PUCRS

ESCOLA DE CIÊNCIAS DA SAÚDE E DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO
BIOLOGIA CELULAR E MOLECULAR

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SOCIAL STRESS: THE GOOD, THE BAD, AND THE NEUROTROPHIC FACTOR:
UNDERSTANDING THE BRAIN THROUGH PET IMAGING AND MOLECULAR BIOLOGY

Porto Alegre
2019

PÓS-GRADUAÇÃO - *STRICTO SENSU*

Pontifícia Universidade Católica
do Rio Grande do Sul

Ficha Catalográfica

L732s Lima Giacobbo, Bruno

Social stress - the good, the bad, and the neurotrophic factor : Understanding the brain through PET imaging and molecular biology / Bruno Lima Giacobbo . – 2019.
176.
Tese (Doutorado) – Programa de Pós-Graduação em Biologia Celular e Molecular, PUCRS.

Orientadora: Profa. Dra. Elke Bromberg.
Co-orientadora: Profa. Dra. Nadja Schöder.
Co-orientador: Prof. Dr. Erik FJ de Vries.

1. Biologia Celular. 2. Biologia Molecular. 3. Neuroimagem. 4. Neurociência. I. Bromberg, Elke. II. Schöder, Nadja. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da PUCRS
com os dados fornecidos pelo(a) autor(a).
Bibliotecária responsável: Clarissa Jesinska Selbach CRB-10/2051

This thesis is valid for a double PhD degree as a collaboration between the University of Groningen, the University Medical Center Groningen, and the Rio Grande do Sul State Pontifical Catholic University. The work on this book has been carried out in the Department of Nuclear Medicine and Molecular Imaging of the UMCG, as well as in the Laboratory of Biology and Nervous System Development of PUCRS.

The research contained in this thesis were financially supported by the Abel Tasman Talent Program (ATTP) of the UMCG and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of PUCRS. The printing of this thesis was financially supported by the School of Behavioral and Cognitive Neuroscience (BCN), and by the Library of the University of Groningen.

Cover design: Bruno Lima Giacobbo
Printing: Boekendeal
ISBN: 978-94-034-2060-8 (e-book); 978-94-034-2061-5 (Printed)
Dissertation of University of Groningen, Groningen, The Netherlands
Dissertation of Rio Grande do Sul State Pontifical Catholic University, Porto Alegre, Brazil

Todos esses aí estão
Atravancando meu caminho,
Eles passarão...
Eu passarinho!

Mário Quintana

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Resumo

Na sociedade moderna, a população vive sob constante pressão. Se por um lado, estressores considerados “leves” podem agir como um motivador, levando a um aumento na produtividade e criatividade, por outro, o constante peso do estresse por um longo período de tempo leva não apenas a um prejuízo cognitivo, mas também a um comprometimento à qualidade de vida da pessoa. Este prejuízo é observado no aumento nos números de problemas de saúde associados ao estresse no mundo, e transtornos de humor e psiquiátricos são uma parcela pequena do amplo leque de doenças do estresse.

Biologicamente, o cérebro é a região mais afetada por situações extremas de estresse. Da sinalização de neurotransmissores, função endócrina e neuronal são diminuídas durante longos períodos de estresse, e se deixados não tratados, estas mudanças neurológicas são fortemente associadas a sintomas depressivos, e o primeiro passo para o desenvolvimento de depressão. Neste contexto, alterações na concentração de fator neurotrófico derivado do cérebro (BDNF) é um dos muitos componentes associados a depressão. Esta tese tem como principal objetivo a melhor compreensão de como situações sociais de estresse são capazes de alterar a concentração de BDNF e modificar fatores relacionados a depressão. Para isto, o uso de tomografia por emissão de pósitrons (PET, em inglês) para a observação destes fatores *in vivo* em modelo animal fora utilizada, com foco na neuroinflamação decorrente do estresse social.

Em conclusão, estímulos sociais tiveram um impacto no cérebro, demonstrado pelas diferenças de comportamento, BDNF, e marcadores sinápticos em situações de estimulação social e isolamento social. Nossos resultados mostram que a duração da estimulação e o tempo ao qual a análise fora realizada são importantes para estas mudanças comportamentais. Protocolos de estresse mais longos são necessários para obter-se um estado crônico de ansiedade e depressão em modelos de estresse em animais. O mesmo padrão encontrado no comportamento destes animais pode também influenciar os marcadores de neuroinflamação e neurotróficos analisados nesta tese.

Abstract

In modern society, people are living under constant pressure. On the one hand, mild “healthy” stressors can be a motivator, leading to increased individual productivity and creativity. On the other hand, however, constant, excessive stress over a long period of time can impair not only the mind but the overall well-being of a person. The burden of aforementioned societal pressure is shown by the increasing number of stress-associated health issues around the globe. Mood- and psychiatric disorders are just a small part of the wide range of diseases related to stress.

Biologically, the brain is the region that is most affected by highly stressful situations. Neurotransmitter signaling, neuroendocrine function, and neuronal signaling are lowered during periods of chronic stress, and if left unchecked, these neurologic changes are amongst the causes of depressive symptoms and the first step towards clinical depression. Alteration of brain-derived neurotrophic factor (BDNF) concentration is one of many biochemical changes associated with depression. The main goal of this thesis is to better understand how socially stressful situations are able to change BDNF concentration and modify depression-related factors. For that the use of Positron Emission Tomography (PET) to observe such factors *in vivo* in an animal model has been used, with a significant focus on social stress-dependent neuroinflammation.

In conclusion, social stimuli had an impact in the brain, as shown by differences in behavior, neurotrophin, and synaptic plasticity markers, both in positive and negative social environments. It appears that the longer the stimulation, and the shorter the analysis period, the more pronounced is the behavioral change. Longer stress protocols are needed in order to achieve a chronic state of anxiety- and depressive-like behaviors in an animal model of stress, allied with possibly a shorter interval between stressor and analysis. The same pattern found in the behavior of these animals can also influence the analysis of neuroinflammation and neurotrophic biomarkers that were analyzed in this thesis.

Chapter 1

Introduction

On stress response

The human body has always been influenced by the environment surrounding it [1,2]. The ability to adapt is the main reason why humans are able to thrive in so diverse environments for such a long time. Since the beginning of mankind, there were several key events that defined the course of human evolution: where to live, what to eat, how to survive, when to run towards or away from something. All the needs one had were based on where one lived and the organism learned, through the course of evolution, that these needs come at a cost.

And that some costs could eventually be more than one could deal with.

By definition, a stressful situation is any given moment where the organism at question is afflicted by a challenge, which could be a one-time-only event (acute stressor) or a repeated event over some period of time (chronic stressor). How the organism is able to cope with the stressor depends on a few factors: the intensity and duration of the stressor; the environment prior to the stressor; and how the organism responds to the first two [3,4]. Intensity and duration are self-explanatory: the stronger and longer the stressful event, the harder for the organism to cope with it. The environment can be a major factor in how the organism deals with stress, as positive or negative events before the stressor can affect the response to the challenge. Lastly, how each individual perceives the stressor in a subjective manner can influence the elicited response towards challenge [1]. Thus, it is possible to create an imaginary threshold of the stressor for each individual (i.e. allostasis), above which the individual is unable to cope with the situation (i.e. allostatic load). If the stressor was not strong – or long – enough to surpass this threshold, the individual will eventually return to physiological levels (i.e. homeostasis) [5].

The most commonly studied stress response system is the Hypothalamus-Pituitary-Adrenal axis (HPA-axis – Figure 1), with key contributions from the hippocampus, prefrontal cortex, and amygdala [6,7]. In a physiological situation, stress elicits a response from the brain by the release of corticotropin-releasing hormone (CRH) from the hypothalamus that signals the pituitary to release adrenocorticotropic hormone (ACTH) in the bloodstream. ACTH stimulates the adrenal cortex to produce and release glucocorticoids – mainly cortisol (in humans) or corticosterone (CORT, in rodents) – in the bloodstream. Cortisol inhibits the further production of CRH from the hypothalamus, thus creating a negative feedback loop, in which cortisol regulates the inhibition of its production.

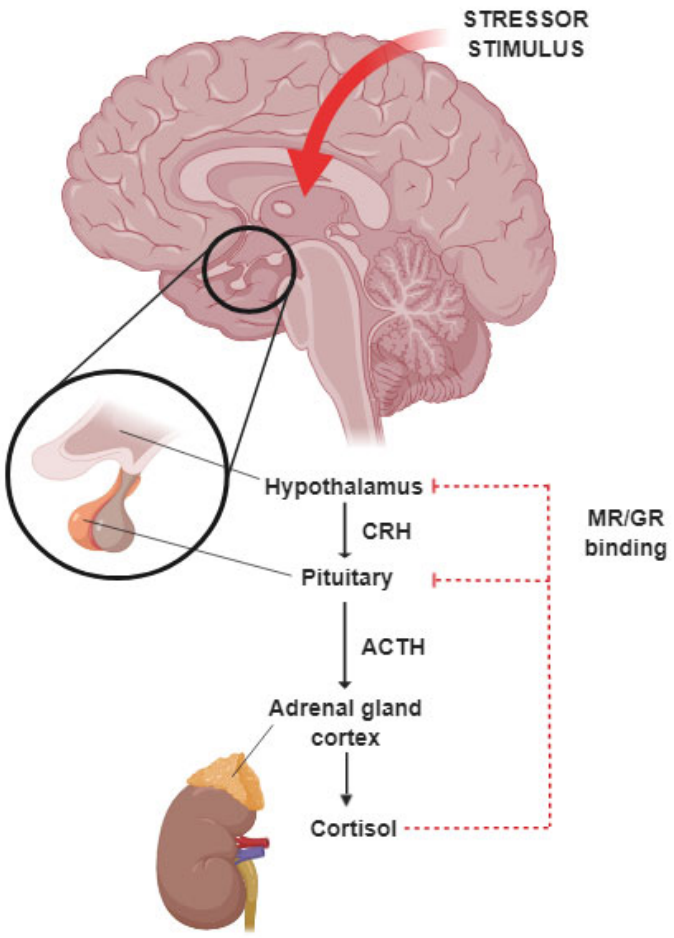

Figure 1: Mechanism of stress response in humans. The presence of a stressor (red arrow) induces the hypothalamus to produce CRH, which stimulates the anterior pituitary to produce and release ACTH in the bloodstream. ACTH reaches the cortex of the adrenal gland and induces the production and release of cortisol in the bloodstream, eventually crossing the BBB and inhibiting the production of CRH and ACTH by the hypothalamus and anterior pituitary, respectively (red dashed arrow). Abbreviations: CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; BBB: blood-brain barrier.

Glucocorticoids easily cross the blood-brain barrier (BBB) and bind directly to intracellular glucocorticoid- or mineralocorticoid receptors (GR and MR, respectively), eliciting a plethora of functions, including regulation of the release of neurotransmitters and neurotrophins, modulation of second messenger signaling and modulation of gene expression [8–10]. At rest, MR are usually close to saturation with glucocorticoids in limbic regions of the brain. Thus, there is always regular, basal signaling from glucocorticoids, while GR are mainly non-active. After activation by a surge of glucocorticoids, usually in the case of stressful situations, MR are quickly saturated by glucocorticoids, while GR are readily activated by the increased concentration of glucocorticoids in the brain. It is assumed that these changes in response to glucocorticoids alter the manner the brain will counter the

stressor in the mid- to long-term, with MR acting as a stimulus of stress-related circuitry, whereas GR act as a suppressor of the very same circuitry [10].

Both MR and GR participate together in the modulation of a successful stress response against challenging stimuli. However, if the stimuli are too strong and persistent or recurrent, the physiological stress response can be unable to deal with the situation, leading to a state where the organism shows long-term modifications. This state of chronic stress happens when there is an allostatic overload, meaning that the organism cannot regulate its allostasis any longer, and the harm of stress becomes constant [6]. The changes are widespread throughout the brain: from DNA regulation to synaptic function in neurons, finally leading to neurotoxicity, apoptosis, behavioral and cognitive changes. Chronic stress eventually becomes a disease condition that needs to be dealt with accordingly.

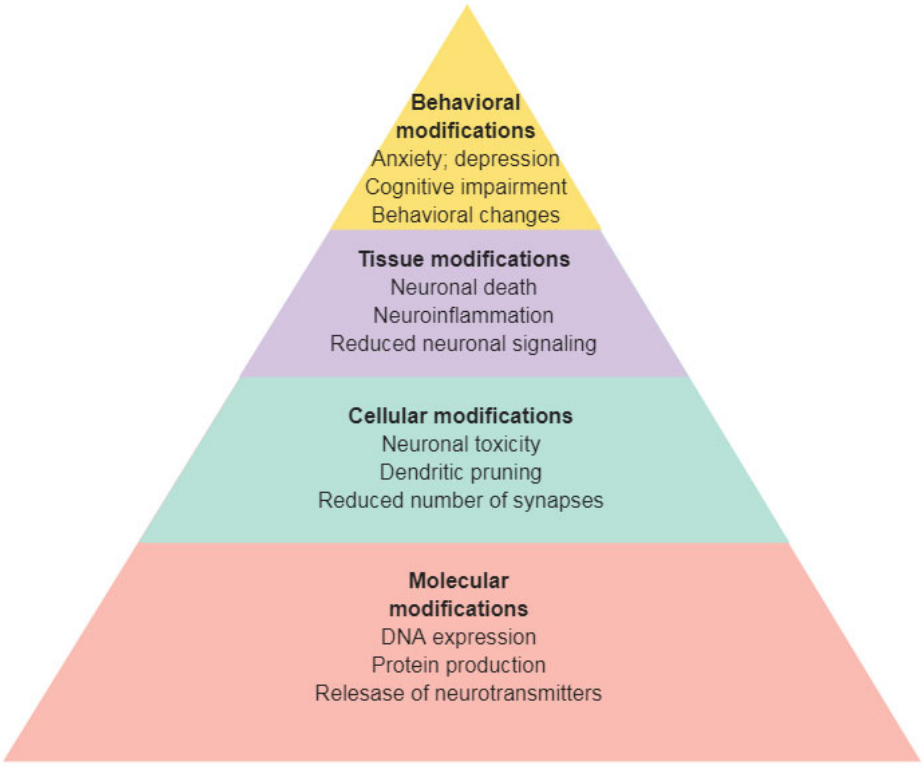

Figure 2: Modifications induced by chronic stress in the brain.

Impaired stress response and depression

In modern-day society, there is a crisis of welfare, with more and more individuals suffering from stress-related disorders. One of the main events caused by social stress is the onset of psychiatric and mood disorders, such as major depressive disorder (MDD). Depression is a concern of its own, turning into a challenge not only for those who suffer from it but also for those around, as well as to governments and healthcare systems throughout the Western world. Of all neuropsychiatric disorders,

depression is the most diagnosed one, being the most prevalent in almost all ages [11,12]. The disease has several main concerns: **1)** depression affects the general quality of patients, leading to familiar and work-related problems; **2)** it is a main healthcare problem by being very difficult to treat and with a fairly low treatment efficacy, as the most frequently used antidepressant drugs only reach 50-60% total remission after months – or years – of treatment; **3)** due to the low efficacy of treatment and a usually long time for antidepressant to act, there is a high number of treatment dropouts, with a significant effect on health quality, and the overall nature of the disease; **4)** depression severity is strongly associated with a higher suicide rate; **5)** depression is present as a comorbidity factor in several other diseases, and most of psychiatric and mood disorders. These factors together contribute to one of the hardest problems in medical research.

The biology of depression is as wide as its symptoms. The genetics of depression are not well understood and there are very few candidate genes that are strongly linked to disease proneness [13–15]. In fact, it is well assumed in the academic field that, even though genetics have a role in the predisposition of the disease (i.e. individuals that are prone to be depressed) and in the relapse of the disease (i.e. epigenetic regulation of gene transcription [16]), environmental effects are the main contributing factors for disease onset, as was shown in several experimental studies with different stressors in humans [6]. The cellular biology of depression is mainly characterized by a decrease of excitatory neurotransmission and, consequently, decreased synaptic activity, especially in dopaminergic, serotonergic and noradrenergic neurons. In fact, these three subpopulations of neurons still play the largest role in treating depression, as the most frequently used antidepressants have a direct effect on the concentration of these neurotransmitters in the synaptic cleft [17]. When synaptic activity is low, there is a decrease in dendritic branching and decreased overall signaling to the affected neuron. As neurons are specialized in receiving and transmitting signals, a constant input of signaling from neighboring neurons and glial cells is needed for these cells to maintain their function (e.g. cytokines, growth factors, neurotransmitters). This transit of information becomes severely impaired during a depressive episode, leading eventually to neuronal apoptosis and disruption of the brain circuitry as the depressive event unfolds.

Impact of depression in neurotrophins

Neurotrophins are a group of proteins that share some structural and functional similarities. This group is comprised of four proteins: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 and 4 (NT-3 and NT-4) [18]. A few other proteins also share similar structure or functionality but are not classified as neurotrophins (e.g. glia-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF)). Neurotrophic factors are expressed with active pro-

domains that can be cleaved by intracellular or extracellular proteinases to its mature form, which is released into the synaptic cleft [19]. These proteins act as a paracrine or autocrine signal inducers by binding to their respective, high-affinity tyrosine kinase receptors (Trk) or by binding to a non-specific, low affinity, $p75^{NTR}$ receptor [20,21]. By binding to Trk receptors, neurotrophic factors participate in the regulation of several neuronal functions, each acting in a different manner.

BDNF is the most studied neurotrophin, and the most known of the four. By binding to its receptor, TrkB, it elicits a wide range of functional modifications, including gene expression, signaling of neuronal survival, regulation of neurotransmitter release, induction of neuronal activity by increase the number of dendritic branches, as well as the surface area of dendrites, allowing a larger range of synapses towards the neuron. As commonly stated in the literature, BDNF is activity driven, meaning that its function and release by neurons is mostly dependent on the health and lifestyle of the individual [22]. Thus, this protein is known as a biomarker of general health quality, but it can also be used to demonstrate when the organism is in a state of disorder. While positive environmental stimuli (e.g. social and cognitive stimulation, physical activity, balanced diet) increase BDNF concentration, negative stimuli (e.g. social isolation, sedentarism, obesity, disease) decrease BDNF signaling in the brain.

In depression, the systemic impairment in neurotransmission leads to decreased concentrations of BDNF in the neurons and therefore impaired BDNF signaling. As BDNF regulates neuronal survival, a lower concentration of the protein is associated with depressive symptoms and, to a lesser extent, to the severity of the disease [18]. Additionally, BDNF seems to be a major factor in the development of depression, as carriers of a single nucleotide polymorphism (SNP) that is associated with a decreased expression of the *Bdnf* gene show a larger susceptibility towards the development of depression [23]. As the protein has a key role in the modulation of neurotransmitter function, situations where neurotransmission is impaired, such as depression, can have a greater effect on subjects with an innate decreased neuronal concentration of BDNF.

Inflammation in stress-induced depression

Whenever a hazard threatens homeostasis, immune cells rapidly migrate to the affected region to resolve the threat and restore the balance by helping in the repairing of the injured tissue. This mechanism has been established through constant evolution for millions of years in different species [24]. More complex life forms, such as vertebrates, would not exist without a specific system to deal with environmental challenges [25,26]. The immune response in the central nervous system (CNS) of mammals is mainly performed by its glial cells (i.e. astrocytes and microglia), whereas neurons and oligodendrocytes contribute in a more indirect manner. Glial cells are present in the whole brain and

perform the first – and last – line of defense against threats to the CNS. In animals, the immune system is highly conserved between species and is ubiquitously present throughout the organism, including the brain.

In a healthy organism, there is a thin balance between pro- and anti-inflammatory cytokines that support brain activity by regulating its microenvironment. In depression though, this system is compromised by the disruption of the brain circuitry, generating a higher pro-inflammatory pattern of immune regulation. This disparity increases as the disease progress in severity [27,28]. In case of disease, two inflammatory processes play a role: peripheral and central inflammation. In the periphery, the activation of immune cells results in the production of pro-inflammatory cytokines and a decrease in anti-inflammatory signaling from their immune counterparts, leading to an inflammatory profile that induces inflammation [29,30]. This signaling pattern is recognized by the brain through stimulation of the peripheral nerve fibers that activate the brain immune system, through the direct crossing of cytokines through the BBB causing glial activation, or through the migration of monocytes into the brain [31]. In the brain itself, decreased neurotransmission, or increased concentrations of glucocorticoids can lead to an impaired neuronal circuitry, which may result in neurotoxicity and neuronal death, release of chemokines by apoptotic neurons and recruitment of glial cells and macrophages to initiate a neuroinflammatory response. This generates positive feedback, as increased inflammation induces the production and release of pro-inflammatory substances (e.g. reactive oxygen species (ROS), interferon, interleukins) and chemokines that stimulate migration of monocytes into the brain. All these factors impact neuronal signaling and generate more toxicity, thus increasing the neuroinflammatory response in the brain.

Even though neuroinflammation has been observed in all kinds of dysfunction of the human brain, surprisingly it has been disregarded for a long time by both researchers and physicians as a potential target for treating brain disorders. Mood disorders can be affected quite significantly by neuroinflammatory processes, and many treatments for these diseases have mid- to low treatment efficacy. Thus, new therapeutic measures to control such diseases are direly needed to further improve the quality of life of patients. In this regard, anti-inflammatory compounds are currently being studied as for their possible antidepressant effect (review in [32]) and might show a promising pharmacological therapy to complement currently used antidepressants.

Depression and its correlates in animal models

Although depression is basically a human disorder, several of its symptoms can be emulated in animals (i.e. depressive-like behavior) [33]. It is impossible, thus, to completely translate what is seen in humans to an animal model and vice versa, especially since the cause of depression in humans is not fully

understood yet. Therefore, researchers created specific models that tackle specific clinical symptoms of the disease, and the sum of all outcomes gives a broad understanding of how the molecular mechanisms of the disease are intertwined (Table 1) [34]. The common approach used to induce depressive behavior in animals is by submitting it to a stressful situation repeatedly. These techniques have been largely used in translational psychiatry as they pose a more "natural" system with some similarities to human depression onset when compared to other methods of induction of depressive-like behavior (e.g.: genetics or pharmacological intervention, or the presence of a stressor over a long period of time), although the amount of animals used is higher due to the inherently high inter-subject variability [35].

However, one of the main concerns on the development and implementation of animal models for stress in psychiatric disorders is the lack of uniformity. Currently, a wide range of methods is applied that vary with regards to the duration of the exposure to stress (minutes to hours), the intensity of the stressor, and for how long the stressor will take place (days, weeks or months). Thus, there is a dire need to standardize the methodologies currently used in order to improve the reproducibility, and consequently reliability, of these models.

Table 1: commonly used stress paradigms to induce depressive-like behavior

Method	Description
Chronic (unpredictable) mild stress - CUMS	Daily use of different stimuli over a long timeframe to induce a recurrent stress response in the animal.
Forced swim test - FST	Considered a behavioral test, can also be used as a hopelessness model, as the animal is submitted to a stressful, unavoidable environment for several minutes.
Chronic immobilization	Considered a mild- to moderate stressor, it places the animal in a frame where it cannot move. Test duration varies greatly in literature.
Social isolation - (SI)	Uses the natural social behavior of rodents by impeding them to socialize with their peers. The time of isolation varies from days to months in the literature.
Repeated social defeat - RSD	Uses the natural territoriality of animals, as animals are presented to the cage of a larger, more aggressive animal each day of the paradigm. The number of days and intensity of aggression varies in the literature.

The use of positron emission tomography to study disease

One of the main concerns of translational research is how to interpret the obtained data, and how to correlate the animal data to what is found in humans. Animal data usually comprises of one

experimental phase, and several ex-vivo molecular analyses performed after termination of the animal. Positron emission tomography (PET) has the advantage that it can perform the very same molecular profiling of animals *in vivo* during the experimental phase without the need for termination of the animal [36,37]. PET imaging in animals has been used in several disease models, adding a tool for translation of the results of animal research, as the same methodology can also be applied in humans.

PET requires the labeling of a molecular marker with an isotope that emits radiation for a short period of time (i.e. minutes to days). This radioisotope emits a positron (β^+ radiation). When the positron collides with an electron, a process called annihilation will occur. This process converts the mass of the positron and electron into two gamma photons that are traveling in opposite directions (180° angle). The photons are captured by scintillation detectors positioned in a ring around the subject and an event is registered if the gamma photons reach opposite scintillators at the same time (coincidence). The coincidences are corrected for attenuation (i.e. absorption of the gamma photons by tissue and surrounding materials before reaching the detector), scatter (i.e. dislocation of photon after interacting with a tissue that eventually bends the photon to a different angle than 180° before reaching detector), random coincidences (i.e. two events that are detected at the same time, but are not related with each other) and decay (i.e. the natural emission of radioisotopes that decreases over time). After correction, the sum of all coincidences will be processed to generate the 3D-distribution of the injected radiotracer over the period of time of the scan. As the injected dose and the bodyweight are variables that affect the tracer uptake in a specific tissue, the images are usually corrected for the injected tracer dose and the bodyweight, giving a standardized uptake value (SUV) for the tracer uptake in a specific region [38]. The advantage of semi-quantifying tracer uptake as SUV is that it is a simple method that does not require any blood input, and thus it can be performed longitudinally without much discomfort to the individual. However, SUV gives only an estimate of where the tracer is present, and it is not possible to obtain quantitative parameters such as volume of distribution (V_t) or binding potential (BP_{ND}). In such case, PET data can be complemented with other molecular analyses to better estimate the amount and placement of the target protein, thus giving a more reliable estimate on its behavior and concentration in the tissue of interest.

Thesis outline

Chronic social stress is one of the main public concerns and has a strong association with depressive symptoms. It is a matter of fact that social stress is a modulator of neuroendocrine, neuroinflammatory, and neurotrophic factors that might be the cause of the depressive symptoms. The main goal of this thesis is to shed light on how social stress is associated with neuroinflammation, cognition and depressive behavior. All of these characteristics are, directly or indirectly, associated with the expression, production, and release of BDNF in the brain, and with the response of the individual towards the stressor. Therefore, BDNF could be a key intermediate in this process. This thesis also intends to assess how beneficial treatments can alter the fate of the stress-induced disease, decreasing or even subsiding it (diagram in figure 3). Therefore, the chapters of the thesis are arranged as follows.

