cost, easier production, increased biosafety and reduced pathogenicity. Non-viral gene delivery systems are engineered complexes or nanoparticles (NPs) composed of the required nucleic acid (plasmid, RNA, oligonucleotides) and a single or a combination of materials such as cationic lipids, surfactants, peptides, among others. Such gene delivery systems have the advantage of offering flexibility in selecting complexing agents and targeting moieties to provide localized gene expression.

In chapter 4, we conducted a pilot study in which we developed and characterized a gene delivery device to carry plasmid BDNF. We reported the synthesis of a liposomal nanocarrier ranging 120-200nm in size, composed by cationic lipids. To enhance the transfection capability, reduce toxicity, and increase the circulating time, we labeled the liposomes with polyethylene glycol (PEG). Furthermore, we functionalized the liposomes, attaching to the PEG chains peptides to promote blood-brain-barrier (BBB) targeting. We used Tf and arginine to enhance BBB crossing via receptor-mediated endocytosis. Finally, the liposomes were tested for their pDNA loading capacity and in vitro transfection efficiency. In conclusion, in this pilot study, we reported that, although there are still features that need to be refined in the liposomes, such as the PEG-peptide conjugation reactions, the development of a gene carrier was feasible. Thus, further improvement of the liposomal gene delivery system could enable BDNF overexpression in targeted brain areas to promote novel treatments for depressive patients.

This thesis opens the way to new molecular and behavioral studies that can be employed to further investigate the effects of BDNF overexpression in a rat model presenting both, decreased BDNF expression and anxiety- and depression-like phenotype, features commonly found in clinical studies of patients suffering from mood-related disorders. We hope that collective efforts in the field will ultimately achieve the goal of promoting alternative therapeutic options for those struggling with resistance to current antidepressant options thus contributing to improvement of human mental health.

Nederlandse samenvatting

Gedefinieerd als een groep van psychiatrische ziekten, beïnvloeden stemmingsstoornissen de emoties, energie en motivatie van het individu. Onder de vele stemmingsstoornissen is depressie een van de meest opvallende stoornissen. Depressie wordt gekenmerkt door depressieve stemming, verlies van interesse of plezier bij bijna alle activiteiten (anhedonie), eetlust- en slaapstoornissen en energieverlies. Deze symptomen leiden meestal tot een aanzienlijke beperking bij het uitvoeren van belangrijke taken thuis, op het werk of op school. Bovendien kunnen depressieve patiënten gevoelens van waardeloosheid en ongepast schuldgevoel manifesteren, resulterend in zelfmoordgedachten of zelfs pogingen daartoe. De etiologie van depressie wordt toegeschreven aan een complexe interactie tussen omgevingsfactoren en genetische kwetsbaarheid, maar specifieke genen zijn nog niet gevonden, waardoor het moeilijk is om de mechanistische oorzaken van een dergelijke complexe ziekte te begrijpen.

Klinische en preklinische studies trachten de onderliggende processen van depressie op te helderen. Deze inspanningen brachten de serotonine-hypothese van depressie aan het begin van de jaren zestig aan het licht. Het verhogen van serotoninespiegels door middel van selectieve serotonineheropnameremmers (SSRI's) bleek depressieve symptomen te verlichten. Uit niet-overtuigende en inconsistente onderzoeken is echter gebleken dat de serotoninehypothese te simplistisch is om de mechanismen te verklaren waarmee deze stemmingsstoornis zich bij sommige individuen ontwikkelt. Eigenlijk werken de huidige antidepressiva niet voor alle individuen, en is het normaal dat na een succesvolle behandeling veel patiënten terugvallen. Bovendien wordt ongeveer 15-30% van de patiënten geclassificeerd als resistent en omdat de antidepressieve effecten zich in families lijkt te clusteren. Dit suggereert dat genetische factoren een rol kunnen spelen in de effecten van antidepressiva. Een voorbeeld van een dergelijke genetische bijdrage aan resistentie is het serotonine transporter polymorfisme (5-HTTLPR). Personen die het korte allel van het 5-HTTLPR-polymorfisme dragen, vertonen een verhoogd risico op non-responsiviteit op SSRI's of hebben meer tijd nodig om op de behandeling te reageren.

Verschillende studies hebben benadrukt dat de 5-HTTLPR de invloed van stressvolle levensgebeurtenissen op depressie kan moduleren. De meest gevestigde en consistente eigenschap die wordt geassocieerd met het 5-HTTLPR korte allel is neuroticisme, de eigenschap om negatieve effecten te ervaren, waaronder woede, angst, zelfbewustzijn, prikkelbaarheid, emotionele instabiliteit en depressie. Het is aangetoond dat korte allel dragers de neiging hebben om selectieve aandacht te ontwikkelen voor negatieve stimuli. Gevoeligheid voor negatieve stimuli kan de kwetsbaarheid voor psychopathologieën vergroten. Klinische studies laten zien dat individuen die de korte allel variant van het 5-HTTLPR droegen, in tegenstelling tot de lange allel variant, het risico liepen depressie te

ontwikkelen wanneer ze op jonge leeftijd werden blootgesteld aan nadelige psychosociale stressfactoren.

Hoewel knaagdieren niet het humane serotonine transporter-gekoppelde polymorfe gebied (5-HTTLPR) dragen, leidt genetische deletie of gedeeltelijke deletie van de serotonine transporter (SERT) bij knaagdieren tot angst- en depressie gerelateerde fenotypen. Er zijn steeds meer aanwijzingen dat met name heterozygote SERT knockout-muizen ($SERT^{-/-}$) reageren op prenatale, vroege levensjaren en psychosociale stress bij volwassenen, zoals te zien is bij korte allel dragers. $SERT^{-/-}$ ratten vertonen anhedonie-achtig gedrag in de sucrose-voorkeurstest, verhoogde immobiliteit in de gedwongen-zwemtest en en brengen minder tijd door in het centrale gebied van een open veld wat aangeeft dat angstniveaus en depressief gedrag toenemen. Bovendien vertonen $SERT^{-/-}$ ratten verhoogde basale niveaus van het stress hormoon cortisol onder controlecondities, en een veranderde basale hypothalamus-hypofyse-bijnier-as (HPA-axis) activiteit.

Aangezien verhoogde serotoninespiegels niet noodzakelijkerwijs leiden tot verbetering van depressieve gedragsfenotypen, werd de neurotrofe hypothese van depressie geïntroduceerd. Neurotrofines zijn betrokken bij synaptische rijping, neuronale groei en synaptische plasticiteit, zowel tijdens ontwikkeling als volwassenheid. Aangenomen wordt dat verminderde productie, afgifte en/of werking van neurotrofines een directe associatie heeft met depressie. De hersenafgeleide neurotrofe factor (BDNF) is de meest voorkomende en een van de meest onderzochte neurotrofines. Verschillende klinische studies rapporteerden een verlaging van de BDNF-eiwitniveaus in het serum van depressieve personen, wat een directe correlatie aantoont tussen behandeling met antidepressiva en een toename van de perifere BDNF-eiwitniveaus van behandelde patiënten, terwijl onbehandelde personen verlaagde niveaus van BDNF-eiwit vertonen. Studies hebben ook melding gemaakt van abnormale mRNA BDNF- of TrkB-expressie in de hippocampus en de prefrontale cortex postmortemweefsel van suïcidale patiënten met ernstige depressie.

Interessant is dat hoewel behandeling met antidepressiva verhogingen van BDNF-niveaus veroorzaakt, genetische manipulatie van de SERT bij ratten een verlaging van BDNF-niveaus veroorzaakt. Daarom, in overeenstemming met de neurotrofe hypothese van depressie, vertonen $SERT^{-/-}$ ratten, onder basale omstandigheden, een downregulatie van BDNF mRNA en eiwitniveaus in de hippocampus en prefrontale cortex. Verder is er een verlaging van de BDNF-genexpressie waargenomen in de prefrontale cortex van $SERT^{-/-}$ ratten.

Depressie wordt geassocieerd met structurele en functionele veranderingen in de hersenen, waaronder een verminderd hersenvolume en activiteit in de prefrontale cortex. Deze structurele veranderingen bij depressieve patiënten zijn bevestigd in postmortem studies die een vermindering van neuronen en gliaverlies in de prefrontale cortex aantonen,