Chapter 2 highlights the state-of-art of BDNF research in health and in many CNS disease conditions and aims to explore the link between BDNF and the presence of neuroinflammation. Both in psychiatric and neurodegenerative disorders, BDNF is associated with predisposition and progression of disease and treatment efficacy, and is considered a non-specific biomarker for a diseased state of the brain. In addition, potential therapies and treatments are considered that could improve the current gold-standard treatment of many diseases.

Chapter 3 delves into one of the major problems of BDNF research: can serum measurements reliably reflect changes in BDNF concentration in the brain? For this purpose, BDNF was analyzed in the brain and serum of animals submitted to a long-term positive, neutral or negative social stimulus. In this study animals of different ages were submitted to a positive (enriched environment), negative (social isolation) and standard social setting and their behavioral pattern and a synaptic (Synaptophysin) biomarker were analyzed. BDNF was analyzed in the serum and hippocampus to observe how the environment affects the expression of this neurotrophin, and determine if the BDNF response differs between brain and serum.

Chapter 4 uses PET imaging with a radioligand for the microglial biomarker TSPO in combination with behavioral tests to answer the question of whether inhibition of the HPA-axis can modulate the stress response and neuroinflammatory response elicited by repeated social defeat. In this study, animals were submitted to adrenalectomy or sham surgery to inhibit the HPA-axis-induced production of corticosterone. Neuroinflammation was measured by PET imaging with the TSPO ligand [^{11}C]PBR-28 two weeks after the repeated social defeat paradigm as a social stressor.

Chapter 5 uses the same social defeat protocol to investigate whether the antidepressant and anti-inflammatory properties of harmine are able to modify the effects induced by the social stressor. Hence, animals submitted by social defeat were treated daily with harmine and behavioral tests were performed to assess their locomotion, anxiety, depressive and cognitive parameters. In addition, BDNF concentration in the hippocampus and frontal cortex were measured, and the effect of harmine on neuroinflammation was assessed with [^{11}C]PBR-28 PET.

In **Chapter 6** a shift is made towards the other side of the social defeat paradigm. In particular, it investigates what the effect of social defeat paradigm is on the reward system of the winning animals? In this study, the animals used as the aggressors in the social defeat paradigm (residents) underwent a [^{11}C]Raclopride PET scan to assess the availability of their dopamine D_2 receptors.

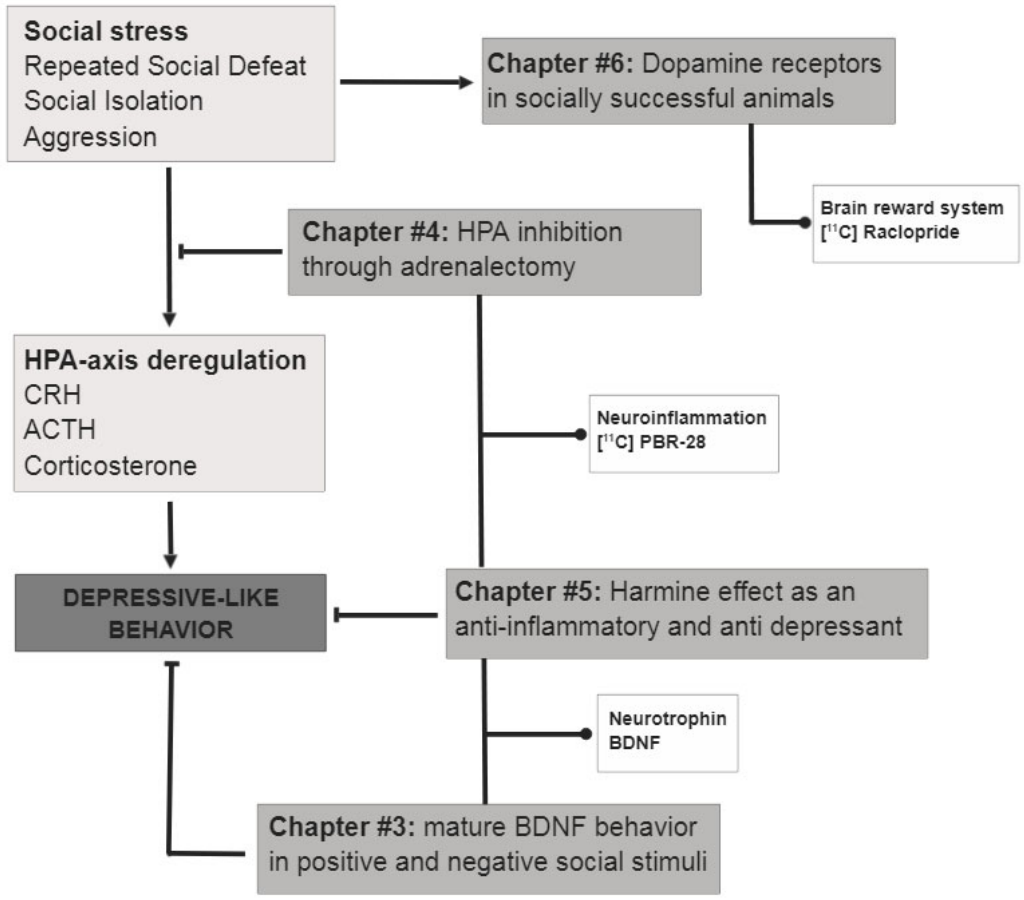

Figure 3: Thesis development. Arrowhead lines present putative direction of events, while blunted lines show tentative to block or treat the occurring event. Abbreviations: HPA: Hypothalamus-Pituitary-Adrenal; CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; BDNF: brain-derived neurotrophic factor; PBR: peripheral benzodiazepine receptor.

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Chapter 2

Brain-derived neurotrophic factor in brain disorders: focus on neuroinflammation

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Mol Neurobiol. 2019 May;56(5):3295-3312. doi: 10.1007/s12035-018-1283-6

Abstract

Brain-derived neurotrophic factor (BDNF) is one of the most studied neurotrophins in the healthy and diseased brain. As a result, there is a large body of evidence that associates BDNF with neuronal maintenance, neuronal survival, plasticity, and neurotransmitter regulation. Patients with psychiatric and neurodegenerative disorders often have reduced BDNF concentrations in their blood and brain. A current hypothesis suggests that these abnormal BDNF levels might be due to the chronic inflammatory state of the brain in certain disorders, as neuroinflammation is known to affect several BDNF-related signaling pathways. Activation of glial cells can induce an increase in the levels of pro- and anti-inflammatory cytokines and reactive oxygen species, which can lead to the modulation of neuronal function and neurotoxicity observed in several brain pathologies. Understanding how neuroinflammation is involved in disorders of the brain, especially in the disease onset and progression, can be crucial for the development of new strategies of treatment. Despite the increasing evidence on the involvement of BDNF and neuroinflammation in brain disorders, there is scarce evidence that addresses the interaction between the neurotrophin and neuroinflammation in psychiatric and neurodegenerative diseases. This review focuses on the effect of acute and chronic inflammation on BDNF levels in the most common psychiatric and neurodegenerative disorders and aims to shed some light on the possible biological mechanisms that may influence this effect. In addition, this review addresses the effect of behavioral and pharmacological interventions on BDNF levels in these disorders.

Keywords:

Brain-Derived Neurotrophic Factor; neuroinflammation; neurological disorders; neurotoxicity

Introduction

Brain disorders are among the major causes of disability and morbidity worldwide. According to recent projections, the incidence of such diseases will increase in the next decades [1]. The lack of adequate treatment turns these diseases into a significant problem worldwide, and the absence of effective treatment can partly be ascribed to our incomplete knowledge of the etiology of most brain disorders. Many mental diseases, however, are tightly associated with environmental stimuli, such as stress [2]. Challenging events can pose a significant burden on individuals, especially those more sensitive to its effects. Although acute stress can have benefits (e.g. enhanced attention, memory), it can also become life-threatening when stressful events become a routine part of the life of individuals. A number of studies have already shown that stress is associated with metabolic changes, cardiovascular risk, endocrine abnormalities, mood changes, and impairment of cognitive functions (i.e. mild cognitive impairment), leading to an increased risk of developing psychiatric and neurologic disorders [2,3]. Chronic stress can lead to the activation of pro-inflammatory microglia, releasing cytokines and pro-inflammatory substances, and recruitment of peripheral immune cells to the brain, thus creating the inflammatory environment that is characteristic for many brain pathologies.

In order to cope with stressful events, brain cells release several substances that can promote neuronal survival, such as anti-inflammatory cytokines, growth factors, and neurotrophic factors. One of the best-studied neurotrophins is the Brain-Derived Neurotrophic Factor (BDNF). Brain pathologies are usually associated with a down-regulation of BDNF release, resulting in reduced BDNF levels in the brain and in blood. BDNF has been suggested as a candidate biomarker of pathological conditions, and therapy efficacy, as most of the current treatments are accompanied by a significant change in blood BDNF levels. However, there is still a gap in our understanding of the physiological mechanisms that lead to changes in BDNF levels under pathological conditions.

This review will summarize our current knowledge of BDNF in the pathophysiology of the most common brain disorders. Since neuroinflammation has been considered an important mediator for the onset and progression of many brain pathologies, this review will also attempt to explore the interaction between neuroinflammation and BDNF expression in the brain.

BDNF expression and function

BDNF is a member of the neurotrophin family, which also includes neural growth factor (NGF) and neurotrophins 3 and 4. The Bdnf gene is comprised of a common 3'-exon that encodes the pro-BDNF region of the protein, and several species-dependent 5'-noncoding, promoter-regulated regions, terminating in a coding 5'-exon that encompasses the gene expression [4,5]. Bdnf gene expression is

strongly regulated by a wide array of endogenous and exogenous stimuli (e.g. stress, physical activity, brain injury, diet). BDNF is translated as a pro-neurotrophin (proBDNF) that can be cleaved into mature BDNF in the cytoplasm by endoproteases or in the extracellular matrix by plasmin or matrix metalloproteinases (MMP). Both mature BDNF and proBDNF can be secreted and bind to the low-affinity p75 neurotrophin receptor (p75NTR), which causes activation of the apoptosis cascade [6,7]. On the other hand, cleaved, mature BDNF binds to its high-affinity receptor Tyrosine Kinase B (TrkB), activating several signaling cascades, including the Ras-mitogen-activated protein kinase (MAPK), the phosphatidylinositol-3-kinase (PI3K) and the phospholipase Cγ (PLC-γ) pathway. These signaling cascades induce an increase in Ca^{2+} intake, phosphorylation of transcription factors and *de novo* expression of the Bdnf gene (Fig. 1) [8]. Although proBDNF can act as a signaling factor for apoptotic cascade, it is not yet clear if proBDNF is secreted by neurons in healthy circumstances as its concentration on presynaptic terminals are relatively low when compared to mature BDNF. Indeed, the concentration ratio of mature BDNF in animal models can be ten times higher than proBDNF [9,10], which posits a question as to the efficacy of proBDNF as a proper signaling factor.

BDNF has a wide array of functions within the brain and is highly abundant in several brain structures. In the brain, BDNF is involved in plasticity, neuronal survival, formation of new synapses, dendritic branching, and modulation of excitatory and inhibitory neurotransmitter profiles[11,12]. BDNF is active at all stages of development and aging [13]. Knockout mice lacking BDNF rarely reach adulthood and, when they do, there is a development of several sensory impairments [14,15]. BDNF is also found in peripheral organs, such as heart, gut, thymus and spleen [16,17]. Around 90% of the BDNF in the blood is stored within platelets [18]. Many brain pathologies cause a reduction of BDNF protein levels both in the brain and serum of patients [19–22]. Unfortunately, it is still unclear whether BDNF protein levels measured in serum samples reflect BDNF levels in the brain, as studies in animal models gave contradictory results so far [23–25].

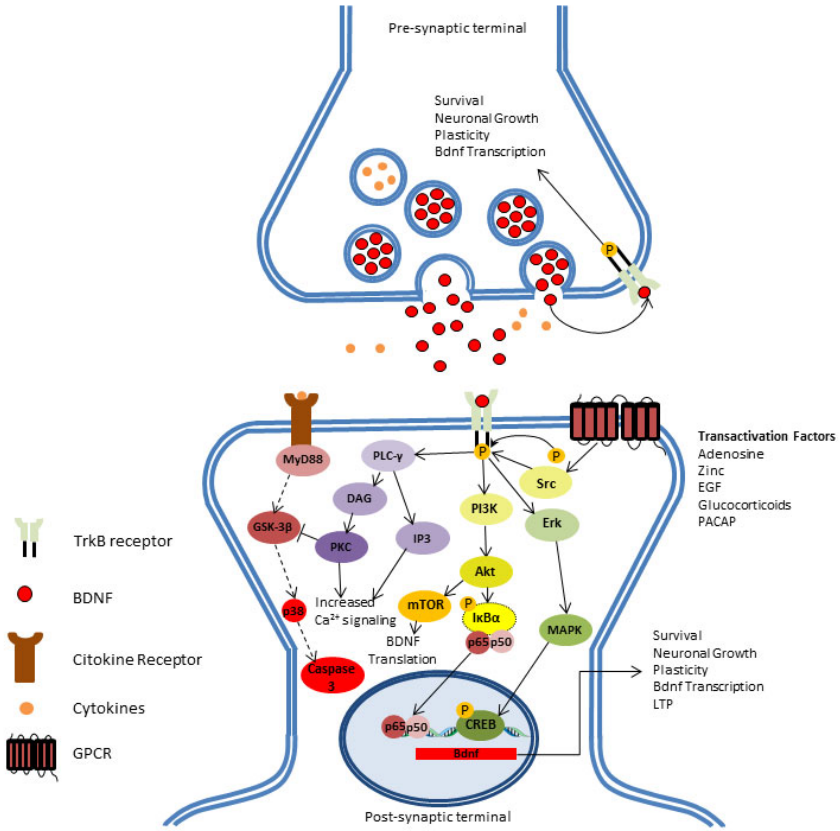

Figure 1: BDNF induces survival-related signaling mechanisms: BDNF induces survival-related signaling mechanisms: In physiological conditions, binding of BDNF to TrkB receptor in either paracrine or autocrine signaling elicits three distinct downstream pathways. BDNF-dependent phospholipase C-gamma (PLC-γ) can induce short-term signaling by increasing Ca2+ neuronal response and inhibit inflammatory-dependent apoptosis cascade (dashed lines) by inhibition of glycogen synthase kinase 3-beta (GSK-3β). Induction of phosphatidylinositol 3-Phosphate (PI3K) induces transcription of BDNF mRNA by activating mTOR-dependent translation of BDNF. Additionally, BDNF can modulate gene regulation by activating NF-κB and CREB transcription factors by inducing Akt and Erk downstream pathways, respectively. Gene modulation induces neuronal survival, growth, long-term potentiation (LTP), and de novo expression of BDNF. In addition, BDNF-independent transactivation of TrkB can also play an important role in the neurotrophic pathway regulation by factors, such as adenosine, zinc, epidermal growth factor (EGF), glucocorticoids, and pituitary adenylate-cyclase-activating polypeptide (PACAP), further enhancing TrkB signaling in the synapse

BDNF in neuroinflammation

After inflammatory signaling (e.g. stress, pro-inflammatory signals), several signaling cascades are changed within the cell. This signaling generates a sequence of events that may eventually lead to neuronal malfunction and apoptosis. Microglia also participate actively in the development of pathological neuroinflammatory process by releasing pro-inflammatory cytokines, which contributes to the neurotoxicity. This cycle is repeated as long as the stressor is present, which can develop into serious consequences (e.g.: cognitive impairment; behavioral dysfunction; neurological and psychiatric disorders). One of the main factors of inflammatory activation is Nuclear Factor-kappa B (NF-κB), a transcription factor that induces the expression of several pro- and anti-apoptotic genes, including Bdnf [26]. Interestingly, binding of BDNF to the TrkB receptor can also induce the expression of NF-κB,

although the pathways for this modulation are yet unclear. NF-κB is closely involved in the innate and adaptive immune response in several psychiatric and neurodegenerative diseases [27]. NF-κB is a regulator of e.g. apoptosis, neuronal survival and proliferation and migration and maturation of immune cells [28]. BDNF-induced NF-κB expression stimulates PLC-γ/PKC signaling through the activation of the kinases IKKα and IKKβ. These kinases phosphorylate the NF-κB inhibitory unit IκBα, resulting in the binding of ubiquitin and subsequent degradation of IκBα by proteasomes [29]. IκBα degradation induces the release of the NF-κB and formation of the p50/p65 dimer, which binds to the DNA and induces the expression of genes related to neuronal proliferation, survival, and inflammatory response [29,30]. Furthermore, it is known that BDNF can also bind to p75NTR receptor. Even though its affinity is several times lower than TrkB [31], a p75NTR-mediated effect on NF-κB expression can be observed. Studies have shown that activation of p75NTR increases apoptotic and inflammatory signaling in neurons and glial cells by activation of c-Jun N-terminal Kinases (JNK) and NF-κB expression, respectively [32,33]. However, the effect p75NTR has on neurotrophic signaling is under debate, as there is no clear evidence on how large the role of p75NTR is in mediating such processes.

Thus, the role of BDNF in neuroinflammation is strongly related to its ability to induce – and being induced by – NF-κB (Fig. 2). However, the exact regulatory mechanisms are not yet clear.

BDNF and aging

The aging process can lead to the impairment of several brain functions. Studies have reported a decrease in whole brain volume in the elderly when compared with young adults, especially in brain regions related to cognition [34–37]. Upon aging, microglia increasingly adopt a pro-inflammatory state due to a decrease in the resting signaling by neurons and astrocytes [38,39]. As a result, external stimuli (e.g. stress, trauma, infection) can submit the aged brain more easily into a state of mild chronic neuroinflammation, making the brain more prone to apoptotic signaling [40]. This can lead to volume loss and the associated cognitive impairment [41]. Animal studies in stress models, such as chronic stress, maternal separation, and social defeat, have confirmed that stress is associated with glial activation and that aging decreases cognitive function [42,43]. The loss of volume is indicative of a reduction in the global neuronal network, and consequently diminished brain plasticity that could support the brain in such events, thus reducing the cognitive function [44,45].

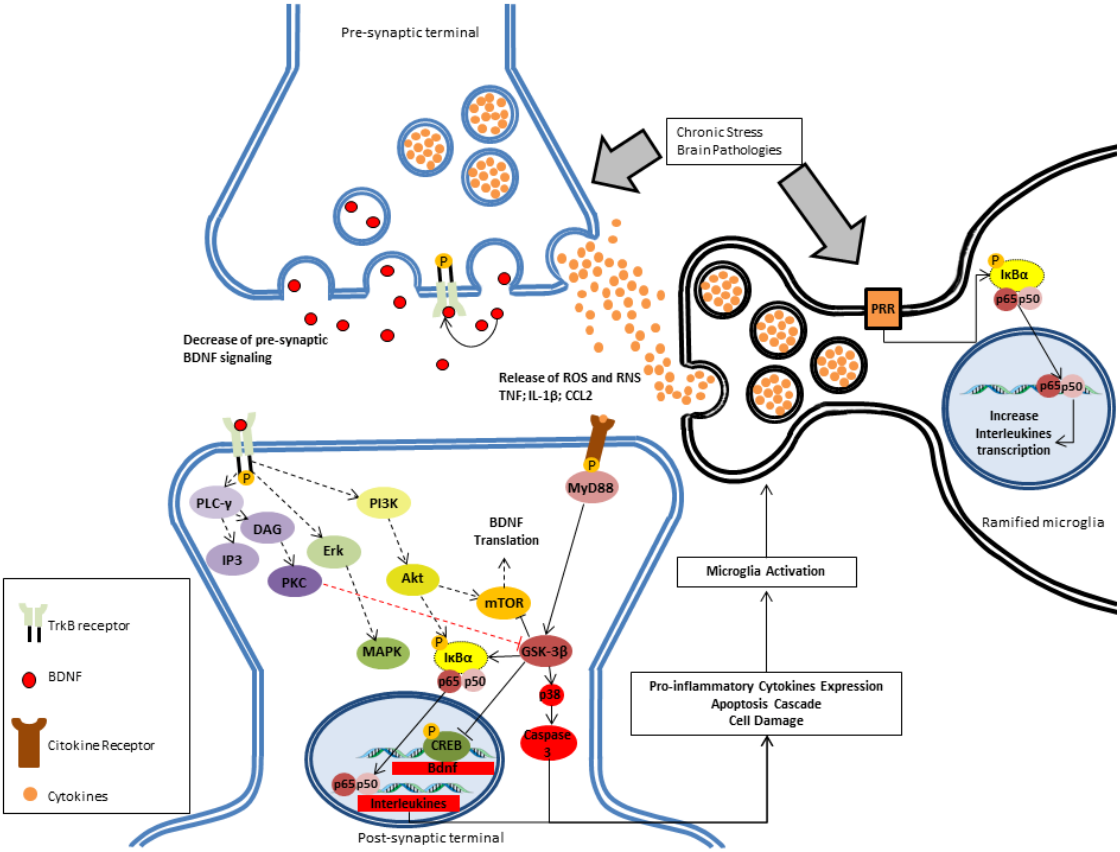

Figure 2: BDNF response after inflammatory brain pathogenicity. In chronically stressful situations, such as brain pathologies, there is an induction of NF-κB-dependent pro-inflammatory activation of microglia after induction of the pattern recognition receptor (PRR) by the challenge (e.g., stress or pathology). Pro-inflammatory cytokines, especially IL-1, can directly bind microglial cells, which result in induction of the expression and release of several mediators, most of which are neurotoxic, including reactive oxygen (ROS) and nitrogen species (RNS), pro-inflammatory cytokines (such as tumor necrosis factor), and chemokines (such as CC-chemokine ligand 2, CCL2; also known as MCP1). Additionally, there is a decrease of BDNF signaling in the synaptic cleft, further reducing BDNF-dependent survival-related signaling (black dashed lines) and inhibition of apoptotic pathways, such as glycogen synthase kinase 3-beta (GSK-3: red dashed line). Such factors will lead to an increase of NF-κB complex binding to genes that express pro-inflammatory cytokines (e.g., interleukin 1-β, IL-6, IL-8, TNF). The effect of transactivation factors on the BDNF-independent maintenance of TrkB is not clear yet

It is known that BDNF plays a role in maintaining brain function by inducing survival signaling and neuroplasticity. Although the effect is more visible in the younger population, elderly subjects also benefit from it, especially those cognitively, physically, and socially active, reducing the risk of age-related comorbidities [46]. Studies in animal models report an increase in brain BDNF levels when aged animals are submitted to protocols such as long-term environmental enrichment [47–49] or physical activity [50,51]. Studies on aged mice demonstrated that animals heterozygous for BDNF showed decreased fear extinction learning [52] and conditioned fear learning [53] when compared with young heterozygous mice, but the mechanism responsible for the behavioral effects is not completely clear yet. A better understanding of the processes that are modulated by BDNF may facilitate the

development of novel therapeutic methods that could help to prevent or counteract the aging effects on the human brain.

BDNF in psychiatric disorders

Whenever the brain is challenged by harmful events, its coping mechanisms are activated in order to revert the system to homeostasis. When these coping mechanisms fail, e.g. due to excessive damage, enhanced sensitivity, chronic or recurrent exposure to stimuli, a disturbance of normal brain function may occur that can lead to the onset of neuropsychiatric disorders. Although psychiatric diseases can display wide spectra of symptoms, common phenomena in these disorders are disarray of excitatory/inhibitory neurotransmitter signaling and loss of neuronal function, leading to mood and behavioral disturbances, and cognitive impairment.

In humans, BDNF is known to be a useful biomarker for several psychiatric disorders [54]. Most chronic psychiatric diseases are accompanied by changes in BDNF levels, but it is still unclear if changes in BDNF levels are the cause or the result of the disturbances of normal brain function. Therefore, the effects of BDNF levels have been investigated in animal models. Heterozygous BDNF mice show increased weight gain, aggressiveness, anxiety, and contextual memory impairment and therefore have been suggested as an animal model for mood disorders [55]. These results suggest that BDNF could be a key player in the development of several symptoms associated with psychiatric disorders. This hypothesis is supported by the fact that effective treatment of these symptoms resulted in the normalization of BDNF levels [56]. In this section, we will further discuss the role of BDNF in the main psychiatric disorders: Major Depressive Disorder, Bipolar Disorder, and Schizophrenia.

Major Depressive Disorder

Major Depressive Disorder (MDD) is a common psychiatric disease characterized by abnormal behavior, anhedonia, sleep, and dietary problems, cognitive impairment and, in more severe cases, suicidal tendencies. Biologically, depression is related to a decrease of neurotransmitter signaling in the brain, dysfunction of Hypothalamus Pituitary Adrenal-axis (HPA-axis), increase in inflammatory signaling and reduction in hippocampal volume. Both MDD patients [57–59] and animal models of depression [60,61] show a remarkable reduction in serum BDNF levels. Karege and colleagues have shown that this decrease is not related to platelet-associated BDNF release in the bloodstream [62], suggesting that reduced BDNF levels in the brain rather than a reduction in the peripheral release of BDNF by platelets, is the cause of altered protein levels in the blood. The magnitude of the decrease in plasma BDNF levels is associated with disease duration [63], but it is not clear yet whether the severity of symptoms is related to BDNF levels. BDNF single nucleotide polymorphism (val66met), however, is

associated with the severity of depression in patients [64]. Successful antidepressant treatment is usually associated with an increase in BDNF levels in serum and plasma, [65,66] whereas treatment failure is associated with a lack of response of plasma BDNF levels. Thus, BDNF seems an important player in the pathophysiology and might be a biomarker for monitoring treatment response in depression [65,67].

Epidemiological studies show that a third of all MDD patients experience no changes in the symptoms when treated with the most commonly used antidepressants. Postmortem studies revealed that these treatment-resistant patients have significantly lower BDNF levels, especially in BDNF-rich brain structures, such as the hippocampus [105–107]. Treatment with the rapidly acting antidepressant ketamine was able to increase plasma BDNF levels to the level of healthy controls [108,109]. Ketamine is an inhibitor of NMDA receptors, inducing rapid, glutamate-dependent Ca^{2+} signaling and activation of cAMP response element-binding protein (CREB). Clearly, there is a need to increase our knowledge of the role of BDNF in MDD.

Induction of a pro-inflammatory response by systemic application of lipopolysaccharide (LPS) causes depressive-like symptoms (i.e., sickness behavior) in rodents [110,111], which may also affect BDNF levels. Increased pro-inflammatory signaling leads to a reduction in the mRNA expression of Bdnf and other neurotrophins in plasticity-related brain structures, especially cortical regions [112]. The effects of reduced BDNF expression levels in mice treated with a systemic LPS injection can be counteracted by induction of TrkB-mediated signaling with the agonist 7,8-dihydroxyflavone, which leads to a reduction of depressive-like behavior [113]. Gibney and colleagues have found increased expression of interleukins IL-1β, IL-6 and tumor necrosis factor-α (TNF-α), and reduced expression of Bdnf genes in depressive-like rats 6 hours after an inflammatory challenge. In this study, expression of cytokines returned to baseline levels after 48 hours, but Bdnf mRNA remained low in frontal cortex and hippocampus [114]. In humans, chronic stress is one of the main precursors of depressive symptoms [115,116]. In laboratory stress-conditioning, depressed patients show a higher inflammatory response, characterized by increased IL-6 release and NF-κB DNA binding, than healthy controls [117]. Depressive symptoms can also be caused by treatment that stimulates the immune system, such as interferon-α (IFN-α). Patients treated with IFN-α were shown to have decreased serum BDNF levels in combination with increased protein levels of the cytokines IL-1 and IL-2 [118,119]. Interestingly, individuals that had higher BDNF levels at baseline showed better resilience to IFN-α-induced MDD.

These preclinical and clinical findings show that long-term exposure to stress or inflammation leads to a decrease in BDNF levels, reducing the capacity of the neurons to cope with further challenges (i.e., neuronal plasticity), and ultimately leading to a decreased function and neuronal death. Interestingly, treatment with antidepressants can result in an anti-inflammatory response throughout

the brain, mitigating the inflammatory unbalance to homeostatic levels and normalizing BDNF concentrations [120,121]. However, further research is needed to understand the mechanisms involved in the regulation of BDNF by neuroinflammation.

Bipolar Disorder

Bipolar disorder (BD) is characterized by fluctuations of mood throughout the lifetime, oscillating between depressive, euthymic and manic episodes. BD is associated with cognitive impairment and other comorbidities that affect the quality of life of the individual [122–124]. BD is characterized by alterations in dopaminergic and glutamatergic neurotransmitter systems, mitochondrial dysfunction and increased oxidative stress, which in turn are related to neuroinflammation, neurotoxicity and eventually neuronal death [124]. Two recent meta-analyses have shown that serum and plasma levels of BDNF in BD patients during depressive and manic episodes are decreased, but no difference in BDNF levels between BD patients in an euthymic episode and healthy controls was found [125,126]. It is known that treatment with mood stabilizers increases BDNF levels in the prefrontal cortex and hippocampus of animals by inducing promoter IV-driven expression [127,128]. Also in humans, treatment for the manic or depressive phases of BD is associated with an increase in serum BDNF levels [129,130].

Recent studies have shown an association between in manic and depressive stages of BD and a pro-inflammatory profile of immune cells [131,132]. Steiner and colleagues have found that suicidal mood disorder patients had a significant increase of microglial cell density in the dorsolateral prefrontal cortex, anterior cingulate gyrus and mediodorsal thalamus compared to healthy controls and non-suicidal, mood disorder patients [133]. The presence of immune cells clusters in these brain regions suggests a strong inflammatory response, which could trigger the suicidal predisposition of these patients [133]. Although plenty of literature is available on BDNF or neuroinflammation in BD, there is a severe lack of studies regarding the association between both mechanisms on BD. Only two studies have analyzed both BDNF and cytokine levels in BD patients, with somewhat different conclusions. Patas and colleagues have shown an association between both serum BDNF and plasma IL-6 levels with a depressive episode associated with melancholic trait [134], while Wang and colleagues have found an association for serum BDNF levels, but not for IL-1β or IL-6 [135]. Clearly, more studies are needed to elucidate the interaction between neuroinflammation, BDNF and disease symptoms in BD.

Interestingly, the most commonly used therapeutic drugs – lithium and valproate – were able to reverse the inflammatory state in mood disorders [136–138]. The most common assumption is that lithium and valproate can inhibit Glycogen Synthase Kinase – 3 (GSK-3) and sodium channel function, respectively [139]. The inhibition of GSK-3 activity by lithium increases cellular levels of BDNF. GSK-3 can

inhibit mammalian Target-of-Rapamycin (mTOR) – an important modulator of BDNF-dependent neuronal plasticity and survival – and thus affect proper BDNF signaling and impair optimal cellular function. In the manic phase of BD, there is a remarkable increase in PKC-mediated signaling, which is associated with BDNF-dependent Ca^{2+} induction. PKC isozymes are involved in the pro-inflammatory response mediated by macrophages [140] and, more recently, microglia [141] through activation of the NF-κB inflammation pathway. Treatment of BD patients with lithium or valproate inhibits PKC upregulation, normalizing its level to that of euthymic subjects, and increases BDNF levels [142]. PKC inhibitor tamoxifen enhances the capacity of lithium to reduce symptoms of mania in BD [142–144]. PKC inhibition probably suppresses the expression of NF-κB and consequently resolves the NF-κB-mediated inhibition of Bdnf expression, resulting in an increase in peripheral BDNF levels in BD. However, the mechanisms underlying the increase in BDNF levels in response to treatment should still be further investigated. Yet, current evidence suggests that a decrease in BDNF levels can be considered as a biomarker for both depressive and manic stages of BD.

Schizophrenia

Schizophrenia is a disease characterized by disturbances in the proper perception of a person's surroundings. Schizophrenia is associated with a high suicide rate and accounts for a large number of hospitalizations, causing a significant burden to healthcare systems worldwide. The symptoms of schizophrenia comprise positive (e.g., hallucinations, delusions, confused thoughts, concentration impaired) negative (e.g., depression, anhedonia, self-neglect) and cognitive effects (e.g., memory, attention, reason impairments). Schizophrenic patients exhibit a decreased activation of γ-aminobutyric acid (GABA) signaling [145], inducing impaired neuronal activation, especially in dopaminergic neurons [146]. The etiology of schizophrenia is not fully understood yet and symptoms can vary between individual patients, making the diagnosis of schizophrenia challenging.

A recent meta-analysis revealed that serum BDNF levels in both drug-naïve and medicated schizophrenic patients are reduced. Serum BDNF levels in schizophrenic patients decrease with age but were independent of the dosage of medication [147]. However, it remains unclear if and how BDNF levels in the brain are altered in schizophrenic patients. Some studies report increased BDNF levels in frontal and temporal structures [148,149], while others report decreased levels in the same brain structures [83,145,150]. Besides clinical observations, *in*-vitro studies using the phencyclidine (PCP) psychosis model also give ambiguous results. Adachi and colleagues reported that exposure of cortical cultures to the non-competitive NMDA agonist PCP initially resulted in an increase in BDNF levels, whereas TrkB, ERK1/2 and Akt signaling was decreased [151]. In contrast, two other studies reported decreased BDNF mRNA expression in cortical slices after exposure to a low-dose of PCP [152]. Taken

together, *in vitro*, *in vivo* and clinical results seem to indicate that plasma BDNF levels are decreased in schizophrenic patients, but data on brain BDNF levels are contradictory.

Schizophrenia is a multifactorial disease, in which both genetic and environmental factors play a role. It is well established that physical and mental distress can trigger psychotic behavior in (genetically) vulnerable patients [153]. These triggers can induce inflammatory changes that are associated with reduced neurotransmitter signaling, increased oxidative stress, and reduced synaptic branching [154]. Mondelli and colleagues have shown in leukocytes of first-episode schizophrenic patients that childhood trauma and the number of recent stressful life events were negatively correlated with BDNF mRNA levels. BDNF levels were also negatively correlated with IL-6 expression, suggesting an inflammation-mediated decrease in BDNF expression, or vice versa. Moreover, BDNF, IL-6 and cortisol levels correlated inversely with hippocampal volume [155]. A postmortem study demonstrated that schizophrenic patients have an increased inflammatory profile in dorsolateral prefrontal cortex. The group of patients with high levels of neuroinflammation had lower expression of BDNF [156]. As psychotic episodes are related to increased neuroinflammation and activated microglia [156,157], it can be hypothesized that pro-inflammatory cytokines may be modulating BDNF mRNA expression via interaction of NF-κB or CREB transcription factors. It is known that schizophrenia patients have upregulated genes for inflammatory cytokines [158,159], and downregulated Bdnf gene transcription [160,161]. Neuroinflammation could be the key factor for the decrease of Bdnf gene expression in schizophrenic patients, as pro-inflammatory cytokines increase methylation of the Bdnf gene, leading to a decrease of CREB binding to the specific Bdnf site.

Remarkably, drug treatment that is effective in controlling disease progression in schizophrenic patients can have diverse effects on peripheral BDNF protein levels [162–164]. In addition, some studies suggest that baseline BDNF levels in schizophrenia patients might reflect the susceptibility towards available drug therapies [165,166]. Clearly, the interaction between neuroinflammation, BDNF levels, and treatment response should be better understood, as this could lead to the identification of new targets for improved therapies.

BDNF in neurodegenerative disorders

Despite research on neurodegenerative disorders has been increasing exponentially, there are still gaps in our knowledge on the etiology, onset, and progression of most neurodegenerative diseases. Treatment is usually restricted to mitigation of the symptoms, rather than cure or delay of progression. Diagnosis of neurodegenerative diseases is usually based on subjective cognitive tests in combination with neuroimaging [167–169], but the current techniques are not able to successfully diagnose

these diseases in their earlier stages when the pathology is already present but does not cause symptoms yet. Attempts to discover new biomarkers for the diagnosis of early stages of the disease are on-going [170–172]. Possibly, BDNF could qualify as such a biomarker.

In the following sections, we will discuss the role of BDNF in neurodegenerative disorders, focusing on Alzheimer's disease, Parkinson's disease, and epilepsy. We will also address the possible use of BDNF as a biomarker for diagnosis. In addition, we describe the role of neuroinflammation in the development of these diseases and explain how BDNF can help the brain to cope with inflammation.

Alzheimer's Disease

AD is characterized by a progressive loss of neurons in the brain, leading to impairment in memory and general cognition. Hallmarks of AD pathology are deposition of amyloid-β plaques in the extracellular matrix, formation of tau-phosphorylated neurofibrillary tangles within the cell and neuritic plaques. Tangles and plaques disrupt the signaling activity of neurons, eventually leading to neuronal apoptosis. As the disease progress, the axonal transport is constantly reduced, and the general function of neurons is impaired. These changes decrease BDNF axonal transport, resulting in a reduced availability of BDNF within the synaptic cleft and consequently diminished signaling through TrkB receptors [173,174]. BDNF mRNA and protein levels in cognition-related structures such as the hippocampus and frontal cortex, which corroborates BDNF depletion to be involved in the cognitive deficit leading to AD dementia [175]. BDNF levels are also reduced in plasma of patients with mild cognitive impairment (MCI) [176] and AD [177]. AD patients with higher serum concentrations of BDNF showed a less cognitive decline after one year; this effect was more pronounced in the more severe stages of the disease [87,178]. These studies suggest that BDNF might be a predictor for the rate of disease progression in AD.

Neuroinflammation is a key factor in the development and progression of AD [179,180]. Amyloid-β deposits induce pro-inflammatory activation of microglia which might be an effort of microglia to mitigate the antigen-related damage [181]. Some studies have reported inflammatory challenges as an associated risk factor for the development of dementia-related symptoms, as they show increased pro-inflammatory cytokines levels [182,183]. On the other hand, treatment with anti-inflammatory medication tends to mitigate cognitive impairment in animal models of amyloid-β injection [184,185]. Interestingly, PET imaging studies have shown that subjects with high amounts of amyloid-β, but no dementia, had decreased microglia activation [186–188], indicating fundamental participation of microglia in the development and progression of AD.

There is no clear evidence on how effective BDNF can be in controlling the neuroinflammation-dependent progression of the disease. Prakash shows in rats injected with Aβ in the hippocampus have a remarkable decrease in BDNF and increased TNF-α, IL-6, and caspase-3 protein levels three weeks after intracerebroventricular injection. These changes were associated with a lack of memory retention in Morris Water Maze test [189]. Another study confirmed the increase in pro-inflammatory cytokines and reduced anti-inflammatory cytokines and BDNF protein levels and gene expression after $A\beta_{1-42}$ injection [190]. In humans, two studies have shown an increase in pro-inflammatory cytokines and a decrease in BDNF levels in the serum of early- and late-onset AD, although there was no correlation with pro-inflammatory and neurotrophin results in both studies [177,191].

It is known, however, that pro-inflammatory cytokines – especially IL-1β – can cause down-regulation of BDNF expression in cognition-related brain structures, such as the hippocampus [192,193]. As these cytokines are upregulated in AD, a decrease of BDNF levels is expected to occur in such brain structures, leading to a decrease in survival signaling and, consequently, neuronal death. Moreover, an increase in hyper-phosphorylated tau impairs anterograde transport of BDNF to the axon, further decreasing BDNF signaling in the synaptic cleft. As AD is a disease that inflicts several different physiological effects in both the internal and external milieu of the brain, efficient analysis of the role of BDNF in these processes is challenging and the overall effect of BDNF on brain function may be variable.

Parkinson's Disease

Idiopathic PD is characterized by movement impairment (bradykinesia, tremors, and rigidity), often combined with mild cognitive symptoms (decreased attention, executive function, memory) and mood disturbances (apathy, aggressiveness, anhedonia, depression) [194,195]. The onset of PD is caused by the formation of aggregated α-synuclein plaques (i.e. Lewy bodies) in the substantia nigra pars compacta, leading to a progressive loss of dopaminergic neurons [196]. It is estimated that clinical symptoms start to appear when more than 50% of the neurons in the substantia nigra are already lost [197]. Movement symptoms are usually the first signs leading to the diagnosis of PD. As the disease progresses, more brain structures become affected by the neuronal loss and non-motor symptoms become evident [196,198]. The severity of symptoms increases as the disease progresses and as a result, the patient loses gradually independence until patients become highly dependent on caregiver support in the late stages of the disease.

PD patients have lower concentrations of BDNF mRNA and protein in the substantia nigra pars compacta than healthy controls [95,199]. Neurons with the lowest BDNF levels were suggested to be most prone to injury. Porritt and colleagues demonstrated that local inhibition of the production of BDNF

with an antisense oligonucleotide leads to a significant loss of dopaminergic neurons in the substantia nigra pars compacta of rats, which suggests that BDNF has an important role in neuronal survival [200]. In contrast to the aforementioned studies, some reports describe that the BDNF levels in serum are increased in PD patients, especially in moderate to severe stages of the disease [96,201]. This could mean that the CNS tries to cope with the loss of neurons by increasing BDNF production, resulting in enhanced serum levels of the protein. However, there is no direct evidence that supports this hypothesis.

The onset and progression of PD are also associated with neuroinflammation. Several studies in animal models of PD have reported increased microglial activation and pro-inflammatory cytokines [202,203]. In humans, PD is associated with neuroinflammation in both post-mortem [204] and in vivo analysis [205,206]. Sawada and colleagues have found a remarkable increase of microglial cells in the hippocampus, amygdala and entorhinal cortex of PD patients, which was associated with a decrease of BDNF mRNA expression and increased IL-6 in those regions [207]. Nagatsu has shown increased levels of IL-1β, IL-2, IL-6, and TNF-α in the striatum of PD patients, associated with decreased BDNF protein levels in the same structure. Aggregated α-synuclein can induce an acute, local neuroinflammatory process in PD-associated brain structures, which suppresses BDNF expression and reduces BDNF protein levels. However, there is no evidence on how changes in BDNF levels in de brain affect the progression of PD and further analysis of the interaction between pro-inflammatory cytokines and BDNF is therefore necessary.

Epilepsy

Epilepsy patients are affected by seemingly unprovoked seizures, but usually, also suffer from significant mood and cognitive changes [208]. The symptoms of epilepsy are induced by a disarray of excitatory neuronal connections, generating deregulated firing, or lack of inhibition of excitatory neurons. Although there are several treatment strategies to reduce seizures, 30% of the patients show little to no response to common antiepileptic drugs.

Seizures have been associated with an increased expression of several neurotrophic-related genes, including transcription factors [209], neuropeptides [210], and growth factors [211]. Two recent reports have shown that BDNF gene expression is increased in the hippocampus and temporal cortex of temporal lobe epilepsy patients [101,102]. Seizures also increased hippocampal and cortical BDNF protein levels in animal models of epilepsy [212–214]. BDNF seems to be involved in epileptogenesis by regulating several signaling pathways within excitatory neurons, increasing Ca^{2+} signaling and glutamate expression [13,215]. Overexpression of BDNF may also contribute to the epilepsy-induced cortical network deregulation by increasing even further the plasticity and dendritic branching signaling and causing an

overexcitement state of glutamatergic neurons, thus reducing seizure threshold [216]. This creates a positive feedback loop: as upregulated glutamate neurotransmitter increases BDNF signaling and expression, it further increases glutamate signaling. Other studies have shown that transient inhibition of the BDNF receptor TrkB after seizure induction prevents the development of temporal lobe epilepsy [217,218]. These findings indicate that BDNF is intimately related to the pathogenesis of epilepsy, and new therapeutic methods should take BDNF into consideration. However, it is worth noting that BDNF may also play a part in protecting neurons against harmful stimuli, therefore treatment aiming to reduce BDNF levels as a whole should be carefully considered.

In epilepsy, chronic seizures lead to excitotoxicity and neuronal apoptosis with associated gliosis [219]. Studies in animal models have reported that seizures are associated with increased activation of microglia, especially of the M1 subtype [220], and an increased release of pro-inflammatory mediators [221,222]. Although studies that link BDNF with neuroinflammation in epilepsy are lacking, a hypothesis for such a link can be formulated based on existing knowledge. In healthy conditions, BDNF signaling induces the activation of transcription factor NF-κB, which in turn induces *de novo* expression of the Bdnf gene [26,223]. It is possible that overexpression of BDNF leads to an inflammatory response mediated by NF-κB, leading to astrocyte activation, increased production of cytokines and neurotoxic reactive oxygen species, and local recruitment of activated microglia [224]. Activated pro-inflammatory microglia are known to elicit apoptotic response after chronic stimulation, which causes neurotoxicity and further neuronal death. As BDNF is overexpressed at the onset of seizures [225], there is also an increase in BDNF-mediated glutamate signaling, contributing to the systemic neuronal imbalance. Although there are several players involved in the development of seizures, BDNF might be an important factor in modulating the disease. BDNF inducing, or being induced, by NF-κB also highlights the importance of neuroinflammation in modulating BDNF-dependent regulation. However, there is a need for further researches to better understand the role of BDNF in epilepsy, especially regarding its role in the modulation of inflammatory processes.

Table 1: Effects of BDNF on several neuropsychiatric and neurodegenerative conditions

CONDITION	PERIPHERAL BDNF	CNS BDNF	REFERENCES
Major Depressive Disorder (MDD)	Decreased serum and plasma levels of BDNF protein, some literature findings showing no change or increased levels in MDD patients; euthymic patients have normalized BDNF levels in serum or plasma; Increased methylation of the Bdnf gene is associated with a decrease in mRNA expression; Treatment-resistant patients show lower BDNF levels in serum compared to treatment-responsive patients;	Decreased BDNF and TrkB mRNA expression in hippocampal slices of MDD patients; Use of antidepressant medication was associated with increased Bdnf mRNA expression;	[68], [62], [69], [70], [71], [72]
Bipolar Disorder (BD)	Decreased serum and plasma levels of BDNF in both manic and depressive stages of BD; euthymic patients show no difference from controls;	Decreased BDNF mRNA expression in the hippocampus of suicidal BD patients No difference in Bdnf expression between different disease stages (euthymic, depressive or manic);	[73], [74], [75], [76], [77]
Schizophrenia (SCZ)	Decreased BDNF protein levels in serum of SCZ patients; No changes in BDNF levels after treatment;	Decreased expression of Bdnf and Trkb genes in Hippocampus and Dorsolateral Prefrontal Cortex of SCZ patients; Increased methylation of Bdnf gene in Prefrontal Cortex of SCZ patients;	[78], [79], [80], [81], [82], [83], [84], [85], [86]
Alzheimer Disease (AD)	Low serum BDNF levels correlate with development of dementia - especially AD; Decreased levels of BDNF in the serum of AD patients. BDNF levels are not related to severity of disease; Successful treatment transiently increases BDNF in AD.	BDNF genotype is related to reduced Hippocampal activity and cognitive function in subjects with high levels of A-β and AD patients; Decreased BDNF mRNA levels in the hippocampus of AD patients; Increase in methylation pattern of the Bdnf gene in the frontal cortex of AD patients	[87], [88], [89], [90], [91], [92], [93]
Parkinson Disease (PD)	Serum BDNF levels are directly correlated with degeneration of striatum in PD; Low serum levels of BDNF is correlated with decreased cognitive function in early PD patients; BDNF decrease in serum is associated with the progression of motor symptoms;	Low BDNF mRNA expression in the striatum of PD patients; Association between BDNF polymorphism and disease progression;	[94], [95], [96], [97], [98]
Epilepsy	BDNF val66met single nucleotide polymorphism is associated with higher BDNF protein expression and an increased risk of developing epilepsy; Increased serum BDNF protein levels after epileptic seizures are associated with increased glutamate signaling;	Increased mRNA expression of Bdnf exons in the hippocampus and cortex of temporal lobe epilepsy patients Increased BDNF protein expression in the hippocampus of temporal lobe epilepsy patients	[99], [100], [101], [102], [103], [104]

BDNF a potential therapy for brain pathologies

BDNF has been regarded as a possible biomarker for monitoring the onset, progression, and treatment of brain pathologies. However, recent studies suggest that BDNF may also be a potential target for new treatment strategies. Some promising results have been published from studies showing that therapeutic use of BDNF can, directly or indirectly, modulate changes within the brain [226]. An important finding is that peripheral BDNF is able to cross the blood-brain barrier [227], which is a prerequisite for BDNF-related therapy. However, the rate at which BDNF is taken up by the brain has not been quantified yet. Nonetheless, experimental therapy has been investigated *in vivo* and *in vitro* with promising results in animal models of AD [228], PD [229] and MDD [230] (a comprehensive review on BDNF drug delivery in brain pathologies and current stage of clinical trials can be found in [231]). For example, BDNF infusion into the hippocampus of adult rats was able to increase neurogenesis and regional neuronal activity [232]. Delivery of the BDNF mimetic 7-8-dihydroxiflavone is able to revert cognitive deficits in an AD animal model [233]. Also, gene transfection of BDNF into a 6-hydroxydopamine-induced unilateral lesion in the striatum was able to revert motor deficits in this animal model of PD [234].

The current need for improved treatments for brain disorders is pressing, and although large amounts of resources are spent on new therapies, few have been able to bring the desired results. The initial findings suggest that BDNF may be more than a biomarker for brain disorders; it may also become a possible target for the treatment of brain disorders. However, there is still a need for more information about the pharmacological features of BDNF-based substances in order to develop new treatments.

BDNF levels are activity-dependent, which means that the expression of BDNF changes under positive (e.g. physical activity, cognitive enhancement) and negative (e.g. obesity, sedentarism) behavioral and environmental stimuli. Several studies on both humans and animals show that physical activity, cognitive stimulation and a balanced diet can stimulate BDNF expression [235–238]. Physical activity is the best studied positive factor for the stimulation of BDNF expression. Thus, exercise can act as an inductor of neuronal plasticity, neurogenesis and neuronal survival [239,240]. Several studies in both animals and humans have assessed the effects of physical activity on BDNF levels in psychiatric [241–244] and neurodegenerative diseases [245–248]. These studies indeed indicated that physical activity augments neuronal protection in brain disorders by stimulating BDNF expression. However, there are

still some questions regarding the required time of the physical intervention, the optimal type of exercise (i.e., strength, endurance, aerobic exercises). Although less studied, diet can also provoke changes in BDNF levels in physiological conditions. BDNF is known to affect the regulation of feeding and energy metabolism [249–252]. Decreased levels of BDNF were found in subjects consuming diets with high sugar and fat [253,254]. On the other hand, dietary restriction (i.e. the maintenance of a balanced diet) or addition of supplementary substances (e.g. omega-3 fatty acids; resveratrol) can induce an increase in BDNF levels and reduce cognitive impairment in animal models [255–257]. It is not clear yet how large the effect of dietary management on BDNF protein levels in psychiatric or neurodegenerative disorders, but it is known that BDNF affects – and is affected by – dietary behavior. More research is needed in order to better understand the dietary influence on brain disorders.

Lifestyle changes that modulate BDNF levels in the brain may prove capable to control symptoms and delay progression in brain pathologies and thus might become a cheap and easily accessible (adjuvant) treatment for these pathologies in the near future. However, a possible concern about such therapies is the compliance of the patients with the treatment. Most of behavioral therapies are based on long periods of treatment with a relatively slow improvement when compared with medications. This may reduce the motivation of patients to continue treatment, especially because most psychiatric and neurodegenerative disorders can be accompanied by mood symptoms (e.g., anhedonia, hopelessness, aggressiveness). Therefore, slow-acting lifestyle therapies could yield better results if they are combined with fast-acting pharmacological interventions.

Concluding remarks

BDNF is involved in several processes that are essential for the optimal functioning of the brain. Several studies report altered brain and plasma BDNF levels in patients with various brain pathologies. However, the pathophysiological mechanisms that underlie these changes are still not fully understood yet. There is also no conclusive evidence that can discriminate whether changes in BDNF levels are a causative or the consequence of the disease onset. Although this paradigm is challenging, there are ways to address this question. In animals, injection of BDNF in the Hippocampus was shown to cause a decrease of depressive-like behavior. In humans, populations with a genetic variant that decreases BDNF concentration appear to be more susceptible to psychiatric disorders. While possibilities to assess the causal relationship of BDNF to psychiatric and neurodegenerative disorders, it is noteworthy that BDNF is highly affected by environmental changes, which in turn generates noise on these genetic findings. In humans, allying genetic of populations together with epigenetics and molecular analysis or molecular imaging (e.g.: Positron Emission Tomography) could improve accuracy of BDNF findings in the future. In animals, the need for an effective disease model – especially for neurodegenerative

diseases – is of utmost importance. Current disease models are not able to properly reproduce human disorders except for a small range of symptoms (i.e.: low predictive validity). This results in a larger noise to the findings, thus decreasing its reliability somewhat. If new and more reliable models are available, eventually findings on BDNF, as well as several other markers, will be more relevant for further clinical therapy.