wat gepaard gaat met een vermindering van BDNF in dit hersengebied. Gezien het verband tussen depressie en prefrontale cortex-veranderingen in studies bij mensen, samen met de verlaagde BDNF-spiegels in de prefrontale cortex van SERT$^{-/-}$ ratten en de rol van BDNF bij het ondersteunen van neuronale plasticiteit, hebben we in **hoofdstuk 2** de effecten van BDNF-overexpressie in de prefrontale cortex van SERT$^{-/-}$ ratten op depressie- en angst-gerelateerd gedrag onderzocht. We toonden aan dat de opwaartse regulering van het BDNF-gen het beste twee weken na de lokale infusie in de prelimbische cortex (een subregio van de prefrontale cortex) werd bereikt, toen de niveaus van totaal BDNF in de prelimbische cortex hoger waren bij met BDNF behandelde dieren dan bij de controle SERT$^{+/+}$ (wild-type) ratten. Onverwacht stelden we vast dat in het algemeen de BDNF-opregulatie de uitkomst van de gedragstesten bij SERT$^{-/-}$ ratten niet veranderde, noch de respons van de wild-type controle-dieren moduleerde. Er waren enkele uitzonderingen. In de sucrosevoorkeurstest leek BDNF-overexpressie bij SERT$^{-/-}$ ratten neofobie te induceren, wat leidde tot een afname van de sucrosevoorkeur, en werd gevolgd door anti-anhedonisch gedrag zoals weerspiegeld door een toename in sucrosevoorkeur. Een andere uitzondering betrof de plasma cortisol niveaus zoals gemeten in de HPA-axis reactiviteitstest. We ontdekten dat BDNF-overexpressie bij wild-type dieren een verslechtering van de HPA-as activiteit veroorzaakte, waardoor de cortisol uitgangswaarden toenamen en de cortisol reactie op stress verhoogde. Verder normaliseerde BDNF-overexpressie bij SERT$^{-/-}$ ratten stress-geïnduceerde cortisol levels , wat suggereert dat verschillende mechanismen ten grondslag liggen aan de HPA-axis reactiviteitsmodulatie door BDNF in SERT$^{-/-}$ en wild-type-ratten.

Omdat BDNF-overexpressie in de prefrontale het angst- en depressie-achtige gedrag bij de SERT$^{-/-}$ ratten niet kon verminderen, was de ventrale hippocampus ons volgende doelwit. De hippocampus speelt ook een sleutelrol bij depressie; het moduleert emotionele verwerking, geheugen en leren, en regelt de secretie van glucocorticoïden door de HPA-as, waardoor dit gebied vatbaar wordt voor de effecten van stress. Stress en andere negatieve stimuli kunnen de plasticiteit van de hippocampus veranderen, waardoor het risico op depressie toeneemt. Deepressie wordt sterk geassocieerd met hippocampale atrofie. Bovendien hebben verschillende onderzoekslijnen met behulp van post-mortem weefsel van suïcidale of depressieve patiënten aangetoond dat BNDF en zijn receptor (TrkB) zijn verlaagd in de hippocampus. Aangezien BDNF-niveaus in de hippocampus van SERT$^{-/-}$ ratten zijn verlaagd, hebben we in **hoofdstuk 3** onderzocht in hoeverre een verhoging van BDNF niveaus in the ventrale hippocampus van SERT-/- ratten depressie-gedrag zou kunnen verminderen. Hiertoe werd dezelfde testbatterij als die gebruikt in hoofdstuk 2 toegepast. We vonden dat BDNF een week na de lentivirus-infusie in SERT$^{+/+}$ ratten tot overexpressie werd gebracht in de ventrale hippocampus, maar neerwaarts werd gereguleerd in de prefrontale cortex. Deze bevindingen geven niet alleen aan dat deze gebieden sterk met elkaar verbonden zijn, maar

ook dat BDNF-transcripten projectie gebieden van de ventrale hippocampus negatief aansturen. Gedragsmatig vonden we dat BDNF-overexpressie in de ventrale hippocampus van $SERT^{-/-}$ ratten een verhoogde inname en voorkeur voor sucrose bevorderde, wat de ratten motiveerde, vooral wanneer ze voor het eerst met deze nieuwe smaak werden geconfronteerd. Evenzo brachten $SERT^{-/-}$ ratten waarbij BDNF verhoogd was in de ventrale hippocampus meer tijd door in het centrum van de nieuwe testkooi dan door controle $SERT^{-/-}$ ratten, wat suggereert dat BDNF angst verminderde.

H**oofdstuk 2** en **3** van dit proefschrift laten zien dat verhoging van BDNF niveaus in de prefrontale cortex en hippocampus verschillende en zelfs tegenovergestelde effecten heeft op gedrag Daarom lijkt het erop dat verhoging van BDNF-eiwitten in het gehele brein mogelijke nadelige gevolgen kan hebben. Hersenspecificiteit is eerder nodig om de gewenste effecten van BDNF te bereiken. Echter, lokale intracerebrale infusie va het BDNF-gen of -eiwit is geen haalbare behandeling voor depressie bij mensen, vooral niet op grote schaal. Ondanks het vermogen van lentivirussen om de bloed-hersenbarrière te passeren bij perifere infusie, hebben de kosten die gepaard gaan met het produceren van voldoende lentivirus voor directe injectie tot nu toe veel klinisch onderzoek verhinderd. Dit belemmerde verdere bevestiging van de veiligheid van lentivirus. Als alternatief zijn niet-virale vectoren naar voren gekomen vanwege de vele voordelen ten opzichte van virale benaderingen, zoals lage kosten, eenvoudigere productie, verhoogde veiligheid en verminderde pathogeniteit. Niet-virale genafgiftesystemen zijn geconstrueerde complexen of nanodeeltjes die zijn samengesteld uit het vereiste nucleïnezuur (plasmide, RNA, oligonucleotiden) en een enkele of een combinatie van materialen zoals kationische lipiden, oppervlakteactieve stoffen, en peptiden. Dergelijke genafgiftesystemen hebben het voordeel dat ze flexibiliteit bieden bij het selecteren van complexvormers.