Evidence from preclinical studies and clinical trials suggests that treatment strategies aiming to increase brain BDNF levels could have a beneficial effect on many brain disorders. In this respect, epilepsy is an exception as it is associated with increased levels of BDNF. At present, pharmacological intervention by the administration of exogenous BDNF is still challenging, but lifestyle changes could provoke the desired effect. Environmental and physiological stimuli, such as physical activity, social interactions, sensory and cognitive stimuli, are powerful modifiers of neurotrophin levels, including BDNF levels, and have been shown to alleviate symptoms of brain pathologies in both humans or animal models. Plasma BDNF can be reliably assayed and samples are easily collected and therefore BDNF has been proposed as a potential peripheral biomarker for the assessment of the status of the brain and the efficacy of treatment. However, discrepancies between brain and plasma BDNF alterations have been observed and need to be further elucidated before plasma BDNF can qualify as a suitable biomarker.

Neuroinflammation is regulated by factors that are also involved in the modulation of BDNF expression. Both neuroinflammation and altered BDNF expression are common phenomena in many brain disorders. Remarkably, there are only a few studies that have investigated the link between BDNF and neuroinflammation. A better understanding of the interaction between BDNF and neuroinflammation could open new ways for therapy management and could facilitate the development of new therapeutic strategies for brain diseases.

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Chapter 3

Long-term environmental modifications affect BDNF concentrations in rat hippocampus, but not in serum

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Behav Brain Res. 2019 October; 372. doi: 10.1016/j.bbr.2019.111965

Abstract

The role of mBDNF on the beneficial effects of cognitive stimulation on the brain remains controversial, as well as the potential of peripheral mBDNF as a biomarker of environmental effects on its central status. We investigated the effect of different environmental conditions on recognition memory, proBDNF, mBDNF and synaptophysin levels in the hippocampus, and on mBDNF levels in the blood. Male Wistar rats (6 and 17 months-old) were assigned to cognitively enriched (EE), standard (SE) and impoverished (IE) environmental conditions for twelve weeks. Novel object recognition was performed at week 10. When the animals were 9 and 20-months old, hippocampus was collected for mBDNF, proBDNF and synaptophysin analysis; serum was analyzed for mBDNF levels. The cognitively EE improved recognition memory resulted in a trend of increased hippocampal mBDNF and augmented synaptophysin levels. Accordingly, hippocampal mBDNF, proBDNF, and synaptophysin were significantly higher in EE than IE animals. Hippocampal mBDNF was positively correlated to proBDNF, cellular and behavioral plasticity markers. No effect of age was seen on the studied variables. Moreover, no significant effects of EE or IE on serum mBDNF were observed. Serum mBDNF also failed to correlate with hippocampal mBDNF, proBDNF and with the cellular and behavioral plasticity markers. These findings indicate that mBDNF is involved in neuronal and behavioral plasticity mechanisms induced by cognitively enriched environments and that peripheral mBDNF may not always be a reliable biomarker of the effects of environmental settings on central mBDNF and plasticity, which is of special interest from a translational research perspective

Keywords:

BDNF; environmental enrichment; hippocampus; serum; social isolation; proBDNF

Introduction

The brain-derived neurotrophic factor (BDNF) has attracted great interest in the last decades because of its broad effects on brain functioning. BDNF is associated with the modulation of neurogenesis, neuroplasticity and neuronal survival [1–3]. As a common trait for all neurotrophins, BDNF is produced as a pro-neurotrophin (proBDNF), being cleaved into its mature form (mBDNF) both at intra- and extracellular compartments [4]. While mBDNF facilitates neuroplasticity, neurogenesis and neuronal survival by means of its interaction with TRKB receptors, binding of proBDNF to the low-affinity p75 neurotrophin receptor (p75NTR) was shown to negatively regulate these functions [5,6]. Thus, since mBDNF and proBDNF may have opposite roles, it is likely that the balance between them plays an important role in physiological and pathological conditions [7–11].

BDNF is thought to have a key role in the beneficial effects of interventions aimed to prevent or rehabilitate age-related cognitive decline [12–14]. As current techniques fail to assess mBDNF levels in the living human brain, peripheral (serum and plasma) measures of mBDNF have been used as an indicator of central (brain) alterations of this neurotrophin. Although physical activity and cognitive stimulation are known to improve cognitive functioning, only physical activity was shown to be associated with increased peripheral levels of mBDNF [15,16]. Thus, there are doubts about the role of mBDNF in the beneficial effects of cognitive stimulation interventions. Besides, the use of peripheral mBDNF as an indicator of brain mBDNF levels, or of mBDNF effects on neuronal and behavioral plasticity, is still seen with skepticism. The dynamics of mBDNF exchange across the blood-brain barrier is poorly understood [17–19]. Moreover, various peripheral tissues, such as the skeletal muscles and the cardiovascular system, are also capable of producing BDNF and contribute to its circulating levels [20–22]. Thus, animal models are needed to properly investigate the effects of cognitive stimulation on brain mBDNF, as well as the relation between central and peripheral mBDNF.

Environmental enrichment protocols for rodents aimed to simulate cognitive stimulation interventions for humans include the traditional components used to create enriched environments (special bedding, toys, tunnels, social interaction), with the exception of running wheels [23]. Although this cognitively enriched environment was shown to confer benefits on learning and memory in young and old adult animals [23–25], its association with central mBDNF alterations is still a matter of debate and was investigated only in young adult animals [23,26].

Environment manipulation is also an interesting paradigm to investigate the relationship between central and peripheral mBDNF levels. In opposition to environmental enrichment, environmental impoverishment (social isolation, lack of sensory-motor stimuli) impairs memory

performance and was shown to decrease the expression and protein levels of mBDNF in the hippocampus, a key structure for memory processing [27–29].Thus, the comparison of experimental conditions expected to have opposite effects on mBDNF levels in the brain, such as enriched and impoverished environments, would be advantageous to evaluate the relations between central and peripheral mBDNF levels.

The present study investigated the effect of three months of enriched and impoverished environmental conditions in younger (6 month-old) and middle-aged (17 month-old) adult rats on: I) central proBDNF and mBDNF levels, as measured in the hippocampus; (II) neuronal plasticity, as assessed by the synaptophysin levels of hippocampal homogenates, considered an indirect biomarker for neuroplasticity [30–32]; (III) behavioral plasticity, evaluated with the novel object recognition task, which is hippocampal-dependent and sensitive to environmental conditions and aging [29,33,34]; (IV) peripheral mBDNF levels, as measured in serum. It is hypothesized that the cognitively enriched environment will increase central levels of mBDNF and that this alteration will be accompanied by increased proBDNF levels, neuronal and behavioral plasticity, improving memory performance in younger and middle-aged adult animals. It is also hypothesized that, if central and peripheral mBDNF levels are correlated, then serum mBDNF will also be a reliable biomarker of the environmental effects on hippocampal neuronal and behavioral plasticity.

Material and methods

Animals

Male Wistar (CrlCembe:WI) rats were bred and housed in the Centro de Modelos Biológicos Experimentais (Center of experimental biological models - CeMBE) of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) until the beginning of experimental manipulation (at 6 or 17 months of age). Animals were maintained in standard transparent individually ventilated cages (Tecniplast), the floor covered with sawdust, under controlled temperature (24±1 °C), humidity (55%), circadian cycle of 12/12 hours (lights on at 7 PM) and ad libitum access to standardized pellet food and water. All experiments were carried out in conformity with the Guide for the Care and Use of Laboratory Animals and performed according to the recommendations of the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI). Experimental protocols were approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University (CEUA, registration No. 7142). All efforts were made to reduce sample size and minimize animal suffering.

Experimental conditions

At the age of 6 or 17 months, animals were moved to CEMBE's research facility and randomly distributed into three groups: standard environment (SE), enriched environment (EE) and impoverished environment (IE). All groups were housed in the same room during the experimental protocol, which lasted 12 weeks. In the SE condition young adult rats (6 to 9 month-old) were housed at three animals/cage, while middle-aged rats (17 to 20 month-old) were housed at two per cage. This difference in the number of animals per cage was required in order to maintain the cage area per animal constant between the groups. Animals of the IE group were singly housed for the entire experimental protocol. The EE condition was adapted from Bruel-Jungerman and colleagues [34] considering the findings of Simpsom and Kelly [35]. Animals were housed in groups as described for the SE condition and placed in a large, one square meter cage-like apparatus with sensory and motor stimuli (e.g.: mazes, toys, bedding material) for 90 minutes per day, six days per week for 12 weeks.

Stimuli were changed every week to encourage exploration. As the middle-aged rats were housed in groups of two animals per cage, we combined animals from two housing cages into one large apparatus for environmental enrichment. As the introduction of new animals can be considered a mild stressor, animal welfare was constantly monitored for signs of fighting and stress. The environmental enrichment exposure was always initiated between 4 PM and 5 PM to avoid circadian influences. After the 90 minutes period, animals were returned to their home cages. In the IE condition animals were single-housed.

Thus, in the SE conditions, animals had social interaction, in the EE condition increased opportunity for sensory-motor experiences was added to the social interaction, whereas in the IE condition animals had no social interaction and sensory-motor experiences were not stimulated.

Behavioral assessment

In the tenth week after the beginning of the experimental protocol, the animals were submitted to the behavioral analysis. Locomotor, exploratory and anxiety behaviors were assessed with the open field test (OF). Memory, an indicator of behavioral plasticity, was evaluated with the novel object recognition (NOR) test. One day before the behavioral tests all animals were handled for 90 seconds for habituation to the experimenter (Figure 1).

The OF arena consisted of a square box (40x40x60) with three wooden walls and one glass wall for animal observation. The floor was divided into 16 symmetrical squares. The four squares in the center of the apparatus were considered the inner zone, while the remaining 12 squares were called

outer zone. The animal was placed with its head towards the glass wall and allowed to explore the open field for 5 minutes. The number of squares the animal crossed (number of crossings), the number of rearings and the proportion of time spent in the inner zone were determined as measures of locomotion, exploratory behavior, and anxiety, respectively.

The NOR task uses the natural preference for novel objects displayed by rats and was used to assess the effects of environmental conditions and age on long term memory. On the first day, animals were habituated to the open field box filled with sawdust. In the training session, performed 24h after habituation, the animal was allowed to explore two identical objects (A and A', Duplo Lego toys) positioned in two adjacent corners, 9 cm from the walls. Animals were left to explore the objects until they had accumulated 30 s of total object exploration time or for a maximum of 10 min. Animals were tested for retention 24 h after training (long-term memory). In the retention test trial, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. Keeping the nose or nostrils on the object, and poking and sniffing of the object were considered as signs of exploration. Trials were videotaped and object exploration was measured by an experimenter blind to group assignment, using two stopwatches to record the time spent exploring the objects. All objects presented similar textures, colors, and sizes, but distinctive shapes. A recognition index calculated for each animal was expressed by the ratio TB/(TA + TB) [TA=time spent exploring the familiar object A; TB=time spent exploring the novel object B]. Between trials, the objects were washed with 10% ethanol solution [36].

Blood and tissue sampling and processing

Animals were euthanized by decapitation 12 weeks after the beginning of exposure to the different environmental conditions and trunk blood and the hippocampus were collected for analysis. Blood was kept at room temperature for 1 h before centrifugation at 1000g for 10 minutes and the supernatant was collected. Hippocampus was separated from the whole brain and snap-frozen in liquid nitrogen. All samples were stored at -80°C for further analysis.

Blood and hippocampus mature BDNF (mBDNF) were measured by ELISA (CYT306 ChemiKine, Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Hippocampal and serum mBDNF concentrations were corrected for the total amount of protein, since it is known that small variations in the levels of proteins can have a significant effect on mBDNF levels, especially in serum samples, which have lower protein levels than hippocampus samples. Total protein levels were measured using the Bradford assay [37] with bovine serum albumin as standard and performed according to a protocol previously described by our laboratory [38]. Briefly, tissue homogenates were prepared by gently grinding hippocampus samples in 0.1M phosphate-buffered saline solution with protease

inhibitor at room temperature. The samples were immediately centrifuged at 2000g for 5 minutes and the supernatant was frozen at −80 °C until further analysis.

For mBDNF analysis, 25 µL of samples of serum or supernatant of brain homogenates (in duplicate) and reference standards of mBDNF with concentrations ranging from 15.63 to 500 pg/mL were added to 96-well flat-bottom microtiter plates. After 24 hours incubating at 4 °C, plates were rinsed four times with the wash buffer provided by the manufacturer. Biotinylated mouse anti-human mBDNF monoclonal antibody (diluted 1:1000 in sample diluent) was added to each well and incubated for 3 hours at room temperature. Wells were once again washed and then incubated with streptavidin–horseradish peroxidase conjugate solution (diluted 1:1000) for 1 hour at room temperature. After the addition of substrate and stop solution (CYT306 ChemiKine, Millipore, Darmstadt, Germany), the amount of mBDNF was determined by a plate reader. Absorbance was set at 450 nm.

Synaptophysin and proBDNF concentrations in the hippocampus were measured by Western Blot with a method previously reported [32]. Briefly, proteins were extracted in homogenization buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, protease inhibitor cocktail (Sigma-Aldrich: 104mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 80µM Aprotinin, 4mM Bestatin, 1.4mM E-64, 2mM Leupeptin, 1.5 mM Pepstatin-A), 0.5% Triton X-100, and 0.1% SDS. After 30 minutes on ice, samples were centrifuged at 14000g for 10 minutes. The supernatant was collected and the protein content was determined using a Bradford assay. Aliquots were stored at −80 °C until further analysis. 25 µg of protein was separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and were incubated overnight with either anti-synaptophysin (1:2500; Abcam, Cambridge, UK) or anti-proBDNF (1:1000; Sigma-Aldrich, São Paulo Brazil). Goat anti-mouse IgG and goat polyclonal anti-rabbit IgG (both from Abcam, Cambridge, UK) secondary antibodies were used and detected using the ECL Western blot Substrate Kit (Abcam, Cambridge, UK). After that, membranes were washed twice with a mild stripping buffer (containing 15 g glycine, 1 g SDS, 10 ml Tween20, pH 2.2, final volume of 1,0L) for 30 minutes, and twice with PBS for 10 minutes at room temperature, before reprobing for loading controls (anti-tubulin, 1:2500; Abcam, Cambridge, UK, for synaptophysin analysis or anti-β-actin, 1:1500; Sigma-Aldrich, São Paulo Brazil, for proBDNF analysis). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine molecular weight corresponding to the detected bands. The densitometric quantification was performed using Chemiluminescent photo finder (Kodak/Carestream, model GL2200). The target-to-control protein ratio was calculated (i.e. synaptophysin/tubulin and proBDNF/β-actin).

Statistical analysis

Data were analyzed using two-way ANOVAs, with the environment (standard, enriched and impoverished) and age (9 and 20-month-old animals) as between-group factors, and Bonferroni as *post hoc* test whenever appropriate.

Pearson's correlation analysis was conducted to verify if the peripheral and central levels of mBDNF could be associated with each other and with proBDNF levels, cellular and behavioral plasticity markers. Results are expressed as mean ± standard error (SE). For all statistical analyses, significance was set at $p<0.05$.

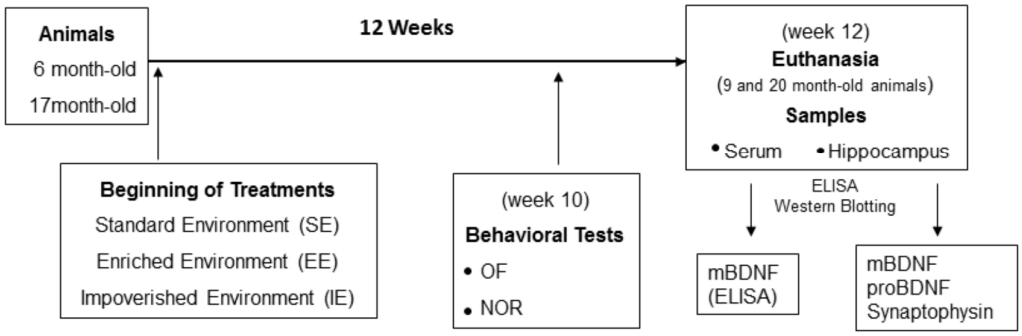

Figure 1: Timeline of experimental procedures. Animals of 6 and 17 months of age were submitted to three different environmental conditions: SE (standard environment), EE (enriched environment), and IE (impoverished environment) for 12 weeks. Behavioral testing on the open field (OF) and novel object recognition (NOR) task was performed during the 10th week after the beginning of the treatments. At the end of the different treatments, 9 and 20-month-old animals were euthanized to collect samples for mBDNF, proBDNF, and synaptophysin analysis.

Results

Open-field behavior

Statistical analysis of the OF results identified significant main effects of the environment on locomotion [number of squares crossed: $F_{(2, 61)}=4.119$; $p=0.021$] and anxiety [time spent in the center of the field: $F_{(2, 61)}=4.691$, $p=0.013$]. However, no significant effects of age or interaction between age and environment were found when the number of crossings and time spent in the center of the arena were compared between groups (all $p>0.05$, Table 1). As can be seen in Table 1, further analysis of the environment effects showed that IE animals showed an increased number of crossings compared to the other two groups ($p=0.048$ for IE vs SE; $p=0.035$ for IE vs EE), and spent significantly less time in the

center of the arena than EE animals (p=0.012). On the other hand, the analysis of the exploratory behavior showed a main effect of age, indicating that younger animals performed more rearings than older animals [$F_{(2,61)}$=13.46, p<0.001]. However, no significant effects of environment or interactions between environment and age (all p>0.05) were found for this behavioral parameter.

Table 1: Open field behavior

Young adult rats (9m)				
Group	**Crossings**	**Rearings**	**Time in center (s)**	**N**
SE	55.93 ± 5.35	26.47 ± 2.60 #	55.87 ± 10.61	15
EE	54.40 ± 4.40	27.50 ± 3.48 #	57.0 ± 10.11	10
IE	70.83 ± 6.49 *	30.0 ± 1.94 #	27.75 ± 6.19 **	12
Middle-aged adult rats (20m)				
SE	52.44 ± 5.39	22.89 ± 3.12	29.78 ± 8.44	9
EE	49.09 ± 3.71	14.18 ± 1.42	47.41 ± 8.25	11
IE	56.65 ± 5.06 *	21.70 ± 3.48	24.85 ± 4.43 **	10

Open-field behavior was analyzed during the habituation session for the object recognition task. Data are expressed as mean ± SEM. Data were analyzed by general linear models (GLMs), with environment (standard - SE, enriched – EE, and impoverished - IE) and age (9 and 20-month-old animals) as between-group factors and Bonferroni as post hoc test. * indicates p< 0.05 when the number of crossings of IE was compared to SE and EE. ** indicates p< 0.05 when the time at the center of IE was compared to EE. # p< 0.05 indicating the main effect of age when the number of rearings of 9 m and 20m rats was compared.

Behavioral plasticity evaluated by the Novel Object Recognition

The long-term memory on the Novel Object Recognition (NOR) task was used as an indicator of the effects of environmental conditions and age on behavioral plasticity. The statistical analysis revealed a significant main effect of environmental conditions on recognition memory retention [$F_{(2, 61)}$=15.548; p<0.001]. However, there was no effect of age or any interaction between age and environment on memory (all p>0.05). As can be seen in Figure 2, the post hoc analysis of the environmental effects on memory indicated that the EE group had the highest recognition memory retention index when compared to the other groups (p=0.003 for EE vs. ES; p<0.001 for EE vs. IE). On the other hand, the IE group had the lowest recognition index when compared to the other two groups (p=0.041 for IE vs. SE; p<0.001 for IE vs. EE). (Figure 2). No statistically significant main effects or interactions of age and environmental conditions were found when recognition indexes of the training session were compared (all p>0.05).

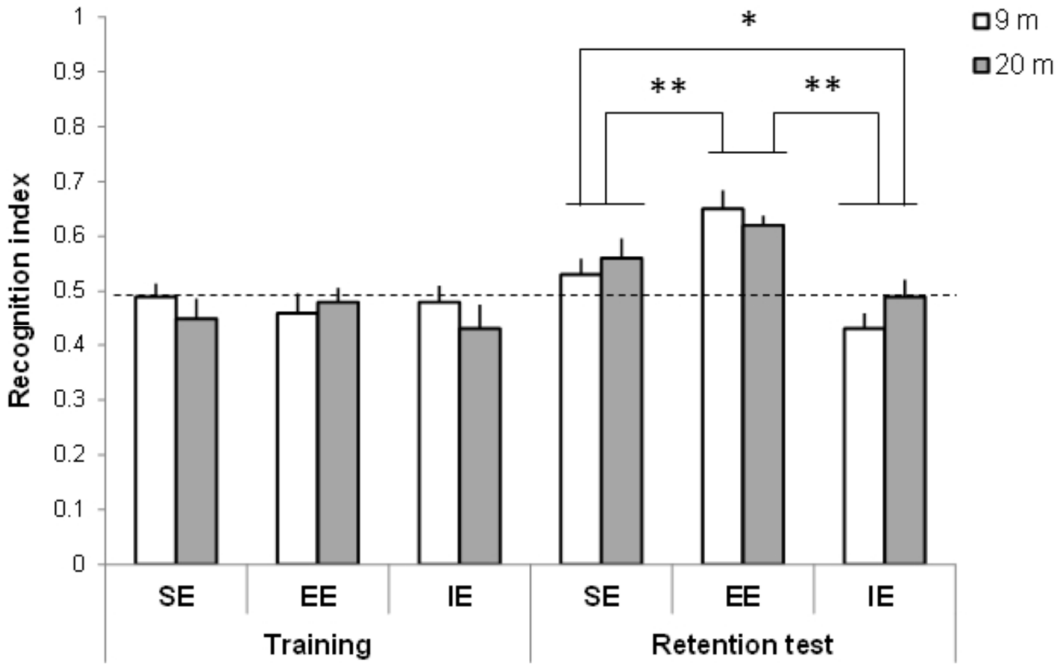

Figure 2: Effects of environmental conditions on recognition memory tested. Data are expressed as mean ± standard error of the mean (SEM). n=9 - 15 per group. Statistically significant differences between EE vs. SE and IE are indicated as ** p<0.001 and between IE vs. SE is indicated as * p<0.05.

Hippocampal mBDNF and proBDNF levels

As can be seen in figure 3A, the statistical analysis indicated significant effects of the environment [$F_{(2, 34)}$=13.31; p<0.001], but not of age, or age x environment interactions (all p>0.05), on hippocampal mBDNF levels. Further analysis indicated higher levels of mBDNF in the EE group in comparison to the SE and IE groups. However, only the difference between EE and EI groups reached statistical significance (p<0.001). EI animals showed significantly lower mBDNF levels than SE animals (p=0.008).

The general pattern of the results obtained for the hippocampal proBDNF levels resembled the results obtained for the hippocampal mBDNF levels. Thus, significant effects of the environment [$F_{(2, 20)}$=7.468; p=0.004], but not of age, or age x environment interactions (all p>0.05), were identified in hippocampal proBDNF levels. Moreover, as previously described for mBDNF, the levels of hippocampal proBDNF were higher in the EE group in comparison to the SE and IE groups. However, in the case of proBDNF, statistical significance was reached both for the differences between the EE and IE groups (p=0.004) and for the differences between the EE and ES groups (p=0.018) (Figure 3B).

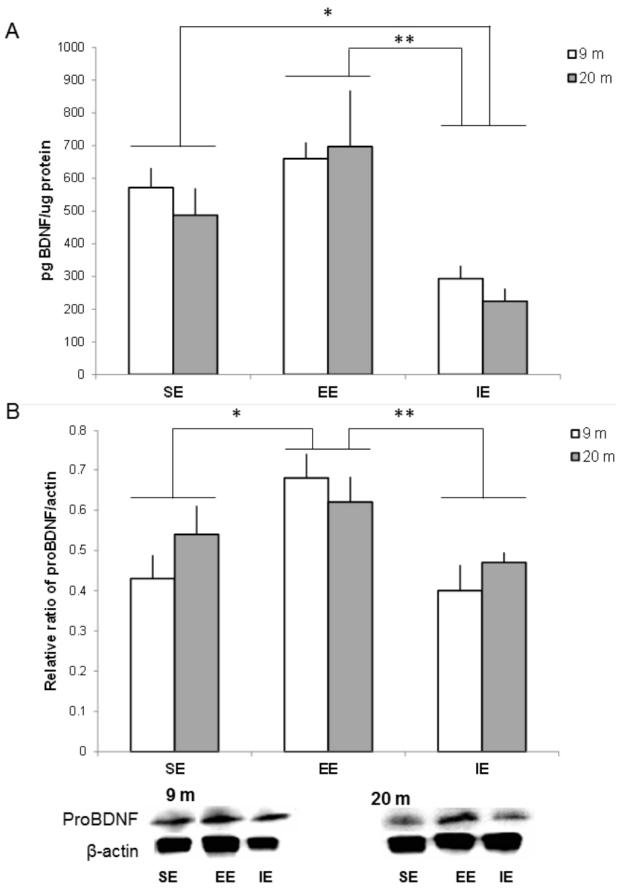

Figure 3: Effects of environmental conditions on hippocampal mBDNF (A) and proBDNF (B) levels in 9 or 20 month-old rats. Representative Western blots for proBDNF and β-actin are shown in the lower panel. Data are presented as mean ± SEM and expressed as pg of mBDNF/ug of protein or the relative ratio of proBDNF/β-actin, n=6-8 (mBDNF) or n=4-5 (proBDNF). Statistically significant differences in mBDNF levels between EE vs. IE is indicated as ** p<0.001 and IE vs. SE is indicated as * p<0.01 and significant differences in proBDNF levels between EE vs. IE is indicated as ** p<0.01 and EE vs SE is indicated as * p<0.05.

Cellular plasticity evaluated by hippocampus synaptophysin levels

As can be seen in figure 4, the hippocampal synaptophysin levels showed the same pattern of results as found for the proBDNF levels. Hence, significant effects of environment [$F_{(2,21)}$=12.066; p<0.001], but not of age, or age/environment interactions (all p>0.05), were identified in hippocampal synaptophysin levels. Post hoc analysis indicated significantly higher levels of synaptophysin in EE animals than in the other groups (p=0.043 for EE vs. SE; p<0.001 for EE vs. EI).

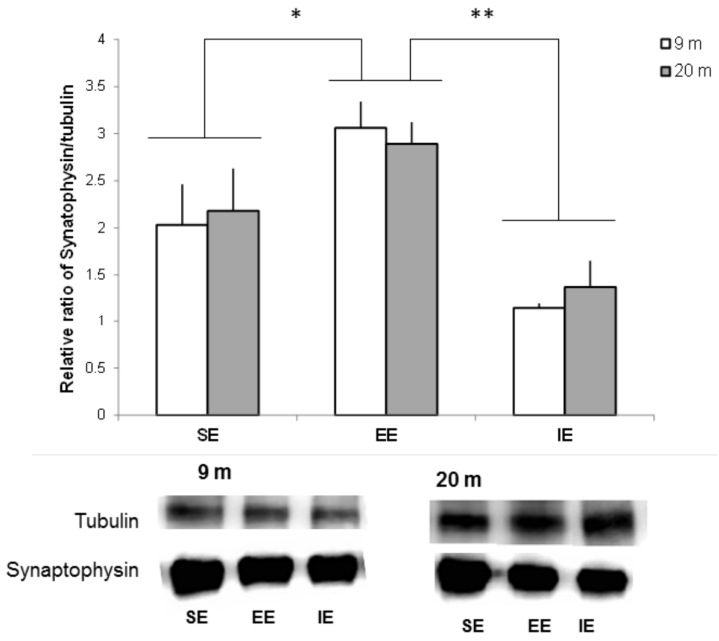

Figure 4: Effects of environmental conditions on hippocampal Synaptophysin levels in 9 or 20 month-old rats. Representative Western blots for synaptophysin and tubulin are shown in the lower panel. Data are presented as mean ± SEM of the relative ratio of synaptophysin/tubulin, n=4-5. Statistically significant difference between EE vs. IE is indicated as ** p<0.001 and between EE vs. SE is indicated as * p<0.05.

Serum mBDNF

The results obtained for serum BDNF levels can be seen in figure 5. Despite the environmental effects seen on hippocampal mBDNF, the two-way ANOVA failed to identify significant effects of environment [$F_{(2,29)}$=1.518; p=0.236], age [$F_{(2,29)}$=0.485; p=0.492], or interactions between age and environment [$F_{(2,29)}$=0.311; p=0.735] on serum mBDNF levels.

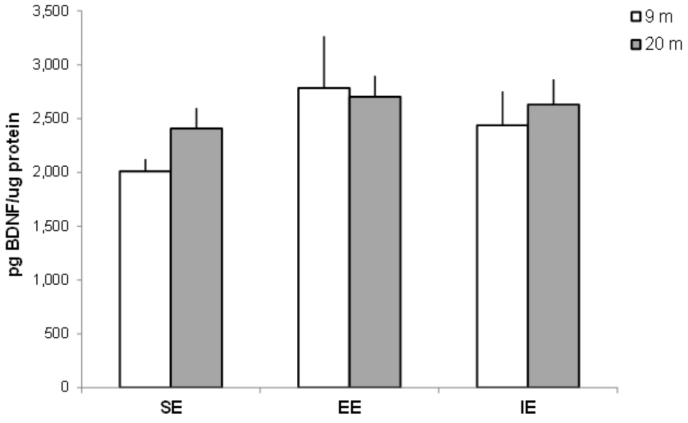

Figure 5: Effects of environmental conditions on serum mBDNF levels in 9 or 20 month-old rats. Data are presented as mean ± SEM of pg mBDNF/µg of protein, n=5-7.

Correlation analysis between peripheral and central mBDNF and proBDNF, cellular and behavioral plasticity markers

Separate Pearson's correlation analysis, including data of all experimental groups, were run to evaluate the relations between (I) hippocampal mBDNF levels and proBDNF, synaptophysin and recognition index in the NOR task; (II) serum mBDNF and hippocampal mBDNF; (III) serum mBDNF and proBDNF, synaptophysin and recognition index in the NOR task. As can be seen in table 2, hippocampal mBDNF levels were significantly correlated to proBDNF and synaptophysin levels, as well as to the recognition index in the NOR task (all $p<0.05$). In clear opposition to these results, no significant associations were found between serum mBDNF levels and hippocampal proBDNF and synaptophysin levels, nor between serum mBDNF levels and the memory index (all $p>0.05$). As already expected from the pattern of the results described above, no significant correlation was found between serum mBDNF levels and hippocampal mBDNF levels ($p=0.48$).

Table 2: Pearson correlation analysis

I)	**Hippocampal mBDNF**	
	R	**Sig.**
proBDNF	**0.82**	**<0.001**
Synaptophysin	**0.50**	**0.047**
Recognition Index	**0.42**	**0.004**
II)	**Serum mBDNF**	
	R	**Sig.**
Hippocampal mBDNF	**0.15**	**0.48**
	Serum mBDNF	
	R	**Sig.**
proBDNF	**0.57**	**0.56**
Synaptophysin	**0.46**	**0.08**
Recognition Index	**0.23**	**0.28**

Separate Pearson's correlation analysis, including data of all experimental groups, were run to evaluate the relations between (I) hippocampal mBDNF levels and proBDNF, synaptophysin and recognition index in the NOR task; (II) serum mBDNF and hippocampal mBDNF; (III) serum mBDNF and proBDNF, synaptophysin and recognition index in the NOR task. Abbreviations: R: Pearson's correlation coefficient; Sig: statistical significance.

Discussion

The present study investigated the effects of enriched and impoverished environmental conditions on hippocampal proBDNF, mBDNF, synaptophysin, and long-term memory, as well as the relation between hippocampal and serum mBDNF levels, analyzing the potential of peripheral mBDNF as a biomarker of central mBDNF, neuronal and behavioral plasticity. Consistent with the working

hypothesis, the EE increased central levels of proBDNF and mBDNF, and these alterations were accompanied by increased neuronal and behavioral plasticity, improving memory performance in younger and middle-aged animals. As expected, IE animals had impaired memory and lower levels of proBDNF, mBDNF and synaptophysin than EE animals. However, no significant effects of environmental conditions were observed on serum mBDNF and no correlation was found between central and peripheral mBDNF levels. Accordingly, serum mBDNF also failed to be a reliable biomarker of the environmental effects on hippocampal neuronal and behavioral plasticity.

In this study we reproduced findings from other research groups, showing that long-term memory for object recognition was improved by the cognitively EE protocol and impaired by the IE condition [23,39–41]. Although the critical elements of the EE condition responsible for the improved performance on the NOR task are difficult to identify, the daily exposure of animals to a variety of toys, objects, and mazes provides an opportunity to improve multiple cognitive abilities, including object memorization [34]. On the other hand, in the IE condition animals are moved from their social environment to a situation characterized by the lack of social interaction with other individuals. It has been demonstrated that social isolation acts as a stressor, affecting multiple behavioral domains, including anxiety behaviors [39,42,43], as suggested in this study by the increased locomotor activity (a sign of hyperactivity) and decreased time spent in the inner zone of the OF. Thus, the role of anxiety on the impairment seen in the NOR task cannot be ruled out for IE animals.

Neurobiological changes associated with beneficial effects of EE and detrimental effects of IE on memory have been extensively studied. Overall, studies suggest that EE increases, while IE decreases the dendritic branching, spine and synapse numbers, as well as the weight and thickness of the cortex and hippocampus [44–46]. EE and IE also have antagonistic effects on neurogenesis and synaptogenesis [25,42,47]. The mature form of BDNF is one of the main candidates to orchestrate all these alterations, because of its multiple functions in neuronal and behavioral plasticity, neurogenesis and neuronal survival [1–3]. In accordance with this concept, studies have demonstrated that hippocampal mBDNF increases in EE [30,31,48,49] and decreases in IE animals [27–29]. However, most of the EE studies include running wheels in the EE cages and the most prevalent view in recent literature is that the physical component of environmental enrichment is responsible for the increase in mBDNF expression and protein levels in the brain [23,26,50]. In fact, physical activity can also increase the levels of other neurotrophins. Twenty two-months-old rats receiving moderate treadmill training had better spatial memory performance associated with increased BDNF and NT3 levels in the hippocampus [51]. Our study shows, for the first time, that a cognitively EE also has the potential to increase hippocampal mBDNF levels.

Although our EE animals showed only a trend towards increased hippocampal mBDNF in comparison to SE animals, it was accompanied by increased proBDNF levels. Moreover, a significant positive correlation was found between hippocampal mBDNF and proBDNF levels. Together these results suggest an increased synthesis of the precursor protein, and thus of mBDNF. Environmental enrichment protocols with running wheels were already the novelty you are reporting. shown to increase mRNA levels for BDNF and promote higher conversion rates of proBDNF to mBDNF [48,52,53]. Prior studies comparing enriched environments with and without the physical activity component failed to find alterations in BDNF expression and protein levels in the cognitively enriched environments [23,26]. However, they analyzed younger animals and submitted them to a shorter time of enrichment than the present study, making direct comparisons difficult.

The IE condition showed opposed and more pronounced effects on hippocampal mBDNF levels than the EE condition, as indicated by the significant decrease of mBDNF in the IE group in comparison to the EE and SE groups. Although social isolation was already shown to reduce BDNF transcription and mRNA expression [42], its effect on proBDNF levels and conversion rates to mBDNF was never explored. However, from studies that evaluated the effects of stress on the proBDNF/mBDNF balance, we can expect that IE, as a stressful condition (for rats are social mammals), also modulates the proBDNF/mBDNF balance [54,55]. Here we show, for the first time, that the decrease in hippocampal mBDNF levels by IE is not accompanied by a significant reduction of proBDNF levels. Thus, it is possible that the conversion rate of proBDNF to mBDNF, catalyzed by intracellular matrix metalloproteinases (MMP) and extracellula plasmin (tPA) [4], is reduced in the hippocampus of IE animals, increasing the proBDNF/mBDNF ratio. However, our experimental design does not allow final conclusions to be drawn about this issue.

The alterations seen for hippocampal mBDNF in animals exposed to the EE and IE conditions were positively correlated with their long-term memory indexes and proBDNF levels. Moreover, hippocampal mBDNF alterations were accompanied by reciprocal modifications of synaptophysin levels. As a synaptic vesicle protein involved in neurotransmission [56], synaptophysin of brain tissue sections or homogenates is commonly used as an indirect marker of neuronal plasticity [30–32]. It is also worth to mention that the hippocampal mBDNF and synaptophysin levels were significantly and positively correlated, further supporting the role of mBDNF on environmental induced neuronal plasticity [30]. Neuroplasticity studies are relatively scarce on cognitively EE protocols, but there is evidence for changes in neuronal morphology and plasticity [56–58]. Our results on hippocampal synaptophysin levels help in expanding the knowledge about the mechanisms proposed to mediate the beneficial effects of this EE protocol on long-term memory retention. Even so, the results obtained

for the synaptophysin levels should be interpreted with caution, since only the analysis of this protein in preparations of purified synaptic fractions can be considered a reliable proof of alterations in synaptic density.

The present findings suggest an association between the environmental condition, hippocampal mBDNF levels, behavioral and cellular plasticity. Thus, a clear difference was observed in these parameters when comparing EE and IE groups. However, these alterations were not accompanied by modifications in serum mBDNF levels. Moreover, no significant correlations were found between serum mBDNF and hippocampal mBDNF, proBDNF, synaptophysin or performance on the NOR task. These results are in clear contrast with the positive correlations found between hippocampal mBDNF and proBDNF, synaptophysin and object recognition performance. Thus, the experimental design of this study compares, for the first time, the different roles of central and peripheral mBDNF levels as biomarkers of neuronal and behavioral plasticity in the hippocampus. Peripheral mBDNF was not able to indicate the ongoing alterations that were occurring in the hippocampus as a result of the different environmental conditions. Therefore, our results suggest that changes in central mBDNF are not always reflected by changes in peripheral mBDNF levels or, in other words, that the lack of alterations on peripheral mBDNF does not signify that mBDNF is not involved in brain mechanisms that induce cellular and behavioral plasticity, having considerable implications from a translational perspective. However, the causes of the discrepancies between central and peripheral mBDNF in the present conditions are not fully understood, and future studies are warranted in order to address this question. The inclusion of positive control for serum mBDNF levels in these studies would be advantageous for strengthening the interpretation of results and draw stronger conclusions.

In fact, only a few studies have tried to correlate peripheral and central BDNF levels in experimental conditions, and the exchanges of central and peripheral mBDNF across the blood-brain barrier are still a matter of debate. Lanz and colleagues induced robust increases of mBDNF in the brain but failed to find detectable changes in plasma [19]. In fact, mBDNF efflux from the brain was measured only under extenuating physical activity [18]. However, other studies suggest that mBDNF influx in the brain is faster than its efflux [17], raising the possibility that blood may function as an mBDNF reserve [59]. Actually, mBDNF can be released by different peripheral tissues [20,21], including skeletal muscle. This tissue could contribute to the build-up of the blood reserve of mBDNF, as well as stimulate BDNF synthesis in brain via proteins released by the active muscle that can cross the blood-brain barrier [60]. These findings could explain why physical activity is more efficient than cognitive stimulation to induce increased central and peripheral mBDNF levels, as already shown by different studies with animal models and humans [15,16,26].

Furthermore, it must be taken into consideration that the biological functions of mBDNF in the brain and its activity-dependent synthesis and secretion by neurons suggest that, under physiological conditions, this neurotrophin is secreted only in amounts necessary to modulate surrounding neuronal populations [3]. Thus, only situations that induce excessive increases in brain mBDNF levels can be expected to contribute to alterations of this neurotrophin in peripheral blood. This seems not to be the case in our cognitively EE condition, as described above. Finally, it cannot be ruled out that the lack of an association between central and peripheral mBDNF levels is caused, at least partially, by the comparison of two different pools of mBDNF, one mostly intracellular (hippocampal BDNF) and the other mainly extracellular (serum mBDNF).

It is also important to note that neither the main effect of age nor an interaction between age and environmental conditions, was seen for the variables investigated in this study. Our results are in line with evidence that there is no age-related change in brain (hippocampus and frontal cortex) and serum levels for this neurotrophin in rats between two months and two years of age [61]. Moreover, former studies suggest that environmental enrichment and social isolation protocols have a conserved pattern of effects on cellular and behavioral plasticity from weaning throughout adulthood in animal models [42,62].

In conclusion, the present findings show for the first time that the beneficial effects of cognitively EE protocols on memory are associated with increased central mBDNF levels and neuronal plasticity. Remarkably, peripheral mBDNF failed to correlate with the central levels of this neurotrophin and with the neuronal and behavioral plasticity induced by the EE and IE protocols. These results suggest that blood/serum mBDNF levels may not always be reliable biomarkers of environmental effects on brain, which is of special interest from a translational research perspective.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – Brasil (CAPES) – Finance Code 001 and by the National Institute for Translational Medicine (INCT-TM, grant #465458/2014-9. E. Bromberg and N.Schröder are CNPq research fellows. V. Naziaseno has a PET/SESu/MEC fellowship; C.S. dos Santos has a CNPq fellowship and J.V. Borges has a CAPES fellowship. B.L. Giacobbo had a CAPES fellowship, L.M. Schlemmer had a FAPERGS fellowship, V. Antoniazzi and D.B. de Lima had a CNPq fellowship.

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Chapter 4

The effect of repeated social defeat on sociability is inhibited by HPA-axis disruption

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Abstract

Introduction: Chronic stress is associated with a deregulation of the hypothalamus-pituitary-adrenal (HPA) axis and can result in behavioral abnormalities, such as depressive behavior. Adrenalectomy (ADX) inhibits the production and release of corticosterone, impairing the response towards stressors, and thus may prevent stress-induced depressive behavior. **Objective:** the goal of this study was to assess if HPA axis disruption by ADX affects depressive- and anxious behavior and stress-induced neuroinflammation in a repeated social defeat (RSD) stress model. **Material and methods:** male Wistar rats were submitted to ADX or sham-surgery. After recovery, the animals were submitted to RSD or control conditions for five days. Depressive-like behavior was assessed by the sucrose preference test (SPT) 1, 7 and 14 days after the RSD paradigm. Anxiety and locomotion were measured by the open field test (OF), and social behavior was measured by the social interaction test (SI). Neuroinflammation was measured 14 days after RSD by [^{11}C]PBR28 PET imaging of the brain and confirmed by Iba-1 immunohistochemistry. **Results:** There was no effect of surgery or RSD in the SPT at any of the time points assessed, nor was there any effect on anxiety and locomotion behavior in the OF. In the SI, sham-surgery animals submitted to RSD spent a significantly shorter time in the interaction zone than the other groups. Neither [^{11}C]PBR28 PET nor Iba-1 immunostaining did not show any significant differences in neuroinflammation between groups. **Discussion and conclusion:** The results of the SI test indicate that animals under social stress tended to have less social interaction. This effect was not observed in ADX animals submitted to RSD, suggesting that ADX inhibited the effect of social defeat in these animals. There was no difference between RSD or ADX animals in the OF test, the SPT tests or the PET imaging results, providing no evidence for anhedonia, abnormal locomotion behavior or neuroinflammation induced by RSD or ADX. A plausible explanation for these negative findings could be that the RSD protocol was not strong enough for its effects to last until the time of assessment. If so, the paradigm of RSD needs to be improved in order to augment the effects of RSD and ADX on stress-induced neuroinflammation and behavioral abnormalities.

Keywords: Adrenalectomy; social stress; social interaction; neuroinflammation; PET imaging

Introduction

Major depressive disorder (MDD) is a main psychiatric disorder, being responsible for more than 40% of the general years with disability associated with psychiatric disorders and drug abuse [1]. Although this depression is observed in almost every stage of life, the highest incidence of depressive disorders is in the periods that the individual is most exposed to environmental challenges (e.g.: societal pressure) [2]. As the stressful situation progresses over time, the organism loses its ability to cope with stressful stimuli and behavioral and physiological changes associated with depression will manifest [3–5]. Therefore, an impaired stress response is regarded as a key factor for the development and progression of depression [6].

Exposure to endogenous or exogenous stressful stimuli triggers the hypothalamus to produce corticotropin-releasing hormone (CRH), which causes the pituitary to produce adrenocorticotrophic hormone (ACTH). ACTH signals the adrenal glands to produce a plethora of hormones, one of which is the stress hormone cortisol (or corticosterone in rodents). After reaching the brain and binding to glucocorticoid and mineralocorticoid receptors, cortisol decreases the expression of many genes associated with cortisol regulation [7]. When this regulatory machinery is impaired, or when the stress stimulus is exacerbated to the point the organism is unable to cope with it, the symptomatology associated with mood disorders arises, as thoroughly described in the literature for humans [8–11] and animal models of depressive-like behavior [12–14].

Chronic stress is characterized by a high concentration of cortisol/corticosterone in the brain, leading to neurotoxicity and eventually neuronal apoptosis, which is accompanied by the release of the intracellular content in the extracellular space [15,16]. This results in microglial activation and release of pro-inflammatory cytokines, generating a regional inflammatory process [17,18]. Neuroinflammation has been related to mood disorders and has become an increasingly relevant target for new therapeutic means to mitigate the symptoms of depression in humans [19,20]. However, the exact nature of the interaction between stress response and neuroinflammation and how it affects mood disorders remains elusive.

Emulating the symptoms of depression from humans in an animal model is extremely difficult and a challenge for behavioral researchers. Several models to mimic depression in rats and mice have been reported in the literature and of those, the ones that show the closest face-validity towards human disease are better suited. Repeated social defeat (RSD) has been shown to induce such effect in small animals by exploiting its natural social behavior of hierarchy and territoriality, and presents an interesting approach to study depressive-like behavior [23]. Additionally, RSD is able to induce an

inflammatory response in the brain [24], which further supports RSD as a viable method for induction of depressive-like behavior.

To better understand the molecular mechanisms of neuroinflammation *in vivo*, imaging approaches, such as Positron Emission Tomography (PET), have become an important tool both in human and small rodent research due to their relative non-invasiveness and applicability in longitudinal studies. A commonly used radiotracer for imaging of inflammation in the brain is [^{11}C]PBR-28. [^{11}C]PBR-28 is a tracer that binds to the 18 kDa translocator protein (TSPO), which is found in the outer membrane of mitochondria of activated microglia, macrophages and astrocytes [21].

The aim of this study was to assess how HPA-axis disruption by bilateral adrenalectomy affects depressive- and anxiety-like behaviors, as well as TSPO imaging as a biomarker for neuroinflammation in socially defeated rats.

Material and methods

Animals

The study protocol complied with European Directive 2010/63/EU and the Law on Animal Experiments of The Netherlands; it was approved by the Central Committee on Animal Experiments of The Netherlands (The Hague, license no. AVD1050020171706) and the Institutional Animal Care and Use Committee of the University of Groningen (IvD 171706-01-004). Male Wistar rats (HsdCpb:WU, 8 weeks old – Envigo, The Netherlands) were placed in groups of four animals per cage and acclimated for at least seven days before any procedure. Animals were maintained in a room with controlled temperature (21±2 °C) and humidity and a light/dark cycle of 12/12 hours. Food was available *ad libitum*. Water was provided as described in the section below. After acclimation, animals were randomly distributed to one of the following experimental groups: 1) ADX + Control; 2) ADX + RSD; 3) Sham-surgery + Control; 4) Sham-surgery + RSD. After surgery, all animals were singly housed until termination.

Surgery

For bilateral ADX, animals were anesthetized with isoflurane (5% induction, 2% maintenance) and placed on a heating pad to maintain temperature, while breathing was monitored constantly. Before the procedure, a veterinary shaver was used to remove the fur from the surgery sites on the dorsal part of the animal close to the kidneys The shaved sites were sterilized with ethanol and injected subcutaneously with analgesia (Carprofen: 5 mg/kg). On each side, an incision was made and the tissue

layer moved until the adrenal glands were visible, then the gland was removed with a cauterizer to stop blood flow. Sutures were applied both in the muscle layer and on the skin. For bilateral Sham surgery the adrenal glands were reached and touched by the experimenter, then suturing was placed. After completion of the surgery, animals were left for one week to recover. One animal of the ADX group had post-surgery complications and died before the start of the RSD protocol.

As a result of bilateral ADX, complete depletion of corticosterone was reported to induce a severe inflammatory response and neuronal death in specific brain regions after a few weeks [25], and also deregulates the salt homeostasis. To avoid this, animals were administered with daily corticosterone and salt replenishment in the drinking water (final concentration: 25µg/ml corticosterone, 0.4% ethanol and 0.5% saline water). This concentration allows the animal to maintain a baseline level of the hormone and salt, while not being assayable to produce a stress response. Animals with a Sham surgery received a water bottle containing 0.4% ethanol and 0.5% saline for the day of surgery onward. Serum corticosterone levels were taken at termination by drawing blood from the heart, in order to confirm the effectiveness of adrenalectomy. Blood samples were centrifuged at 10000 rpm for three minutes. Samples were kept at -20 °C until being analyzed with an ELISA (K014-H1, Arbor Assays, U.S), according to the instructions of the manufacturer. Samples were read at 450 nm. As expected, serum corticosterone levels were negligible for ADX animals when compared with Sham.

Repeated social defeat

Training phase: 12-weeks old male Long Evans (residents: HsdBlu:LE – Harlan, The Netherlands; n=6; weight: 450-500 grams at the beginning of RSD protocol) were paired with surgically sterilized females of the same age in a large wooden cage (80x50x40 cm) with plastic sliding doors on the investigator side. Male residents were trained on 5 consecutive days by introducing a Wistar rat in their home cage according to the same procedure as described below for the experimental phase. Residents that showed an attack latency (i.e.: time to initiate the first attack) of less than 60 seconds and no signs of violent behavior (i.e.: attack latency of fewer than three seconds without threatening behavior before the first attack) were selected as residents for the experimental phase of the study. Residents that showed non-aggressive or over-aggressive behavior were not used for RSD in this study.

Experimental phase: One hour before the beginning of RSD, female rats were removed from the resident cage. Then the experimental animals (intruders) were placed in the resident cage to begin the defeat protocol. Attack latency and submission time (i.e.: time the intruder takes to show a submissive posture for at least three seconds) were timed. After the intruder displayed a submissive posture, a

barrier was placed dividing the cage into two equal parts, each part containing one animal. By splitting the cage with the barrier, there is no physical contact between intruder and resident but animals can still share sensorial stimuli. After 60 minutes had elapsed, the intruder was removed from the RSD cage and placed back to its home cage, and the female is placed back in the resident cage. For the control group, the animals were placed in large, plastic cages for 60 minutes until they were placed back to their home cages. This protocol was repeated on five consecutive days, and the intruder was always introduced to a different resident during this period.

Sucrose preference test (SP)

Before the first RSD trial, animals were habituated to the sucrose solution. On three consecutive days, a bottle of water with 1% sucrose was placed in the home cage of the animal for one hour and the replace again by normal drinking water. For the SP test, animals received two water bottles: one containing their usual drinking water (as stated in the surgery section), and the other containing the same drinking water supplemented with 1% sucrose. The SPT was performed overnight on days 1, 7 and 14 after the last RSD trial. Both bottles were weighed before and after the test, and the amount of drinking water supplemented with sucrose ingested divided by the total amount of drinking water ingested provides the percentage of sucrose consumption as a measurement of anhedonic behavior.

Open field test (OF)

The open field test was performed 24 hours after the last RSD trial. For the OF test, a round wooden arena of 80 cm diameter was used. The animal was placed in the room one hour before the experiment and left alone during this period to acclimate to the environment. After one hour, the investigator placed the animal in the arena facing the wall and started recording the exploratory behavior of the animal on video for six minutes, after which the animal was returned to its home cage. After each test, the arena was cleaned with ethanol 70% and wiped with dry paper. For analysis of the videos, an inner and outer zone were delineated using Ethovision XT 14.0 software (Noldus, The Netherlands), and the setup was replicated for all trials. The distance traveled by the animal was automatically generated by the software, while the ratio of the time spent in the inner zone by the total time spent in both zones was calculated manually after the data was acquired. These parameters are measures of the general locomotion and anxiety-like behavior of the animals.