In **hoofdstuk 4** hebben we een pilotstudie uitgevoerd waarbij we een genafgifteapparaat hebben ontwikkeld en gekarakteriseerd om plasmide BDNF te dragen. We rapporteren de synthese van een liposomale nanodrager van 120-200nm groot, samengesteld uit kationische lipiden. Om het transfectievermogen te verbeteren, de toxiciteit te verminderen en de circulatietijd te verlengen, hebben we de liposomen gelabeld met polyethyleenglycol (PEG). Verder hebben we de liposomen functioneel gemaakt door Tf en arginine eraan te hechten en zodoende de bloed-hersenbarrière te passeren. Ten slotte hebben we de liposomen getest op hun pDNA-laadcapaciteit en in vitro transfectie-efficiëntie. Deze pilot data laten zien dat het mogelijk is om een gendrager te ontwikkelen. Een verdere verbetering van het liposomale genafgiftesysteem zou BDNF-overexpressie in gerichte hersengebieden mogelijk kunnen maken om de behandeling van depressieve patiënten te bevorderen.

Dit proefschrift opent de weg naar nieuwe moleculaire en gedragsstudies die kunnen worden gebruikt om de effecten van BDNF-overexpressie verder te onderzoeken in een ratmodel dat beide vertoont, verminderde BDNF-expressie en een angst- en depressie-achtig fenotype, kenmerken die vaak worden aangetroffen in klinische studies bij patiënten die lijden aan stemmingsstoornissen. We hopen dat collectieve inspanningen in het veld uiteindelijk het doel zullen bereiken om alternatieve therapeutische opties te promoten voor diegenen die worstelen met resistentie tegen de huidige antidepressiva, om de menselijke geestelijke gezondheid te verbeteren.

Resumo

Definidos como um grupo de doenças psiquiátricas, os transtornos do humor afetam as emoções, a energia e a motivação do indivíduo. Entre os muitos transtornos do humor, um dos mais destacados é o transtorno depressivo maior. A depressão é caracterizada por humor deprimido, perda de interesse ou prazer em quase todas as atividades (anedonia), distúrbios de apetite e sono, e perda de energia. Esses sintomas geralmente levam a um comprometimento significativo na realização de tarefas importantes em casa, trabalho ou escola. Além disso, pacientes depressivos podem manifestar sentimentos de inutilidade e culpa inadequada, resultando em pensamentos ou tentativas suicidas. A etiologia da depressão é atribuída a uma interação complexa entre fatores ambientais e vulnerabilidade genética, mas genes específicos ainda não foram encontrados, dificultando a compreensão das causas mecanicistas dessa doença complexa.

Estudos clínicos e pré-clínicos estão tentando elucidar os processos moleculares da depressão. Esses esforços trouxeram à luz a hipótese monoaminérgica da depressão no início dos anos 60. Foi demonstrado que o aumento dos níveis de serotoninas por meio do uso de inibidores seletivos da recaptação de serotonina (ISRSs) aliviava os sintomas depressivos; no entanto, estudos inconclusivos e inconsistentes indicaram que a hipótese monoaminérgica da depressão parece ser simplista demais para explicar os mecanismos pelos quais esse transtorno de humor se desenvolve em alguns indivíduos. Na verdade, os tratamentos antidepressivos atuais não funcionam para todos os pacientes e é comum que, após um tratamento bem-sucedido, os sintomas reapareçam. Além disso, cerca de 15 a 30% dos pacientes são classificados como resistentes e, como a resposta antidepressiva parece agrupar-se nas famílias, sugere-se que fatores genéticos possam desempenhar um papel fundamental. Um exemplo dessa contribuição genética para a resistência é o polimorfismo funcional do transportador de serotonina humana. Este polimorfismo gera alelos longos e curtos na região promotora do gene SLC6A4. Indivíduos portadores do alelo curto (“short”) do polimorfismo 5-HTTLPR apresentaram maior risco de não responderem aos ISRSs, ou demonstraram exigir mais tempo para responder ao tratamento com dificuldades para alcançar a remissão.

Várias pesquisas destacaram que o 5-HTTLPR pode modular a influência de eventos estressantes na depressão. A característica mais bem estabelecida e consistente associada ao alelo 5-HTTLPR é o neuroticismo, que é a disposição da característica para experimentar efeitos negativos, incluindo raiva, ansiedade, autoconsciência, irritabilidade, instabilidade emocional, e depressão. Foi demonstrado que os portadores do alelo curto (s-carriers) tendem a desenvolver atenção seletiva a estímulos negativos. Consequentemente, a sensibilidade a estímulos negativos pode aumentar a vulnerabilidade para psicopatologias. Estudos clínicos revelaram que indivíduos portadores da variante s-alélica do 5-HTTLPR, em

oposição à variante l-alelica (alelo longo), corriam risco de desenvolver depressão quando expostos a estressores psicossociais adversos no início da vida.

Embora os roedores não possuam a região polimórfica ligada ao transportador de serotonina humana (5-HTTLPR), a exclusão genética ou a exclusão parcial da 5-HTT nos roedores leva a fenótipos relacionados à ansiedade e à depressão. Há evidências crescentes de que camundongos heterozigotos contendo 5-HTT gene nocaute ($SERT^{+/-}$) respondem ao estresse psicossocial na vida pré-natal, no início da vida e na vida adulta, como observado nos portadores do s-alelo. Os ratos $SERT^{-/-}$ apresentaram comportamento do tipo anedonia no teste de preferência pela sacarose, aumento da imobilidade no teste de nado forçado, e diminuição do tempo gasto na parte central da arena no teste de campo aberto, indicando aumento nos níveis de ansiedade e o comportamento do tipo depressivo. Além disso, os ratos $SERT^{-/-}$ apresentaram níveis aumentados de corticosterona (CORT) basal sob condições de controle positivo, mostrando atividade alterada do eixo hipotálamo-hipófise-adrenal basal (eixo HPA).

Levando em consideração que simplesmente o aumento dos níveis de serotonina não leva necessariamente à melhoria dos fenótipos comportamentais depressivos, foi introduzida a hipótese neurotrófica da depressão. As neurotrofinas estão envolvidas na maturação sináptica, no crescimento neuronal e na plasticidade sináptica, tanto durante o desenvolvimento quanto na idade adulta. Acredita-se que um comprometimento na produção, liberação e/ou ação das neurotrofinas tenha uma associação direta com a depressão. O fator neurotrófico derivado do cérebro (BDNF) é o mais abundante e uma das neurotrofinas mais investigadas. Vários estudos clínicos relataram uma redução nos níveis de proteína BDNF no soro de indivíduos depressivos, mostrando uma correlação direta entre o tratamento antidepressivo e um aumento nos níveis periféricos de proteína BDNF nos pacientes tratados, enquanto indivíduos não tratados apresentam níveis reduzidos de proteína BDNF. Os estudos também relataram expressão anormal de mRNA BDNF ou TrkB no hipocampo e no tecido post-mortem do córtex pré-frontal de pacientes suicidas com histórico prévio de depressão.

Curiosamente, embora o tratamento antidepressivo induza aumentos nos níveis de BDNF, a manipulação genética do SERT em ratos causa uma diminuição nos níveis de BDNF. Em concordância com a hipótese neurotrófica de depressão, os ratos $SERT^{-/-}$ apresentam, em condições basais, uma regulação negativa do mRNA do BDNF e dos níveis de proteína no hipocampo e no córtex pré-frontal. Além disso, os níveis totais de mRNA de BDNF (exon IX) apresentaram-se significativamente reduzidos e a redução da expressão gênica de BDNF observada no córtex pré-frontal de ratos $SERT^{-/-}$ mostrou ser devida, pelo menos em parte, a alterações epigenéticas que afetam as regiões promotoras de éxons IV e VI.