Social interaction test (SI)

The social interaction test was performed 48 hours after the last RSD trial. For the test, a 50^2 cm arena was used with a fixed wire mesh cage in front of one of the walls of the arena. The test was

performed in two stages: in the first stage, experimental animals were placed in the corner of the arena facing the wall opposite to an empty wire mesh cage and left exploring the environment for five minutes. After the time elapsed, another male Wistar rat (stimulus animal) was placed inside the wire mesh cage, and the procedure is repeated for five minutes. By the end of the experiment, both experimental and stimulus animals were placed back in their respective home cages. The arena cleaned with ethanol 70% and wiped with dry paper after each session. The time spent interacting with the stimulus animal (i.e. time spent in the interaction zone) and the time spent outside the interaction zone in both the training session and the experimental test were automatically quantified by Ethovision software. The ratio between the time spent in the interaction zone and the total time in the arena were calculated manually, as a measure of the sociability of the animals. During the automatized analysis, one animal from group 1 (adrenalectomy and without RSD) showed no movement on the arena, neither in the presence nor in the absence of the stimulus animal, and thus was excluded from the statistical analysis.

[^{11}C]-PBR28 positron emission tomography (PET) imaging

[^{11}C]-PBR28 scans were acquired with a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA), while the heart rate and blood oxygen levels of the animals were constantly monitored. Before tracer injection, anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane. After induction, a tail cannula was inserted in the lateral tail vein of the animal and held in place until tracer injection. [^{11}C]-PBR28 was then injected as a bolus and the animal was allowed to wake up and left to rest for 30 minutes until the second induction of anesthesia was done. Before data acquisition, a transmission scan was performed for 515 seconds with a Co-57 source for correction of attenuation and scatter. A 30-minute emission scan was started 45 minutes after tracer injection. Images were reconstructed (OSEM2D, 4 iterations and 16 subsets) after correction for attenuation, scatter and radioactive decay. Reconstructed images were co-registered automatically to a [^{11}C] PBR-28 rat brain template and volumes of interest (VOI's) were generated by applying a template using PMOD software (PMOD Technologies LLC, Switzerland). The VOI analyzed were: amygdala; cerebellum; corpus callosum; entorhinal cortex; insular cortex; prefrontal cortex; hippocampus; brainstem; hypothalamus; striatum. The standard uptake value (SUV) was calculated for each brain region by correcting the average radioactivity concentration (in kBq/cc) in each VOI for the injected dose and the body weight of the animals.

Immunohistochemistry for Iba-1

Animals were terminated immediately after the PET scan by transcardial perfusion with PBS. Then the brains collected and stored in 4% paraformaldehyde at 4 °C. Three days before being analyzed, brains were placed in a solution of PBS with 30% sucrose. Brain tissue was cut sagittally and frozen in a sagittal position in a cryostat at -50 °C. Frozen slices of 30µm thick were cut and stored at -80 °C until further analysis.

For Iba-1 staining, slides were left at room temperature for 30 minutes, before being incubated with 0.3% hydrogen peroxide for 30 minutes. After washing with PBS, slides were incubated in 5% goat serum in PBS^+ (PBS with 0.3% Triton X-100) for 30 minutes and then incubated overnight at 4 °C with Iba-1 antibody in PBS^+ with 1% normal goat serum (goat anti-mouse, 1:1000). Slides were washed with PBS and incubated with the secondary antibody (IgG, 1:400) for one hour. Slides were incubated for 30 minutes with Avidin-Biotin Complex (ABC – Vector Labs, USA) and stained using 3,3'-diaminobenzidine (DAB – Vector Labs, USA) for one minute. The slides were then washed with PBS, mounted and digitalized using a slide scanner (Hamamatsu, Japan). For image analysis, each slide was transformed into grey-scale image and circular areas of interest were drawn on the frontal cortex, hippocampus, and hypothalamus, based on coordinates from Paxinos and Watson rat brain atlas. From these ROI's, the optical densities for each specific area were calculated and corrected for background staining. The average of all circular areas was calculated for each region of interest using Fiji software [26,27].

Statistical analysis

Behavioral and PET data were analyzed using a multifactorial generalized linear model (GLM), with surgery (adrenalectomy or sham-surgery) and social defeat (RSD or control) as factors. A repeated-measure GLM was performed with time as a within-subject factor for the SPT analysis. The main effects of RSD and ADX were evaluated, as well as the interaction between both factors and time, whenever needed. For all tests, $p<0.05$ was used as the threshold of statistical significance. SPSS 23 (IBM, United States) was used for statistical analysis.

Results

No effect of social defeat or adrenalectomy on anxiety

Anxiety was measured by the percentage of time the animal spent in the center of the arena of the OF test. There was no main effect of adrenalectomy or RSD on the time the animal spends in the center and neither was there any interaction between factors (RSD vs. Control; p=0.67; ADX vs. Sham, p=0.65; Interaction, p=0.92 – Figure 1). Additionally, there is no effect of both factors on the distance traveled by the animal (RSD vs. Control; p=0.89; ADX vs. Sham, p=0.91; Interaction, p=0.97 – Figure 1).

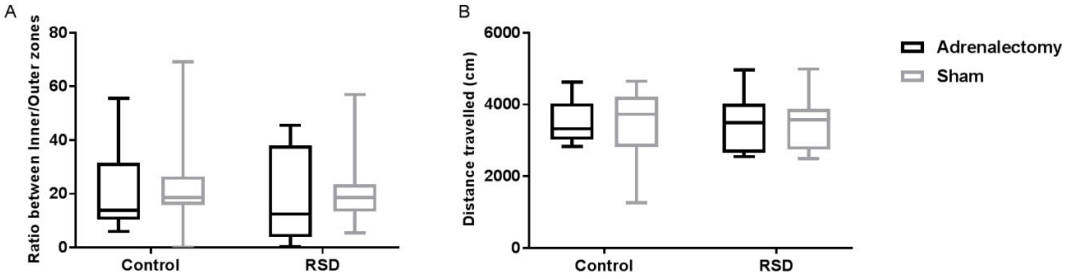

Figure 1: No effect of surgery or RSD on the percentage of time spent in the center of the arena (A) or the total distance traveled (B). Boxes show the mean ± interquartile range; whiskers represent minimum and maximum values. N=9-10 animals per group.

No effect of social defeat or surgery on anhedonia

Anhedonia was measured by the sucrose preference of the animals, which is defined as the percentage of intake of drinking water supplemented with sucrose compared to the total intake of drinking water. There was no significant effect of ADX or RSD on the percentage of drinking water with sucrose consumed, neither was there any significant interaction between factors (RSD vs. Control; p=0.82; ADX vs. Sham, p=0.06; Interaction, p=0.285). Additionally, no significant effect of time in the preference for water with sucrose was observed (Time, p=0.891; time and RSD vs Control, p=0.974; time and ADX vs Sham, p=0.427; time and interaction between surgery and social defeat, p=0.881 – Figure 2).

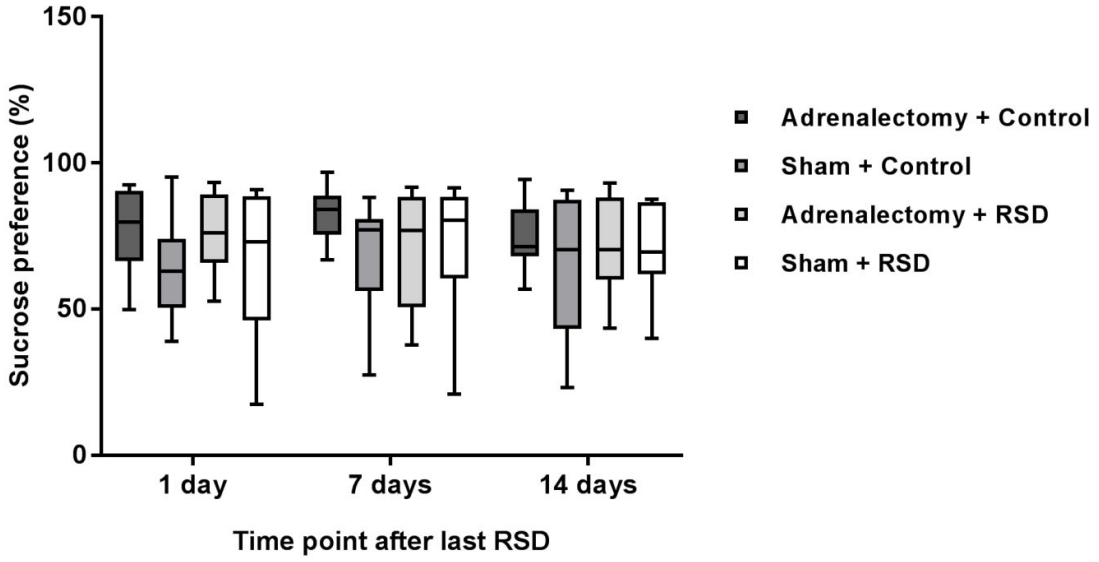

Figure 2: No effect of ADX, RSD, or time of sucrose preference in SPT 1, 7 and 14 days after RSD. Boxes represent the mean ± interquartile range; whiskers represent minimum and maximum values. N=9-10 animals per group.

Effect of surgery and social defeat on animal sociability

Social interaction was measured by the ratio of the time the animal spends interacting with the stimulus animal and the total time in the arena (figure 3). There was a marginal but significant effect of ADX ($F_{(1,34)}$=4.183; p=0.049), RSD ($F_{(1,34)}$=4.672; p=0.038), and a significant interaction between factors ($F_{(1,34)}$=4.456; p=0.042). Bonferroni correction for multiple comparisons shows that animals that were submitted to a sham-surgery and to the RSD protocol had a significantly lower interaction to the stimulus animal when compared to the other groups (Control + ADX, p=0.032; Control + Sham, p=0.023; ADX + RSD, p=0.035).

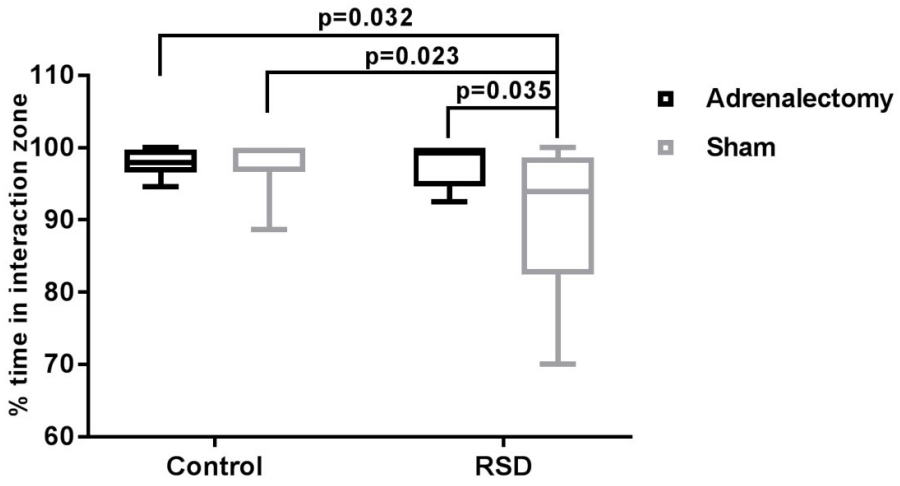

Figure 3: Effect of ADX and RSD on the time spent interacting with stimulus animal. Boxes represent the mean ± interquartile range; whiskers represent minimum and maximum values. N=9-10 animals per group. Numbers over lines indicate significant p-value when comparing Sham animals submitted to RSD to each group. N: 9-10 animals per group.

No effect of ADX or RSD on neuroinflammation

Neuroinflammation was measured by the uptake of [^{11}C]PBR28 in different brain regions 14 days after the last social defeat. The results showed no significant main effect of adrenalectomy, social defeat, or the interaction between factors in any of the brain regions assessed (all $p>0.05$ – figure 4). As [^{11}C]PBR28 is not completely specific for activated microglia, Iba-1 immunohistochemistry was performed to confirm the PET imaging results Iba-1 staining also could not reveal any significant difference in microglia density between the groups in the hippocampus, frontal cortex or hypothalamus (Figure 5).

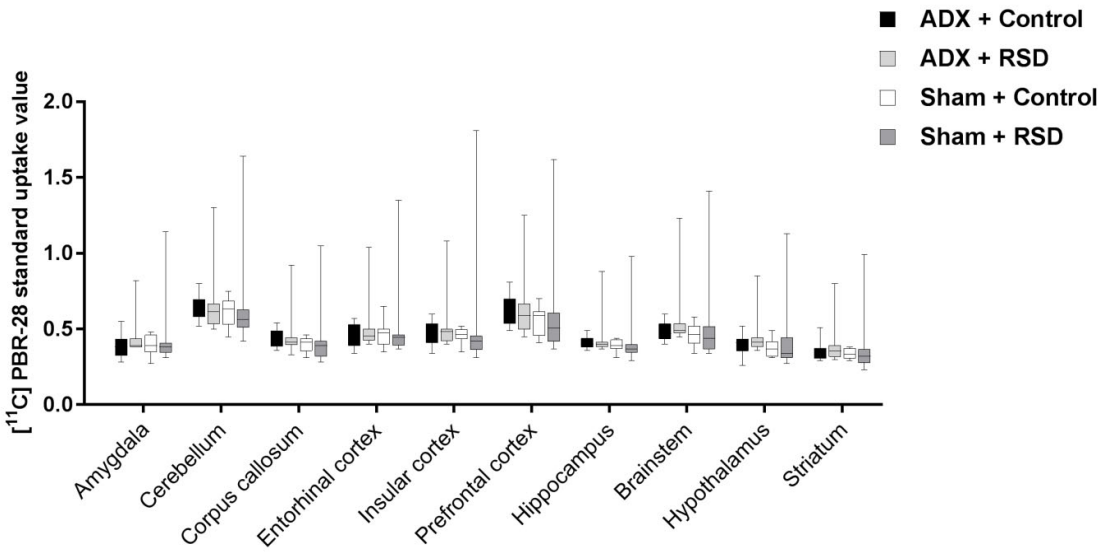

Figure 4: No significant effect of ADX or social defeat on the uptake of [^{11}C]PBR28 in various brain regions. Boxes represent the mean ± interquartile range; whiskers represent minimum and maximum values.

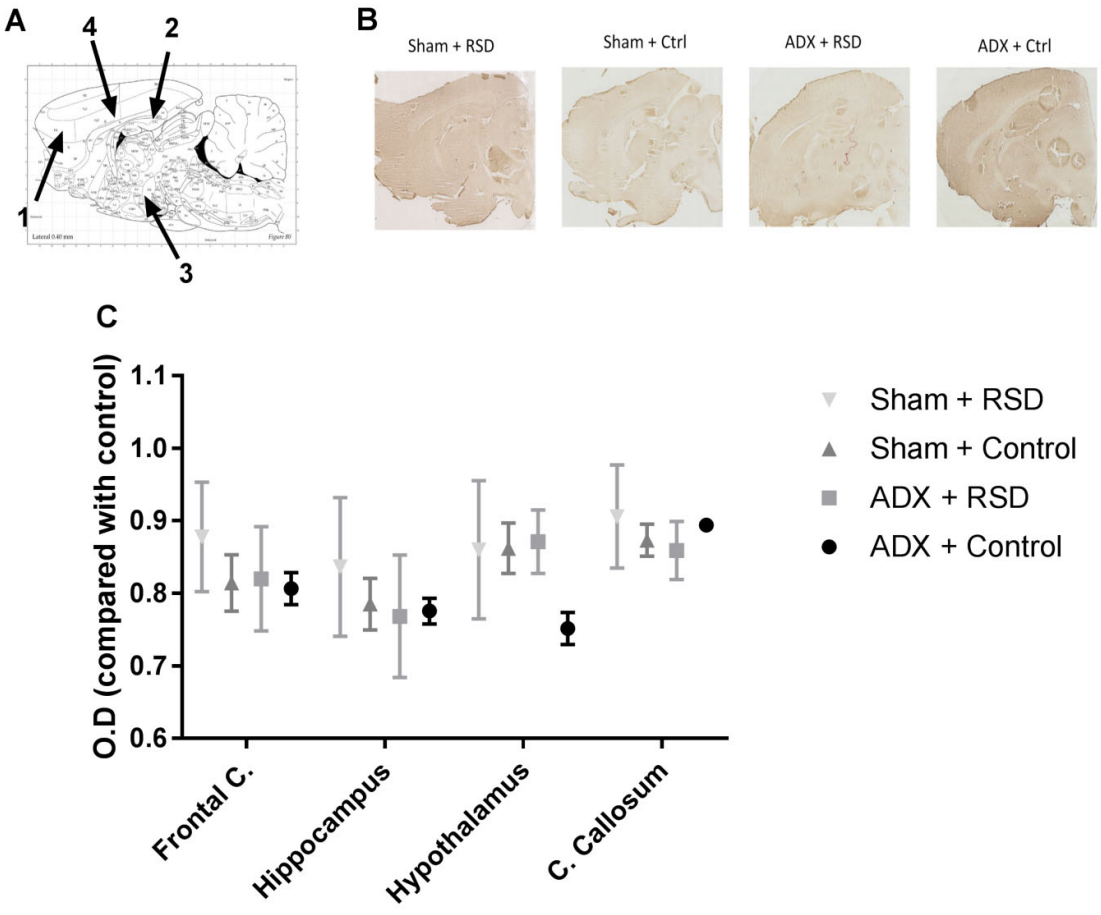

Figure 5: Iba-1 Immunohistochemistry. A) Regions of interest were delineated following the Paxinos atlas for rats; 1: Frontal cortex; 2: Hippocampus; 3: Hypothalamic area; 4: Corpus callosum. B) Low magnification (1.5x) representative

images of each group. C) Optical density of each assessed area after correcting for background and negative control. Data represent the average of 4 random selections at each region of interest. N: 3-4 animals per group.

Discussion

This study aimed to evaluate how disruption of the HPA-axis through corticosterone depletion would affect the sub-chronic stress response inflicted by a 5 days RSD protocol. This study has shown a significant effect of RSD on social interaction of animals 2 days after RSD. This effect was not present anymore when the HPA-axis was disrupted by adrenalectomy before RSD. In contrast, no effects of RSD or adrenalectomy on anxiety, anhedonia, or neuroinflammation outcomes were observed on the time points of assessment.

Altered social behavior in RSD animals is normalized by ADX

The main effect seen in this study was the effect of RSD and ADX on the social behavior of rats. There was a significant decrease in the time spent in the interaction zone together with a stimulus animal for Sham-surgery animals submitted to RSD when compared to all other groups. This result supports the idea that RSD inflicts a fear response towards a completely novel – and thus unfamiliar – individual [28–31]. Interestingly, adrenalectomized animals submitted to RSD did not show alteration in social behavior when compared with their controls for social defeat. The effect of stress on HPA-axis physiology and consequently behavior is already reported in the literature [15,32,33]. Previous research from our laboratory has shown a significant increase in corticosterone levels induced by RSD [24]. Thus, by inhibiting corticosterone response through ADX, the stress response is impaired, making the animal less reactive to stressors, such as a novel environment and novel animal interactions. One study has shown that ADX animals were less responsive to novel environments when compared to controls. In this study, ADX animals showed a lower heart rate in the presence of an unfamiliar animal, and a decreased interaction with other animals in the social interaction test, as compared to controls. [34] In addition, the study showed that ADX animals - unlike control animals – did not show an increase in heart rate in the open arms of the elevated plus maze. Although the authors on the manuscript suggest that this might be an effect of an anxiety-like behavior of ADX, it is also possible that the impaired stress response modulates the reaction towards novel and unusual situations, leading to an anhedonic behavior towards social stimuli. Another study using mice found similar results [35]. Thus, we suggest that fear response is mainly compromised in animals submitted to ADX, which explains the lack of avoidance towards other animals in the social interaction test.

No effect of RSD or ADX in anhedonia

Anhedonia was measured at three different time points: 24 hours, one and two weeks after RSD. There was no effect of time, RSD or ADX on the sucrose preference of the animals. There are studies showing an effect of RSD on anhedonia, especially right after the last RSD [36–38]. Two studies from the same group reported normalization of sucrose consumption in adrenalectomized animals submitted to stress when compared with controls [39,40], suggesting an antidepressant effect due to the decreased production of corticosterone. A plausible explanation for the absence of any effect of RSD and ADX in the SP test in our study could be the fact that in order to maintain basal levels of corticosterone and salt replenishment during the night of the test, the drinking water also included 0.5% NaCl and 0.4% ethanol. This might have masked the sucrose taste, thus making these measurements less reliable. This hypothesis is supported by the relatively low sucrose preference of the animals (around 70% of preference in control animals with sham-surgery). Thus, in order to properly assess anhedonic behavior in this specific case, a shorter SPT protocol with a sucrose solution without additives should be used, which allows the abstinence of corticosterone for a short period of time.

No effect of RSD or ADX in anxiety behavior

Anxiety behavior, measured by the time the animal spent in the center of the open field arena, was not significantly different between groups after the last RSD trial. Although surprising, the results are not entirely unexpected. Reports using the open field test as a measure of anxiety-like behavior in the RSD protocol have shown conflicting results with some studies showing increased anxiety-like behavior [24,41], while others showed no effect whatsoever [31,42]. The effect of adrenalectomy on anxiety behavior is less well studied. Two studies have shown that there was no effect of ADX in the open field measurement of anxiety [43,44]. To the best of our knowledge, this is the first attempt to see the combined effect of RSD and ADX in the open field. Others have attempted to do so, using different behavioral paradigms. Lehmann and colleagues reported a significant effect of ADX in mitigating the stress caused by RSD in the light/dark box test, with adrenalectomized mice with or without submission to RSD showing a similar trend to explore the brighter side of the arena. In contrast, sham animals submitted to RSD preferred the darker side, indicating their unwillingness to explore the brighter and unsafe environment [35]. A potential explanation why no effects of RSD were observed in our study could be that the stressor in our study was too weak to evoke a measurable effect in the OF test, or that the test was too insensitive to detect the effect. In this respect, the light/dark box could be a good alternative.

No effect of RSD or ADX in neuroinflammation

This is the first attempt of *in vivo* PET imaging to assess microglial activation in adrenalectomized animals. In this study, [^{11}C]PBR28 PET could not detect any difference in microglial activation between any of the groups two weeks after the end of the RSD protocol. The results found by the [^{11}C]PBR28 were confirmed by the immunostaining of Iba-1 in different brain regions. There was no observable difference in the number of Iba1-positive cells between groups in three different regions of interest (frontal cortex; hippocampus and hypothalamus). It is worth noting that, in our findings, there was a large variance within the defeated animals, which might suggest that some animals were more susceptible to develop the negative effects of RSD than others within the same group. Studies focusing on these variances and with a larger sample size might be able to define better the effect size of RSD on social interaction of animals.

A likely cause for the lack of neuroinflammation observed by PET is the timing of the scan. The PET imaging of this study was performed two weeks after the last RSD protocol, thus the inflammatory process involving microglial activation might have resolved, and resolution of inflammation (astrocyte activation) is already underway [47,48]. This would imply that the effect of RSD was not strong enough to cause inflammation that lasts long for 2 weeks. The best timepoint to assess neuroinflammation is a matter of debate, as it is influenced by the type of cells that are assessed. Some cell types are more or less active during a certain period of time than others [48]. In this case, PET imaging at an earlier time point might have been better to show the influence that RSD has on microgliosis. One study from our laboratory has shown a significant effect of RSD at more acute stages of the stressor (i.e.: six days after last RSD trial) [24]. Increasing the number of RSD trials, or using a priming effect – e.g. by performing an additional RSD trial at a time point closer to the PET scan – might be additional options to enhance the stress-induced inflammatory response.

The main limitation of this study is the high variance of animals submitted to RSD, suggesting that some animals might be more affected by the protocol while others show no effect whatsoever. Thus, we suggest that increasing the sample size could largely benefit TSPO imaging in this specific model, especially in order to assess if the resilience/susceptibility hypothesis is valid.

Conclusion

Sham animals submitted to RSD had lower time interacting with a stimulus animal in the social interaction test, which was not found in adrenalectomized animals. This result shows that HPA-axis signaling disruption is able to counterbalance the impairment on the social behavior of these animals.

In contrast, no effects of ADX or RSD were observed in other behavioral paradigms, which could be due to the sensitivity of these tests and the relatively mild nature of the stressor. Neuroinflammation was also not observed two weeks after the RSD, suggesting that the inflammatory response of microglial cells is too mild to be detected by PET or Iba-1 staining or that neuroinflammation was only transient and already resolved at the time of measurement. Thus, future studies might consider different timepoints and RSD protocols (or other stressors) in order to evaluate the effect of stress in ADX animals by PET imaging.

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Chapter 5

Short- but no long-term effect of social stress in rodent depressive-like behavior is not affected by chronic treatment with Harmine

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Abstract

Introduction: Depression is characterized by behavioral, cognitive and physiological changes, imposing a major burden to the overall wellbeing of the patient. Some evidence indicates that social stress, changes in growth factors, like brain-derived neurotrophic factor (BDNF) and neuroinflammation, are involved in the development and progression of the disease, suggesting a potential role for anti-inflammatory drugs. The monoamine oxidase A inhibitor drug harmine was suggested to have both antidepressant and anti-inflammatory properties and may, therefore, be a potential candidate for treatment of depression. **Aim:** The goal of this study was to assess the effects of harmine on depressive-like behavior, brain BDNF levels and microglial activation in a rat model of social stress. **Material and methods:** Rats were submitted to 5 consecutive days of repeated social defeat (RSD) or control conditions. Animals were treated daily with harmine (15mg/kg) or vehicle from day 3 until the end of the experiment. To assess the effects of RSD and harmine treatment on behavior, the sucrose preference test (SPT) was performed on days -1, 6 and 15, the open field test (OFT) on days 6 and 14 and the novel object recognition test (NOR) on day 16. Brain microgliosis was assessed using [^{11}C] PBR-28 PET on day 17. Animals were terminated on day 17 and BDNF protein concentrations in the hippocampus and frontal cortex were analyzed using ELISA. **Results:** Both RSD and harmine treatment caused a significant reduction in bodyweight gain. The OFT and SPT showed that RSD significantly increased respectively anxiety and anhedonia related parameters on day 6, but these effects were not observed anymore on day 14/15. Harmine treatment induced anhedonia in the SPT on day 6 and significantly reduced the mobility and exploratory behavior of the animals in the OFT. PET imaging and the NOR test did not show any significant effects on microglia activation and memory, respectively. BDNF protein concentrations in the hippocampus and frontal cortex were not altered by either RSD or harmine treatment. **Discussion:** The main finding of the study was the short- but not long-term effect of RSD on anxiety and depressive-like behavior, and the long-term effect of harmine on the general locomotion and weight of the animals. RSD stress was not strong enough to induce a long-term effect on the behavior of the animals. Additionally, there was no difference between groups in the [^{11}C] PBR28 uptake and BDNF protein concentration. Thus, the long-term effect of treatment with harmine could not be properly evaluated in this model, and therefore, stronger stressful stimuli are needed in order to draw further conclusion on the effectiveness of harmine as an antidepressant and anti-inflammatory drug.