O transtorno depressivo maior está associado a alterações estruturais e funcionais da imagem cerebral, incluindo redução do volume e atividade cerebral no PFC. Essas alterações estruturais em pacientes deprimidos foram confirmadas em estudos post-mortem demonstrando uma redução nos neurônios e perda glial no PFC, o que é acompanhado por uma redução no BDNF nessa área do cérebro. Dada a associação entre depressão e alterações do PFC em estudos em humanos, juntamente com os níveis reduzidos de BDNF no PFC de ratos SERT$^{-/-}$ e o papel do BDNF no apoio à plasticidade neuronal, que é particularmente afetada nos transtornos depressivos, no **capítulo 2**, foram investigados os efeitos da superexpressão do BDNF no PFC de ratos SERT$^{-/-}$. Demonstramos que a regulação positiva do gene BDNF foi melhor alcançada duas semanas após a infusão local no córtex pré-límbico (PrL), quando os níveis de BDNF total no PrL foram mais altos em animais tratados com BDNF do que nos ratos SERT$^{+/+}$ tratados com controle – GFP. Inesperadamente, observamos que, em geral, a regulação positiva do BDNF não alterou o resultado dos testes comportamentais em ratos SERT$^{-/-}$, nem modulou a resposta dos animais controle. Algumas exceções foram encontradas. Por exemplo, no teste de preferência pela sacarose, a superexpressão do BDNF em ratos SERT$^{-/-}$ parecia induzir neofobia, levando a uma diminuição na preferência pela sacarose, que foi seguida pelo comportamento anti-anedônico, refletido por um aumento na preferência pela sacarose. Outra exceção envolveu os níveis de CORT, medidos no teste de reatividade no eixo HPA. Descobrimos que a superexpressão de BDNF em animais controle mediou um comprometimento na atividade do eixo HPA, aumentando os níveis basais de CORT e diminuindo os níveis de CORT em resposta ao estresse. Além disso, em ratos SERT$^{-/-}$, a superexpressão do BDNF normalizou os níveis de estresse do CORT, sugerindo que diferentes mecanismos estão implicados na modulação da reatividade do eixo HPA por BDNF em ratos SERT$^{-/-}$ e seu respectivo controle.

Como a superexpressão do BDNF no PFC falhou em resgatar os comportamentos semelhantes à ansiedade e à depressão nos ratos SERT$^{-/-}$, nosso próximo alvo foi a área ventral do hipocampo (vHIP). O hipocampo também desempenha um papel fundamental na depressão; modula o processamento emocional, a memória e o aprendizado, e controla a secreção de glicocorticóides pelo eixo hipotálamo-hipófise-adrenal (eixo HPA), tornando essa área suscetível aos efeitos do estresse. O estresse e outros estímulos negativos podem alterar a plasticidade do hipocampo, aumentando o risco de depressão; além disso, o transtorno depressivo está muito associado à atrofia do hipocampo. Várias linhas de pesquisa mostraram que o BNDF e seu receptor de alta afinidade (TrkB) estão diminuídos no tecido post mortem do hipocampo de pacientes suicidas ou deprimidos. Conformemente, considerando que os níveis de BDNF estão diminuídos no hipocampo de ratos SERT$^{-/-}$, no **capítulo 3**, realizamos a mesma bateria de experimentos mencionados no **capítulo 2**, para avaliar se a superexpressão de BDNF no vHIP de SERT$^{-/-}$ ratos remediariam seu fenótipo

comprometido. Curiosamente, uma semana após a infusão de lentivírus no $SERT^{+/+}$, o BDNF foi superexpressado no vHIP, mas com regulação negativa no PrL e IL. Esses achados indicam não apenas que essas áreas estão fortemente conectadas, mas também as transcrições do BDNF forneceram feedback negativo para áreas distais do vHIP. Comportamentalmente, descobrimos que a superexpressão de BDNF no vHIP de ratos $SERT^{-/-}$ promoveu aumento da ingestão e preferência de sacarose, motivando os ratos, especialmente quando eles foram confrontados com esse novo sabor pela primeira vez. Da mesma forma, no teste locomotor induzido pela novidade, os ratos $SERT^{-/-}$ infundidos com lentivírus BDNF passaram mais tempo no centro do novo campo de teste do que os ratos $SERT^{-/-}$ que receberam controle, sugerindo que o BDNF diminuiu o comportamento semelhante ao da ansiedade que é expressado por ratos $SERT^{-/-}$, porém somente quando o BDNF foi superexpresso no vHIP.

Portanto, como mostrado nos **capítulos 2** e **3** desta tese, a injeção de BDNF no córtex pré-límbico promoveu uma resposta comportamental diferente em ratos $SERT^{-/-}$ quando comparados aos animais injetados na área ventral do hipocampo. Da mesma forma, outros estudos mostraram efeitos distintos do BDNF, dependendo da região alvo no cerebro. Portanto, parece que a regulação positiva global da proteína BDNF pode ter desvantagens potenciais. Pelo contrário, a especificidade na região cerebral é necessária para alcançar os efeitos desejados do BDNF. No entanto, embora possível, a infusão intracerebral local do gene ou da proteína BDNF não é um tratamento viável para a depressão em humanos, principalmente em larga escala. Além disso, apesar da capacidade dos lentivírus de atravessar a barreira hematoencefálica (BBB) após infusão periférica, os custos envolvidos na produção de lentivírus suficiente para injeção direta impediram muitas investigações clínicas, dificultando a confirmação da segurança do lentivírus. Alternativamente, os vetores não virais surgiram por suas muitas vantagens sobre as abordagens virais, exibindo baixo custo, produção mais fácil, maior biossegurança e menor patogenicidade. Os sistemas de entrega de genes não virais são complexos manipulados ou nanopartículas (NPs) compostos pelo ácido nucleico necessário (plasmídeo, RNA, oligonucleotídeos) e um único ou uma combinação de materiais, como lipídios catiônicos, surfactantes, peptídeos, entre outros. Tais sistemas de entrega de genes têm a vantagem de oferecer flexibilidade na seleção de agentes complexantes e porções de direcionamento para fornecer expressão gênica localizada.

No **capítulo 4**, demonstramos um estudo piloto no qual desenvolvemos e caracterizamos um dispositivo de entrega de genes para transportar um plasmídeo contendo a sequencia genética do BDNF. Relatamos a síntese de um nanocarreador lipossômico de 120-200 nm de tamanho, composto por lipídios catiônicos. Para melhorar a capacidade de transfecção, reduzir a toxicidade e aumentar o tempo de circulação, adicionamos polietilenoglicol (PEG) a camada experior dos lipossomas. Além disso, funcionalizamos os

lipossomos, aderindo peptídeos as cadeias de PEG para promover a permeabilidade seletiva da barreira hematoencefálica (BBB). Usamos Tf e arginina para melhorar a travessia da BBB via endocitose mediada por receptor. Finalmente, os lipossomas foram testados quanto à sua capacidade de carga de pDNA e eficiência de transfecção in vitro. Em conclusão, neste estudo piloto, relatamos que, embora ainda existam características que precisam ser refinadas nos lipossomas, tal como as reações de conjugação PEG-peptídeo, o desenvolvimento de um nanocarreador genético foi possível. Assim, uma melhoria adicional do sistema de entrega de genes lipossômicos poderia permitir a superexpressão do BDNF em áreas cerebrais direcionadas para promover novos tratamentos para pacientes depressivos.

Esta tese, portanto, abre caminho para novos estudos moleculares e comportamentais que podem ser empregados para investigar mais os efeitos da superexpressão de BDNF em um modelo de rato apresentando ambos, expressão diminuída de BDNF e fenótipo de ansiedade e depressão, características comumente encontradas em estudos clínicos de pacientes que sofrem de transtornos relacionados ao humor. Esperamos que os esforços coletivos nesta área de pesquisa atinjam o objetivo de desenvolver terapias alternativas às opções atuais de antidepressivos para aqueles que lutam contra a resistência ao tratamento depressivo, contribuindo assim para a melhoria da saúde mental humana.

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Tenho certeza que muitos outros nomes de colegas e amigos deveriam ser citados aqui. O doutorado é o mais alto título acadêmico que um profissional pode conquistar, mas eu não teria chegado até aqui, por exemplo, sem a ajuda dos meus professores da minha escola querida, Carmela Veloso, ou dos professores do curso de Farmácia da UEPB. O meu muito obrigada a todos esses profissionais que contribuíram de diversas maneiras para construir a minha carreira acadêmica. E a todos os amigos e colegas que passaram pela minha vida deixando a sua contribuição, o meu muito obrigada!

Last, but (definitely) not least. Minha profunda gratidão ao amor da minha vida, Eduardo Lincoll. O que seria de mim sem você ao meu lado. No meio turbulento desse doutorado eu me tornei a senhora Danielle Alves, e que presente maravilhoso foi isso! Você enxugou minhas lagrimas, me abraçou, me levou todos os dias para o trabalho quando eu não tinha mais energia para ir de bicicleta. Obrigada por sua paciência, pelas noites que você foi para a cama sem mim porque eu estava no computador até tarde, obrigada pelas caminhadas noturnas para refrescar minha cabeça. Muito obrigada pelos sacrifícios que você fez por mim. Você largou sua vida no Brasil e veio se aventurar ao meu lado. Ficamos mais fortes! Com a ajuda de Jeová, nosso casamento e todos os desafios serviram de treinamento para os próximos que virão. Essa experiência com toda certeza do mundo não teria sido a mesma sem você! Te amo!