Keywords: Major depressive disorder; neuroinflammation; harmine; monoamine oxidase inhibitors; behavior; PET imaging

Introduction

Major depressive disorder (MDD) is a psychiatric disorder that affects the daily life of millions of people and poses a burden to healthcare systems worldwide [1]. Depression is mainly characterized by the loss of willingness to perform activities, sleeping and eating problems, sadness and social isolation. Clinical and preclinical research indicates that decreased neurotransmitter and growth factor activation, microgliosis and astrocytosis are involved in the pathogenesis of depression [2–4]. Neuroinflammation was suggested to play a major role in stress response to internal and external challenges, and increased inflammatory markers have been reported in MDD patients [5,6], leading to the hypothesis of neuroinflammation-derived depression. Although the mechanisms are not completely understood, it is possible that brain inflammation may be caused by severe or prolonged stressful events and in turn, cause some of the symptoms associated with MDD.

The involvement of neuroinflammation in depression was corroborated by the results of imaging studies. Positron emission tomography (PET) using [^{11}C]PBR28 or [^{11}C]PK11195 as a microgliosis marker has been used to assess neuroinflammatory processes throughout the brain in a non-invasive manner [7]. Both tracers bind to the mitochondrial receptor 18kDa translocator protein (TSPO), which is expressed mainly in endothelial cells, glial cells and astrocytes [8]. Although the baseline expression of TSPO in these cells is low, there is a strong increase in the expression of this protein when an inflammatory challenge to the brain occurs [9–11]. PET studies showed increased TSPO binding in the brain of MDD patients [5,12], although another study failed to show any changes [13]. A few preclinical studies in animal models of depression have also shown increased binding of TSPO radiotracers [14,15]. These results suggest that neuroinflammatory processes may be associated with depressive behavior, which opens the possibility for the use of anti-inflammatory drugs as therapeutic candidates for mitigation of MDD symptomatology, either as monotherapy or in combination with conventional antidepressants.

Monoamine oxidase-A (MAO-A) inhibitors have been used as a treatment for MDD and other mood disorders for a long time. In the brain, the main function of MAO-A is the degradation of neurotransmitters, such as serotonin (5-HT), dopamine and norepinephrine, and blocking their release into the synaptic cleft [16]. Like many other interventions used for depression, however, there is a large variability of treatment efficacy of MAO-A inhibitors, with a large percentage of MDD patients showing partial or no remission of symptomatology [17]. Harmine is a β-carboline alkaloid derived from *B. caapi* (Malpighiaceae) found mainly in the Amazon rainforest of South America. Its main mechanism of action is through reversible inhibition of MAO-A [18]. Harmine may be an interesting candidate drug as it shows not only antidepressant [19–21], but also anti-inflammatory properties [22,23].

The goal of this study is to assess the anti-inflammatory and antidepressant effects of the MAO-A inhibitor harmine in rats submitted to repeated social defeat (RSD). RSD is considered a model of MDD for its ability to emulate psychosocial stressors of human depression in an animal model by using territoriality and hierarchical status as motivators. To verify the effect of RSD and harmine treatment on the animals, anhedonia, explorative behavior, anxiety, and memory were measured with the sucrose preference test (SPT), the open field test (OFT) and the novel object recognition test (NOR) respectively. [^{11}C]PBR28 PET of the brain was performed to assess stress-induced neuroinflammation in various brain regions and the modulating effect of harmine thereon. To further understand the effect of harmine on the brain, we also assessed the concentration of brain-derived neurotrophic factor (BDNF) – a protein associated with neuronal survival and maintenance. BDNF was shown to be decreased in MDD patients [24,25] and animal models of depression [26,27] and models of neuroinflammation [28,29]. BDNF concentration was quantified *ex vivo* in the frontal cortex and hippocampus – two of the main brain structures associated with the cognitive outcome of depressive-like behavior.

Material and Methods

Animals and drug

The study protocol complied to European Directive 2010/63/EU and the Law on Animal Experiments of The Netherlands; it was approved by the Central Committee on Animal Experiments of The Netherlands (The Hague, license no. AVD1050020171706) and the Institutional Animal Care and Use Committee of the University of Groningen (IvD 171706-01-006). Male Wistar rats (HsdCpb:WU, 8 weeks old – Envigo, The Netherlands) were housed individually at the Central Animal Facility (CDP) of the University Medical Center Groningen (UMCG). Prior to the experiments animals were habituated to the facility for at least 7 days. Animals were maintained in rooms with controlled temperature (21±2 °C) and humidity in a 12/12 hours cycle (lights off at 08:00 P.M.), with food and water provided *ad libitum*. After acclimatization, animals were randomly divided according to harmine treatment (harmine or vehicle: n=10 per group) and social defeat protocol (RSD or control: n=10 per group). Harmine hydrochloride (Santa Cruz biotechnology; sc-295136B) was diluted in saline water to the desired concentration of 15 mg/kg in a volume of 1ml. The solution was then heated up to 50 °C and stirred with ultrasound until it became a clear solution (about 10 minutes). When injected in the animals, the solution was at room temperature.

Study design

A summary of the experiment is presented in figure 1. Five days before the beginning of the RSD animals were daily trained for the SPT for 1h (SPT – training). The first SPT (day 0) was performed on the night before the first day of RSD. Animals were then submitted to the RSD protocol daily for 5 days. The second SPT and the first OFT were performed one day after the last RSD trial (day 6). On the third day of RSD, harmine or vehicle administration was started, which lasted until the end of the experimental phase (day 3-17). Nine days after RSD (day 14), animals were submitted to the second OFT. On day 15, the third SPT test and the training for the NOR test were performed. On day 16, the NOR test was done. and finally, on day 17, a [^{11}C]PBR28 PET scan was acquired before termination of the animals and collection of brain tissue for further analysis. Animals were weighed daily from day 1 to 17, always before the drug administration.

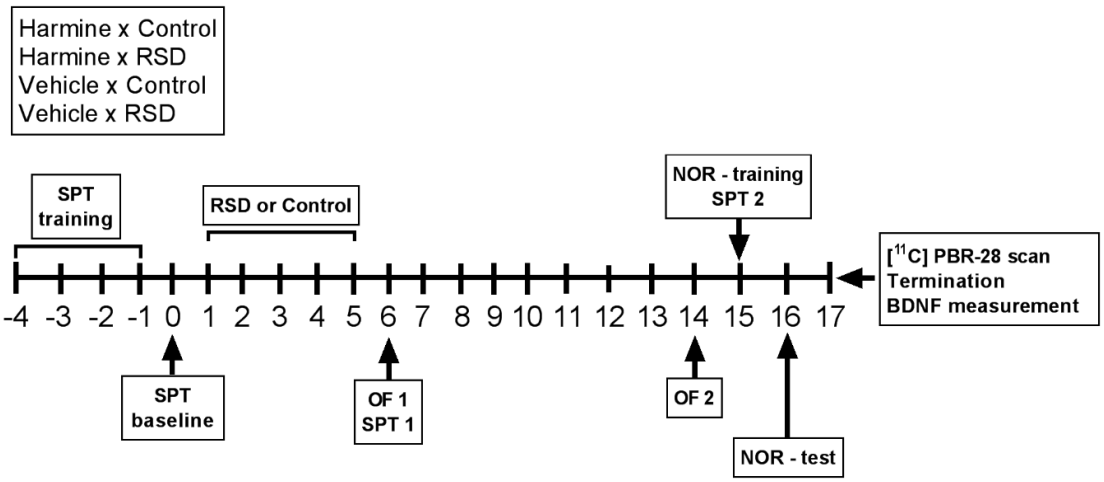

Figure 1: Design of the study. Abbreviations: SPT: Sucrose Preference Test; RSD: Repeated Social Defeat; OF: Open Field test; NOR: Novel Object Recognition

Repeated social defeat

For the social defeat, 12-weeks old male Long-Evans (residents: HsdBlu:LE – Harlan, The Netherlands; n=6; weight: 450 to 500 grams at the beginning of RSD protocol) were paired with females of the same age in a large wooden cage (80x50x40 cm) with a plastic lid. This setup allowed the resident to develop territorial behavior over a large area. The Long-Evans rats were submitted to a training social defeat protocol to allow for the selection of their aggressiveness prior to the beginning of the first RSD protocol. Animals that showed an attack latency (i.e.: time to initiate the first attack) of 60 seconds or less and no signs of violent behavior (i.e.: attack latency of fewer than three seconds without threatening behavior before the first attack) during the five days of training were selected for

the study. Long-Evans rats that showed non-aggressive or over-aggressive behavior were excluded from the study.

One hour before the beginning of RSD, the female rats were removed from the resident cage. Then the experimental animals (intruders, Wistar rats) were placed in the resident cage to begin the defeat protocol. Attack latency and submission time (i.e.: time the intruder takes to show a submissive posture for at least three seconds) were measured. After the intruder displayed a submissive posture, it was placed in a wire mesh cage (40x20x20 cm) inside the resident cage for 60 minutes. By placing the intruder in a wire mesh, there is no physical contact between intruder and resident anymore, but the intruder is still aware of the presence of the aggressive resident. After 60 minutes, the intruder is removed from the RSD cage and placed back to its home cage, and the female is placed back in the resident cage. Control animals were placed in a large, plastic cage for 10 minutes without resident and subsequently in the wire mesh cage for 60 minutes. Then the animals were placed back to their home cages. This protocol was repeated on five consecutive days, and the intruder was always introduced to a different resident.

Drug administration

From day 3 to 17, defeated and controls submitted to a daily intraperitoneal injection of either harmine (15mg/kg) or vehicle solution. Harmine caused slight tremors in the animals one minute after injection, as was previously described in the literature [30]. In our study, the effect lasted for 45-60 minutes, and the behavior of the animals returned to normal after this period. To avoid the motor effects on the RSD or behavioral parameters, all interventions were performed at least one hour after the injections.

Sucrose preference test (SPT)

Animals were habituated to the SPT protocol by replacing their water bottle for a bottle containing 1% sucrose for 1h on 4 consecutive days. For the test SPT, 2 identical bottles– one containing drinking water and the other containing 1% sucrose solution – were placed in the cage of the rat and left overnight (placement of bottles at 03:00-04:00 P.M.). The next day, the bottles were removed (at 10:00 A.M.) and weighed to estimate the amount of fluid consumed by the animal. The percentage of sucrose intake was calculated from the weight difference of the 1% sucrose bottle divided by the sum of the weight differences of both bottles.

Open field test (OFT)

OFT were performed on day 6 and 14 to observe the acute and delayed effects of RSD and harmine treatment on the animals. To avoid habituation effect, two different arenas were used for the trials. For the first OFT, a round wooden arena of 80 cm diameter was used, whereas a square arena of 50x50 cm^2 was used for the second trial. For both tests, the animal was placed in the room 1h before the experiment and left alone during this period. After 1h, the investigator placed the animal in the arena facing the wall and started recording its exploratory behavior for 6 minutes, after which the animal was placed back into its home cage. The arena was cleaned with ethanol 70% and wiped with dry paper after each test. Analysis of the total distance the animal moved, its velocity, the time spent in the center and in the periphery of the arena, and time moving was performed using Ethovision XT 14.0 software (Noldus, The Netherlands). The number of times the animal explored the environment (rearing), the number of times the animal spent grooming and the time the animal spent immobile (freezing) were measured manually by the investigator.

Novel object recognition test (NOR)

The NOR test was performed in the circular OFT arena. The test was performed on day 15 (training) and 16 (Long-term memory – LTM). For training, two identical objects (A and A' – plastic cylinders) were placed 20 cm from the wall and 20 cm from the center on opposite sides of the field. Thus, the animal had plenty of space to explore the environment and interact with the objects separately. For training, the animals were placed in the arena and allowed to explore the objects. When the animal had explored both objects for 30 seconds, the training protocol was ended, and the animal was returned to its cage. If the animal did not reach the exploration criteria after 8 minutes, the training protocol was also ended, and the animal was returned to its cage.

For the long-term memory test, the animals are placed back into the arena 24 hours after training, but with one object being replaced by an object with a different shape and color (A' replaced by B – piled Lego bricks). The animal was placed on the corner of the arena facing towards the objects and left to explore freely, during which the animal was recorded. After 6 minutes, the animal was retrieved and placed back in its home cage. After each trial, the objects and apparatus are cleaned with 70% Ethanol and wiped dry with paper. Analysis of the time spent exploring objects A and B were analyzed automatically with Ethovision XT 14.0. The recognition index (RI) was defined as the time spent exploring object B divided by the total amount of time exploring both A and B. Animals that explored the objects for less than five seconds were excluded from data analysis.

Positron emission tomography (PET)

[^{11}C]PBR28 was performed on small animal PET scanner (Focus 220, Siemens Medical Solutions, USA) with constant monitoring of the animal's heart rate and blood oxygen levels. Anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane. After anesthesia induction, a cannula was inserted in the lateral tail vein for tracer injection. [^{11}C]PBR28 (49.62 ± 3.33 MBq) was injected as a bolus and the animal was placed in their home cage for 30 minutes. Then the animals were anesthetized again and a transmission scan with a Co-57 source was performed for the correction of attenuation and scatter. A 30-minute emission scan was started 45 minutes after tracer injection.

Images were iteratively reconstructed (OSEM2D, 4 iterations and 16 subsets) after correction for attenuation and radioactive decay. The reconstructed PET images were automatically co-registered to a [^{11}C]PBR28 rat brain template using PMOD software (PMOD Technologies LLC, Switzerland). Regions of interest (ROI's) were delineated for the following regions: amygdala, cerebellum, corpus callosum, midbrain, frontal cortex, temporal cortex, dorsal cortex, hippocampus, hypothalamus, brainstem, olfactory nucleus, thalamus, and striatum. The average uptake in the ROI's (in kBq/cc) was corrected for the injected tracer dose and the bodyweight of the animals and expressed as standard uptake value (SUV).

BDNF analysis

After the PET scan, the animals were transcardially perfused with cold phosphate-buffered saline pH 7.4 (PBS) and the brain was removed for tissue extraction. The frontal cortex and hippocampus were excised from the brain, placed in ice-cold PBS solution, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. RIPA buffer (Sigma-Aldrich, R0278 – containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) was added to the brain tissue (50 µl/mg tissue) and cooled on ice. The tissue was pounded until no solid fragments were visible anymore. The homogenized tissue was centrifuged at 12000 rpm for 15 minutes. The supernatant was collected for total protein quantification by the bicinchoninic acid assay (BCA) using bovine serum albumin as a standard. Then, BDNF was measured with ELISA (Cloud-clone, SEA011Ra) according to the manufacturer instructions. Intra-assay precision was <10%. Tissue lysate was diluted 1:5 in PBS (five samples were diluted 1:6 due to a low amount of lysate). Samples were read at 450nm and corrected for the total amount of protein.

Statistical analysis

Analyses were performed using the two-way generalized linear model (GLM) with RSD and harmine treatment as factors. A within-subject factor (time) was added to the SPT analysis. The main effects of RSD and harmine were evaluated, as well as the interaction between both factors and time, whenever needed. For all tests, $p<0.05$ was considered statistically significant. SPSS 23 (IBM, United States) was used for all statistical analyses.

Results

Social defeat and harmine treatment decrease bodyweight gain

Figure 2 depicts the effect of RSD and harmine on bodyweight gain over time. As expected, there was a significant effect of time on bodyweight within animals ($F=11.380$, $p<0.001$). Additionally, there was a significant effect of RSD and harmine treatment on bodyweight gain (RSD: $F=3.275$, $p=0.040$. Harmine: $F=0.192$, $p<0.001$), but no interaction between RSD and harmine treatment ($p>0.05$). RSD induced a significant reduction in bodyweight gain compared to the control group ($F_{(1,25)}=12.123$, $p=0.002$), and also harmine treatment caused a significant reduction in bodyweight gain when compared to vehicle-treated controls ($F_{(1.25)}=28.624$, $p<0.001$).

The significant reduction in bodyweight induced by RSD lasted until day 12 ($p<0.05$), after which the effect of RSD had resolved. Harmine treatment seemed to have a stronger effect on bodyweight, as harmine-treated animals showed a significant difference when compared to vehicle-treated animals until the end of the experiment (day 17 – $p<0.05$ at all time points).

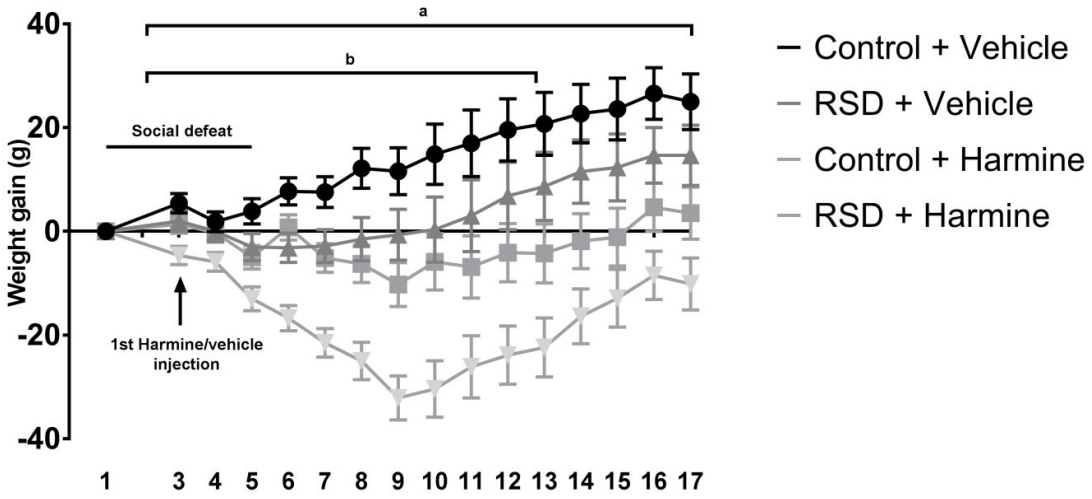

Figure 2: Bodyweight change over time. a: p<0.05 between harmine and vehicle treatment for each time point until day 17. b: p<0.05 between RSD and control for each time point until day 12. Points and whiskers represent mean ± SEM.

RSD affects sucrose intake on a short-, but not long-term

The sucrose preference test was performed on days -1, 6 and 15. There was a main effect of time ($F_{(2,54)}$=17.270, p<0.001) and a main effect of RSD (F=3.797, p=0.036) on SPT. The between-group analysis, however, did not show any significant differences at any time point (p >0.05).

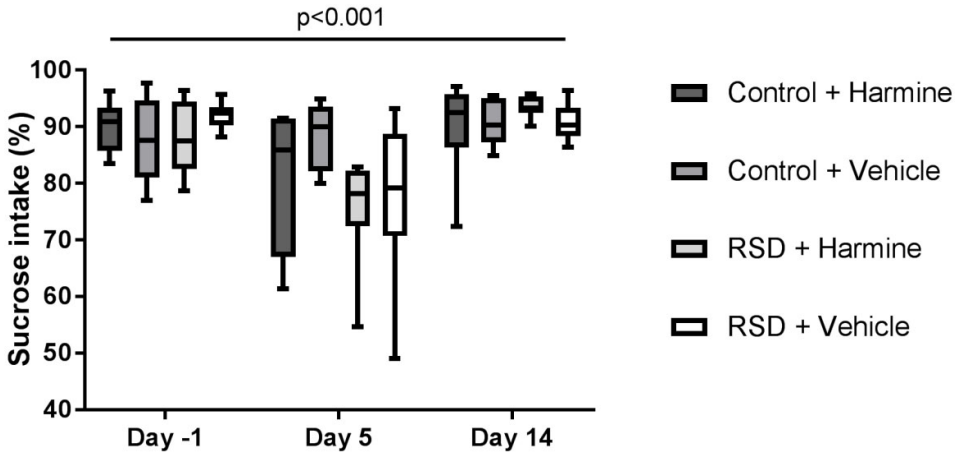

Figure 3: Sucrose preference at different time points. Baseline: 1 day before RSD; SPT 1: 1 day after RSD; SPT 2: 10 days after RSD. Horizontal lines and whiskers represent median ± 95% CI, respectively.

Transient effect of RSD on anxiety-like behavior

The effect of RSD and harmine treatment on anxiety was assessed by the time the animal spent in the center of the arena during the OFT. The OFT showed a significant main effect of RSD on the time

the animal spent in the center of the arena in the OFT performed on day 6 (F=4.747, p=0.038 – Figure 4), with defeated animals spending less time in the center when compared with control ones. However, this effect of RSD was not observed anymore on day 14.

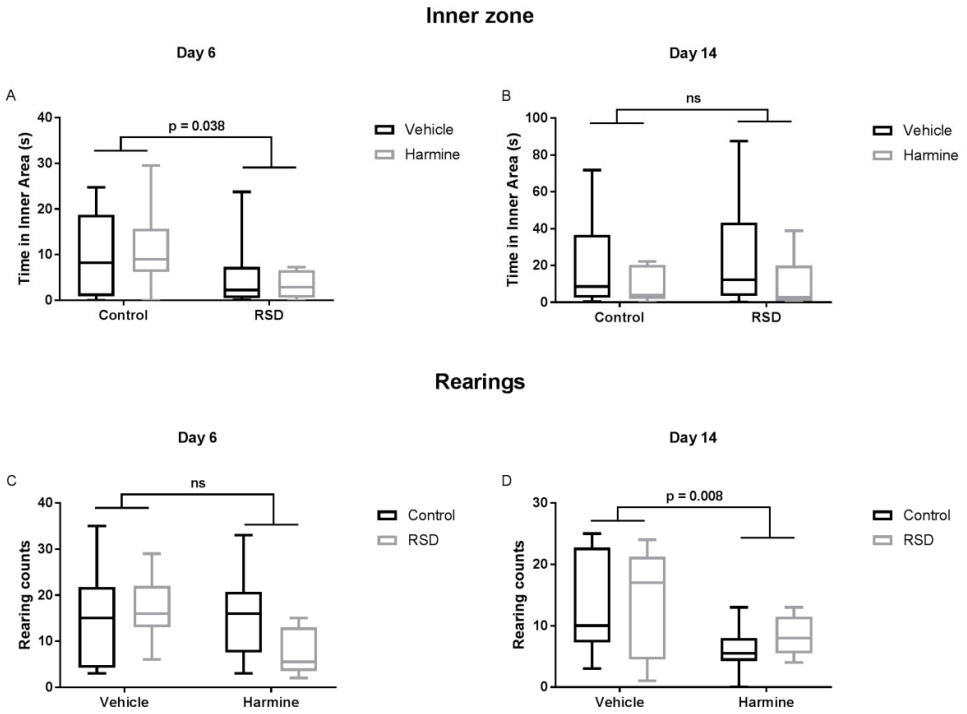

Figure 4: Effect of social defeat on the time spent in the inner zone of the arena (A-B) and the number of rearings (C-D) in the OFT on day 6 (left panel) and day 14 (right panel). Horizontal lines and whiskers indicate median ± 95% CI, respectively; sample size: 7-8.

Long-term effect of harmine on mobility

Harmine treatment significantly reduced the time the animal spent moving in the OFT on day 6 (F=6.356, p=0.018) and day 14 (F=7.283, p=0.012 – Figure 5). Likewise, the total distance moved by harmine treated animals was significantly smaller than the distance traveled by vehicle-treated animals (F=7.283, p=0.012). RSD did not have any effect on mobility neither on day 6 nor on day 14.

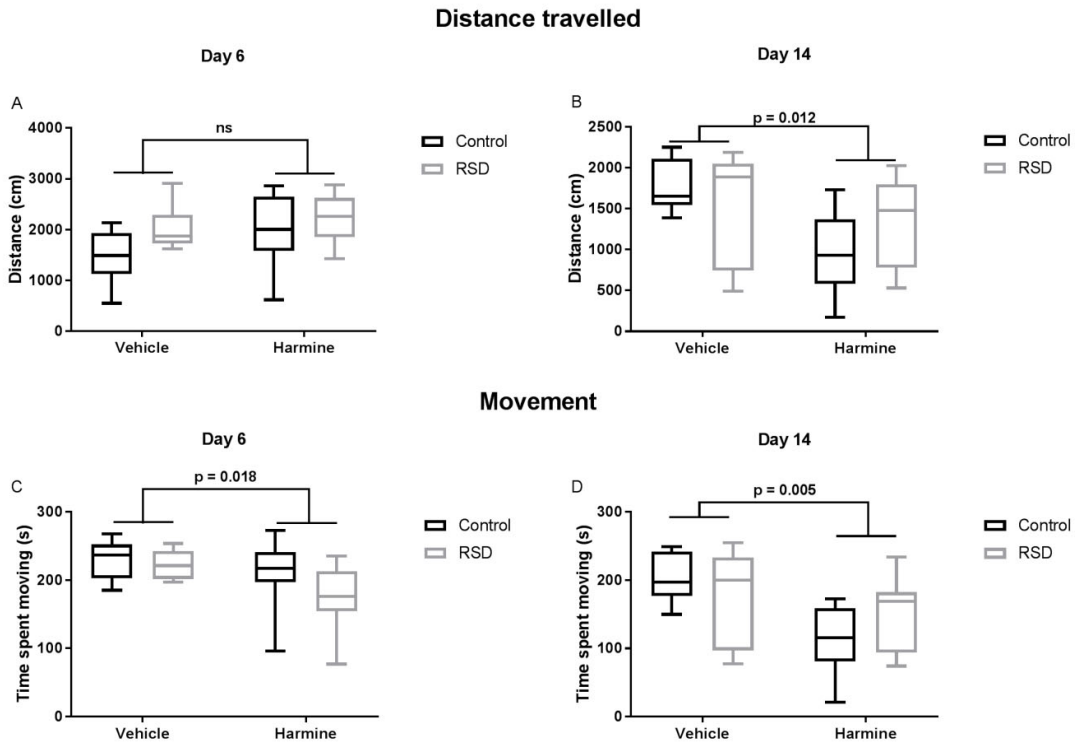

Figure 5: Short- (left panel) and long-term (right panel) effect of harmine treatment on the distance traveled (A-B) and time on movement (C-D). Horizontal lines and whiskers indicate median ± 95% CI, respectively; sample size: 7-8 animals per group.