Curriculum Vitae

Danielle Mendes Diniz was born on the 4th of March 1991 in Campina Grande – Paraiba, Brazil. After finishing high school at the Escola Carmela Veloso in 2008, she passed in the very competitive exam to pursue her fully-funded Bachelor in Pharmaceutical Sciences at the Paraiba State University. During her bachelors, Danielle has always been engaged in scientific research. In the second year, she received funding to research the topic of mutagenesis, and later she switched to the topic of pharmaceutical nanotechnologies, where she started to develop nanoparticles to be used as drug carriers. In 2013, she was awarded with the Brazilian Science without Borders scholarship exchange program, and she moved to Toronto-CA, where she joined York University and, as an extracurricular activity, had the opportunity to work at a Canadian pharmacy. On coming back to Brazil in 2015, Danielle defended her Bachelor thesis on the topic of pharmaceutical nanotechnologies to improve the delivery of topical drugs. Afterward, Danielle engaged as an invited student in the Master for Pharmaceutical Sciences at Paraiba State University, during which she came in contact with Prof.dr. Judith Homberg and applied for a scholarship to pursue her PhD. In 2016, under the funding provided by the Science without Borders Brazilian scholarship program, Danielle started her PhD at the Radboud University. In 2018, Danielle spent about four months at the Universita' degli Studi di Milano, where the molecular analysis of the data presented in chapters 2 and 3 were performed in collaboration with the department of Pharmacological and Biomolecular Sciences under the supervision of Prof.dr. Marco A. Riva and Dr. Francesca Calabrese. Additionally, parts of the data presented in chapter 4 were obtained in collaboration with the department of Pharmaceutical Sciences at the Universita' degli Studi di Milano under the supervision of Dr. Silvia Franze. In 2018, Danielle was also a Co-Chair in a session held at the Dutch Neuroscience Meeting entitled "Depression: new molecular and therapeutic insights". Overall, the research presented in this thesis was conducted between February 2016 and February 2020 under the supervision of Prof.dr. Judith Homberg.

Completed graduate courses:

The Art of Presenting Sciences – 1.5EC
Laboratory Animal Course – 3EC
Animal Models for Brain function and Disorders – 6EC
Education in a Nutshell – 1EC
Writing a Review Article – 1EC
Statistics for PhDs using SPSS – 1.5EC

Research data management

Chapters 2 and 3 of this thesis are based on animal research, which was conducted in accordance with the research proposal 2016-0036 approved by the Animal Ethics Committee (Dier Experimenten Commissie, RU-DEC, Nijmegen, The Netherlands). The data obtained during the experiments conducted at the Radboud university medical center (Radboudumc) was stored on university servers belonging to the department. Data is additionally captured and stored on Labguru, a digital lab book client, which is centrally stored and daily backed-up on the local Radboudumc server. Published data generated or analyzed in this thesis are part of preprint articles, and its additional files are available from the associated corresponding authors on request. To ensure the interpretability of the data, all filenames, primary and secondary data, metadata, descriptive files, and program code and scripts used to provide the final results are documented along with the data.

Donders Graduate School for Cognitive

For a successful research Institute, it is vital to train the next generation of young scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School for Cognitive Neuroscience (DGCN), which was officially recognized as a national graduate school in 2009. The Graduate School covers training at both Master's and PhD level and provides an excellent educational context fully aligned with the research program of the Donders Institute.

The school successfully attracts highly talented national and international students in biology, physics, psycholinguistics, psychology, behavioral science, medicine and related disciplines. Selective admission and assessment centers guarantee the enrolment of the best and most motivated students.

The DGCN tracks the career of PhD graduates carefully. More than 50% of PhD alumni show a continuation in academia with postdoc positions at top institutes worldwide, e.g. Stanford University, University of Oxford, University of Cambridge, UCL London, MPI Leipzig, Hanyang University in South Korea, NTNU Norway, University of Illinois, North Western University, Northeastern University in Boston, ETH Zürich, University of Vienna etc... Positions outside academia spread among the following sectors: specialists in a medical environment, mainly in genetics, geriatrics, psychiatry and neurology. Specialists in a psychological environment, e.g. as specialist in neuropsychology, psychological diagnostics or therapy. Positions in higher education as coordinators or lecturers. A smaller percentage enters business as research consultants, analysts or head of research and development. Fewer graduates stay in a research environment as lab coordinators, technical support or policy advisors. Upcoming possibilities are positions in the IT sector and management position in pharmaceutical industry. In general, the PhDs graduates almost invariably continue with high-quality positions that play an important role in our knowledge economy.

For more information on the DGCN as well as past and upcoming defenses, please visit:
http://www.ru.nl/donders/graduate-school/phd/

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