Additionally, there was a significant effect of harmine treatment on rearing (exploratory behavior). Animals administered with harmine display less frequently a rearing posture ($F=4.475$, $p=0.012$).

No chronic effect of treatments on long-term memory

The NOR test did not show any effect of harmine or RSD on long-term memory, as no significant differences in recognition index between groups were observed ($p>0.05$).

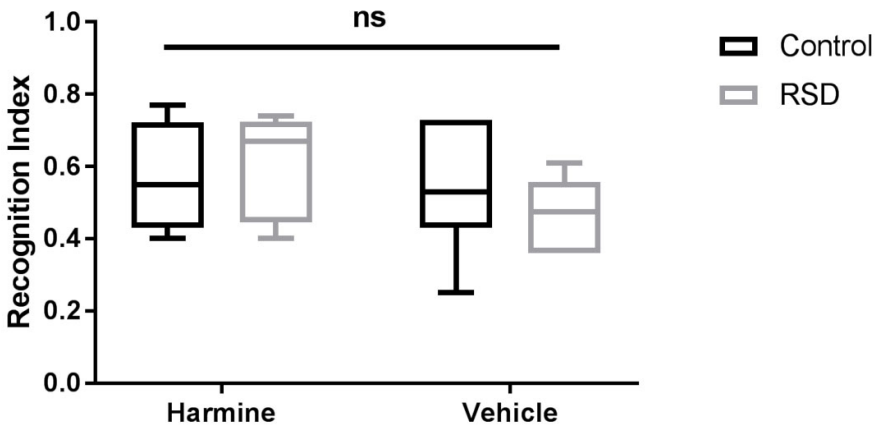

Figure 6: Results of the NOR test on day 16, showing no effect of RSD or harmine treatments on long-term memory. Horizontal lines and whiskers indicate median ± 95% CI, respectively; sample size: 6-7 animals per group.

No chronic effect of treatments on neuroinflammation

For all groups, [^{11}C]PBR28 PET showed the highest tracer uptake in olfactory nucleus, frontal and dorsal cortex and cerebellum. However, stress-induced neuroinflammation could not be detected on day 17, as there was no significant effect of RSD or treatment with harmine on the uptake (SUV) of [^{11}C]PBR28 in any of the brain regions assessed (all $p>0.05$ – figure 7).

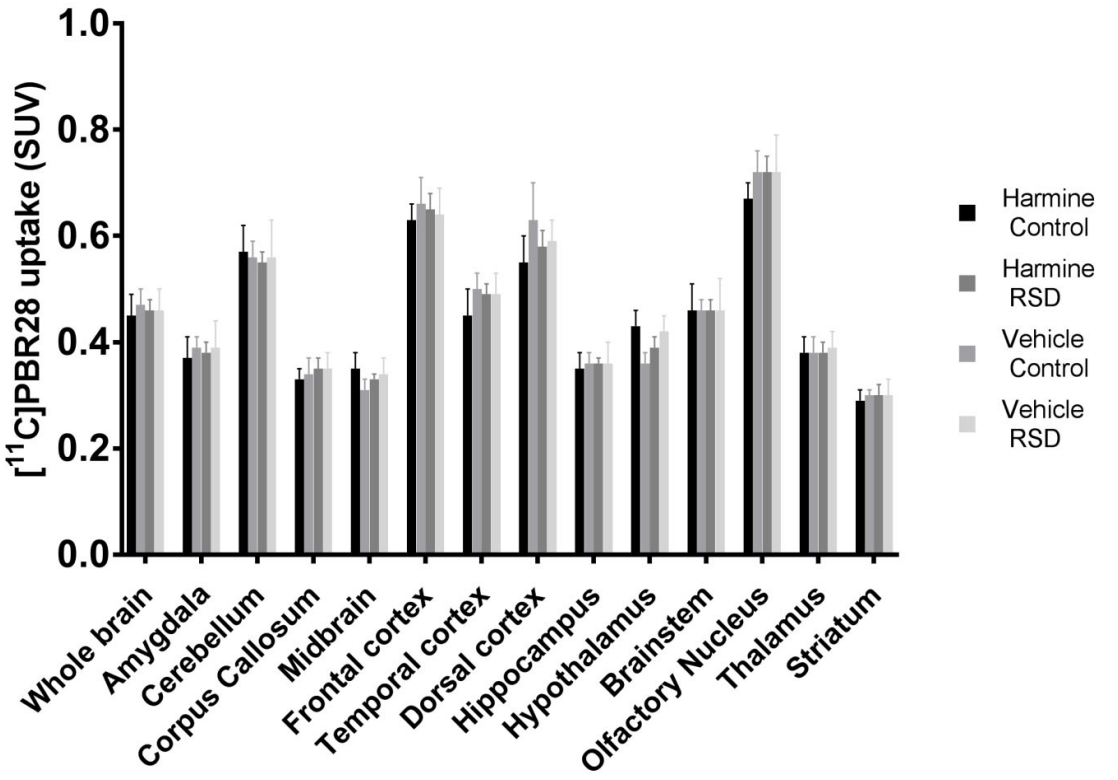

Figure 7: Results of [11C]PBR28 PET, showing no significant effect of RSD or harmine treatment on tracer uptake (SUV) in any brain region of interest. Bars and error bars represent mean and SEM, respectively; sample size: 7-8 animals per group.

No chronic effect of treatments on BDNF concentration

There was no significant main effect of RSD or treatment with harmine on the BDNF concentration in the hippocampus or frontal cortex ($p>0.05$ – figure 8). As BDNF is highly correlated with cognitive parameters, we also assessed if there was a significant relationship between the memory and the concentration of BDNF in either brain region using linear regression.

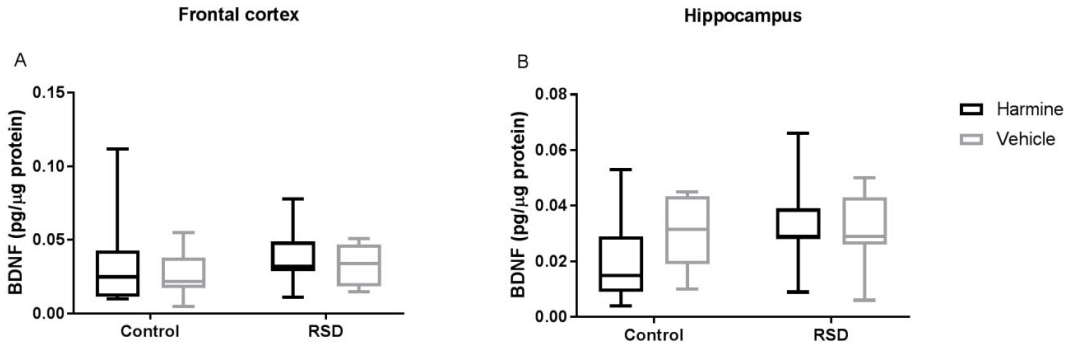

Figure 8: BDNF concentration in the hippocampus and frontal cortex on day 17, showing no significant effects of RSD or harmine treatment. BDNF corrected for total concentration of protein. Horizontal lines and error bars represent mean ± 95% CI, respectively; sample size: 5-8 samples per group

Discussion

This study aimed to assess the antidepressant and anti-inflammatory properties of harmine in rats submitted to RSD. We found that RSD was able to induce a transient depressive-like state in defeated animals. Harmine, on the other hand, negatively affected the bodyweight gain, general movement and explorative behavior of the animals. No delayed effects of both RSD or harmine on the memory performance, BDNF levels or the neuroinflammation were observed, probably due to the transient nature of the five-day RSD protocol.

RSD and harmine reduce bodyweight

Both RSD and harmine treatment caused a reduction in bodyweight. The effect of RSD is in line with previous literature describing that animals submitted to the RSD show a transient weight loss – or a lower weight gain – during the period of such protocol [15,31]. Stress can increase brown adipose tissue thermogenesis and hyperthermia and thus cause a reduction in bodyweight [32]. The effect of harmine treatment on bodyweight might be due to a similar mechanism, as harmine is able to induce adipose tissue thermogenesis by blocking *Ucp1* gene inhibition by chromodomain helicase DNA binding protein 4 (CHD4) [33].

Harmine reduces locomotion

Our findings show that harmine significantly decreased the general movement of the animals, as seen by their immobility time on days 6 and 14. Unlike our study, others have not observed any differences in locomotion after acute or chronic administration of harmine for 12 days [20,34]. However,

another study showed that the harmine analogs, harmane, and norharmane, induced a significant decrease in the distance traveled by the animals, but no differences in anxiety or motor coordination outcomes [35]. Harmine has been suggested as a potential metabolite of harmane [36].

Previous research has shown that acute administration of harmine causes tremorgenic effects on rats [30]. In this study, we also found that harmine administration caused transient tremors, which lasted for approximately 60 minutes (data not shown). Although the tremors were not visible anymore after 1 h, it may have had some lingering effect on the general locomotion. Indeed, one of the main side-effects of monoamine oxidase inhibitors is movement impairment due to increased serotonin neurotransmission (i.e.: serotonin syndrome) [37]. Likewise, one could speculate that the reduced mobility induced by the administration of harmine in our study could be the cumulative effect of the daily treatment on serotonin neurotransmission. However, further investigation of the mechanisms for the effect of harmine on general locomotion is required.

RSD transiently induces anxiety and depressive-like behavior

Animals submitted to RSD showed more anxiety (time spent in the center of the open field arena) and depressive-like behavior (preference of sucrose solution over water) than controls. These results are supported by literature showing that several stressors can induce anxiety and depression-associated parameters in animals [21,38,39] [40,41]. Although our results show an acute increase of anxiety and depression-associated measures in animals submitted to RSD, this effect did not last until 9 days after RSD. This transient effect has previously been observed in various RSD protocols, using different species, number of defeats, RSD duration and evaluation period. Kopschina Feltes and colleagues observed in Wistar rats that the effect of a similar RSD protocol was observed one week after, but was resolved after 90 days [15]. Martin and colleagues used a modified 10-day RSD protocol on C57BL mice and found that the transient effect of RSD had normalized after 18 days [42]. We found that the effect of RSD was already gone 9 days after the last RSD protocol, suggesting that the effect of RSD as a stressor in our study might be lower than expected. Thus, a priming effect (e.g. adding another RSD trial before the behavioral paradigm) might be needed in order to obtain a stronger effect in future studies.

Our study shows that acute administration of harmine was unable to improve the acute depressive-like state of the animals subjected to RSD. Although there are no studies on the therapeutic effect of harmine after social stress, it is known that mid- to long-term administration of harmine improves depressive symptomatology in animals submitted to a chronic unpredictable stress protocol [43]. One study reported that chronic administration of harmine prior to application of chronic unpredictable stress was able to mitigate the stress-induced depressive-like behavior [21]. Another study

investigating the therapeutic effect of chronic harmine administration for 1 week in chronically stressed rats showed similar results, using the preference for sugary food as outcome measure [44]. Both studies showed the effect of harmine after chronic administration of the drug, whilst in our study, the effect of RSD on anhedonia was observed only 1 day after the completion of the RSD protocol. Consequently, the treatment period may have been too short for harmine to become effective. Unfortunately, the anhedonia effect of RSD had already resolved 9 days after RSD and therefore the effect of chronic harmine treatment could not be assessed in our study.

RSD and harmine do not affect long-term memory

There was no significant effect of harmine or RSD on the long-term memory 11 days after RSD. This is similar to what was found previously in our laboratory [15]. Other studies, however, showed that different subtypes of memory are affected by RSD. McKim and colleagues found that a six-days RSD protocol was able to impair spatial memory recall, as assessed with the Morris and Barnes mazes [45]. Wohleb and colleagues found that this effect lasts for up to eight days, suggesting a subchronic effect of RSD [41]. It is worth noting that memory tests can pose stressful environments, the NOR test is considered a substantially less stressful event than the Barnes maze, or Morris water maze; comparison between the tests is therefore difficult.

To the best of our knowledge, this is the first attempt to assess the effects of harmine on memory performance of socially defeated animals. A similar study using chronic unpredictable stress for 40 days also did not show any significant effect of harmine on memory [43]. In our study, however, the effect of the stressor seemed to be transient and on the day of the memory test the effect of the social stressor likely already had resolved. Consequently, no conclusion can be drawn on the protective effect of harmine. However, harmine by itself was unable to modify – positively or negatively – the long-term memory. Further studies are needed with stressors that are able to induce long-term cognitive impairment to assess the effect of harmine administration on stress-induced cognitive impairment.

RSD and harmine do not affect glial activation

[^{11}C]PBR28 PET imaging showed no significant effect of the social stress protocol or harmine treatment on glial activation. It is known that microglial activation starts hours after exposure to the stressor and can last for several days or even weeks, decreasing gradually as the resolution of neuroinflammation begins [46]. Kopschina Feltes and colleagues found a significant effect of RSD protocol on [^{11}C] PK11195 6 days after RSD in several key regions associated with depressive behavior (e.g. medial prefrontal cortex, entorhinal cortex, and insular cortex), but this effect was not observed

anymore 3 weeks after RSD [15]. In our study apparently either the neuroinflammatory process was not severe enough to be shown by PET imaging or the neuroinflammatory response had already resolved 11 days after the last RSD trial. The latter option is in line with the results of the behavioral studies, which also did not elicit long-term changes in behavioral parameters associated with depressive-like behavior. [^{11}C]PBR28 PET also did not show any significant effect of harmine treatment on tracer uptake, neither in controls nor in defeated animals. The latter is most likely due to the lack of an effect of RSD glial activation at the time of the PET scan.

RSD and harmine do not affect BDNF concentration

Reduced levels of BDNF protein in specific regions of the brain have been associated with cognitive impairment [47–51]. Treatment of brain disorders is generally accompanied by an alteration – usually an increase – in BDNF levels [24,52,53]. In this study, there was no effect of RSD or treatment with harmine on BDNF concentration in frontal cortex or hippocampus. The absence of an effect of RSD on BDNF levels might be explained by the transient effect of the stressor used in this study, resulting in a normalization of BDNF levels at the time of assessment. Other studies suggest that assessment at an earlier time point may have shown changes in BDNF concentration [54]. However, an earlier assessment may potentially have obscured the effect of harmine treatment, as treatment with antidepressant drugs often take at least one week to induce behavioral changes.

Conclusion

RSD generated a fluctuation on a short-term (here observed by the behavioral outcome of the first OF and the SPT after RSD), but not a long-term effect, as seen by the lack of difference in uptake of [^{11}C] PBR28 between RSD and control groups, together with unaffected long-term behavioral alterations on anxiety and depressive-like behavior. The changes of RSD or harmine did not alter BDNF concentration in the frontal cortex or hippocampus, regions that are key for stress regulation and further brain homeostasis. With the results reported in this study, it is not possible to infer the effect of harmine as an antidepressant and anti-inflammatory drug, only to conclude that the treatment had no long-lasting harm towards the organism. To better assess the influence of harmine in depression and its effect as an antidepressant, there is a need for further studies using different stressors or longer time RSD protocols in order to induce a chronic stress response in the organism, thus allowing a more efficient analysis of how harmine could act under such conditions.

Acknowledgments

The authors thank the staff of the animal facility (CDP) at the University Medical Center Groningen. Also, the staff responsible for the production, synthesis and quality control of the radiotracer. This paper would not be possible without their contribution.

E. Bromberg has a CNPq research fellowship.

This research was supported by the Stichting de Cock-Hadders grant (grant number: 681717)

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Chapter 6

Dopaminergic receptor D_2 contribution on aggressive behavior: new insights about acute and chronic conditions

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Abstract

Introduction: Aggression is one of the most basic components of the limbic system, especially in social beings. Aggression is associated with the production and release of several neurotransmitters, and one tightly related to it is dopamine (DA). Binding of DA to dopamine receptor type 2 (D_2) receptors was suggested to be involved in pathological aggression in chronically aggressive humans and animals. The aim of this study was to evaluate changes in D_2 receptor availability with [^{11}C]-raclopride PET in a rat model of aggression. **Methodology:** Long-Evans rats from 2 independent studies with a repeated social defeat protocol (RSD) were used and screened for aggression. Controls and aggressive rats were stratified based on their attack latency during the training phase. Aggressive animals were further used as aggressors in a series of repeated social defeat trials. After RSD trials, the 2 cohorts of aggressive animals and unexposed non-aggressive controls were scanned for D_2 receptor availability using binding potential (BP_{ND}), as determined by dynamic [^{11}C]-raclopride PET scans. Caudate and putamen (striatum) and nucleus accumbens (NAc) binding potentials were calculated by kinetic modeling using a reference region (cerebellum). **Results:** Surprisingly, aggressive animals from cohort 1 showed a shorter attack latency in the period immediately before the PET scan, when compared aggressive animals from the second cohort (last trial: 42.84 ± 44.67s vs. 12.41 ± 13.60s; p=0.03). [^{11}C]-raclopride PET showed that these animals had also a significantly higher BP_{ND} in the striatum when compared with cohort 2 of aggressors ($p<0.001$) and the control group ($p<0.001$). However, there were no differences in BP_{ND} in the NAc between any of the groups. **Discussion:** Attack latency throughout several exposures is a parameter used to discriminate the level of aggression in animals. Our results for cohort 1 - but not for cohort 2 - agree with literature suggesting a relation between DA and aggressive behavior. The differences found in the D_2 receptor binding between cohort 1 and 2 of aggressive animals might be related with differences in their level of aggression at the time of the PET scan, which may be due to some experimental differences between the cohorts, such as the total number of RSD trials the aggressors were exposed to (29,4±4,9 vs 14,1±4,8 for cohorts 1 and 2, respectively), the frequency of the RSD trials (1.5 trial/week vs. 0.9 trial/week for cohort 1 and 2, respectively), or the interval between the last RSD trial and the PET scan (1 day and 14 days for cohort 1 and 2, respectively). To exclude the last parameter, the PET scan was repeated 4 weeks after the last RSD trial in cohort 2 (for practical reasons), but results were similar to those of the PET scan performed immediately after the last RSD trial. **Conclusion:** Our results suggest that aggressive behavior is associated with an increase in D_2 receptor availability in striatum, but not in NAc. The discrepancy between the 2 cohorts of aggressive animals in this study seems to be related to the aggressiveness of the animals at the time of the PET. This difference in aggressiveness between the cohorts may be due to overexposure of the animals of cohort 2 to aggressive events, which could have blunted the

rewarding reaction of the animals. Nonetheless, the results suggest a participation of D_2 receptors in aggressive behavior, which might, therefore, be a potential pharmacological target for diseases associated with aggressive events.

Keywords: aggressive behavior; dopamine; D_2 receptor; positron emission tomography; repeated social defeat

Introduction

Aggression is a functional behavior that is affected by a wide variety of experiences [1,2], such as possession of a resource [3], increase the likelihood of gene transmission over generations [4,5], and social dominance. Although aggression is important for the survival of an organism, and *per se* is considered normal behavior, excessive aggression and violence are associated with brain disorders, such as personality disorders, schizophrenia, depression, Alzheimer's disease, brain injury, and others [6–9]. Pathological aggression is the leading cause of all child and adolescent referrals to mental health clinicians [10,11], and therefore a serious concern for society. Aggressive behavior can be classified in two different subtypes: 1) controlled-instrumental, or the aggressive behavior needed in order to achieve a specific goal; and 2) reactive-impulsive, which is mostly driven by emotional behavior (e.g. anger). In humans, aggression can be seen as adaptive behavior, which can develop from reactive and instrumental to appetitive or rewarding aggression [12,13], which activates the reward circuitry in the brain, mimicking the effects of drugs. Indeed, appetitive aggression shows some core similarities with addiction, like the relapse (recidivism) rates between aggressive offenders and drug addicts, and the desire for aggressive-events despite short or long-term harmful consequences [14].

Molecular mechanisms involved in normal and abnormal aggression, including its relationship with dopaminergic (DA) transmission, have been studied in several species. Literature suggests that dopamine release is associated with aggressive behavior [15–17], an idea which is supported mostly by pharmacological studies showing that drugs that modulate dopaminergic transmission are able to contain aggressive-like behavior [18–20]. Additional studies using positron emission tomography (PET) have also shown the relationship between aggression and dopamine synthesis and dopamine receptor D_1 availability [21–26]. PET imaging studies on aggression mainly reflect the chronic disposition of dopamine concentration in patients who have shown recurrent aggressive behavior, with no data regarding the acute effect of an aggressive episode on dopamine levels. Understanding the contribution of the dopaminergic system to aggression can lead to alternative treatments for people suffering from pathologies associated with aggressive behavior. Additionally, understanding how brain dopamine release and binding to its receptors is changed after acute aggressive life events can contribute to better understand how normal aggression becomes pathological, thus helping in the prevention of this kind of antisocial behavior. In this regard, dopamine D_2 receptors are associated with the addiction pathway and can be a possible factor of turning aggressive behavior into a more pathological state [27,28].

Therefore, the aim of this study was to evaluate how dopaminergic D_2 receptors behave in animals showing aggressive behavior using [^{11}C]-raclopride PET imaging. To achieve this, animals were

trained using the repeated social defeat protocol to develop appetitive aggression through repetitive winning confrontations [29] and divided into groups according to their aggression level (aggressors or controls). Both controls and aggressors were scanned for D_2 receptor availability.

Materials and Methods

Subjects

Outbred 16-weeks old male Long Evans (LE) (Harlan, Indianapolis) from 2 studies (6828B and 171706-01-004) undergoing the same repeated social defeat protocol were studied. The animals were divided into 3 groups: Non-aggressive controls (n=6), aggressive animals from study 171706-01-004 (cohort 1, n=13) and aggressive animals from study 6828B (cohort 2, n=12).

One week after arrival, the male LE rats were paired with tubal-ligated LE female rats in a large cage (80x50x40cm), with *ad libitum* access to water and food. Animals were maintained in a controlled room with a 12:12 hour light/dark cycle (lights off at 8 PM), regulated temperature (21±2°C) and humidity controlled environment. Male and female LE rats were paired and housed together at least one week prior to the screening phase of the experiment in order to increase male territoriality. All animal experiments were performed according to the Dutch Law for Animal Welfare and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, under protocols 171706-01-001 and 171706-01-004.

Study design

The study was conducted in 3 different stages (see Figure 1). In the first stage, LE males were trained and screened for aggression during 5 consecutive days. During the screening sessions, females were removed from the cages 1 hour before an intruder was introduced inside the resident's cage. To avoid habituation, each day of aggressive trial consisted of a different intruder being presented to the resident. Time measurements for the latency to attack of the resident and the time of successful submission (i.e. when intruders submitted for more than 3 consecutive seconds) were performed. After intruders submitted, or after 10 minutes if they did not, intruders and residents were separated by a barrier placed inside the resident's cage. Sixty minutes after the introduction, the intruders were removed from the resident's cage.

Residents were classified according to their average attack latency over the 5 consecutive screening sessions as follows: not aggressive (more than 60 seconds), aggressive (between 10 and 60 seconds), and violent (less than 10 seconds, accompanied with violent behavior, i.e. direct attack to

vital zones of the body, no threatening before attacking [29]). Violent animals were excluded. After this selection, aggressive animals were used for the second stage, consisting of several trials of repeated social defeat (RSD) over six months (cohort 1: 14,1±4,8; cohort 2: 29,4±4,9). On the third stage, aggressive animals and controls were submitted to a dynamic [^{11}C]-raclopride PET scan either 1 day (cohort 2) or 2 weeks (cohort 1) after the last exposure to a resident-intruder paradigm. In cohort 2, the PET scan was repeated after 4 weeks to exclude any time interval effects.

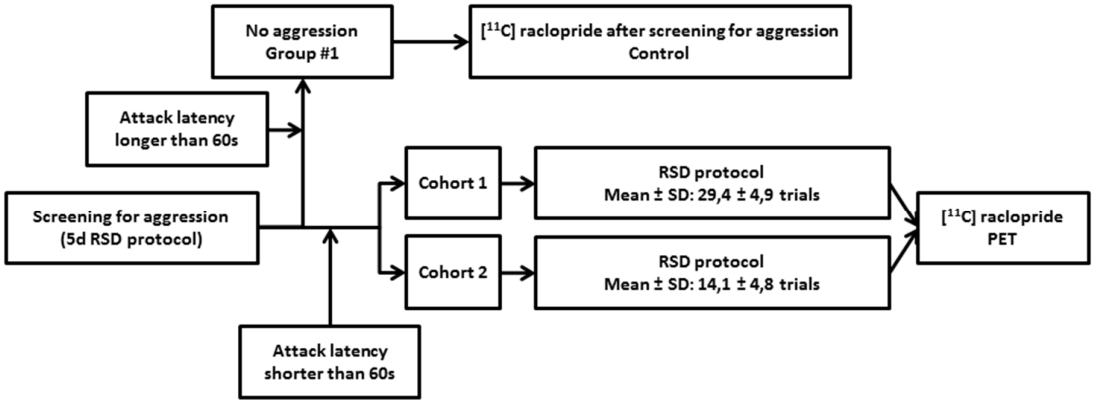

Figure 1: Study development protocol. Abbreviations: RSD: Repeated social defeat; PET: Positron emission tomography imaging.

Repeated social defeat (RSD)

This protocol was performed as described elsewhere [29,30] Wistar rat intruders of smaller size were introduced into the resident's cage, and after the residents explored the intruder and threatened them, latency to attack and latency of submission was measured. The submission was achieved when the intruder assumed a supine (submissive) position for at least 3 seconds. Animals were allowed to fight until the intruder was submitted, or when the maximum time of 10 minutes since the introduction of the Wistar animals was reached. Either after submission or 10 minutes of the encounter, intruders and residents were separated by a physical barrier in the resident cage that allows visual, auditory and olfactory (but not physical) contact until a period of 60 minutes since the first introduction was complete. After completion of the 60 minutes period, animals were returned to their own cages and females placed back into the resident cages. All RSD experiments were performed between 14:00 and 18:00 hrs.

Radiotracer preparation and dynamic PET imaging.

Brain imaging of D_2 receptors was performed using the radiotracer [^{11}C]-raclopride. The synthesis of the radiotracer was performed through alkylation of S-(+)-O-desmethylraclopride (ABX, Radeberg, Germany) using ^{11}C-methyl triflate, as described previously [31]. PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Prior to PET imaging, animals were anesthetized using a mixture of isoflurane and oxygen (5% for induction, 2% for maintenance) and a tail vein cannula was placed for tracer injection. Heating pads were used to maintain body temperature throughout the experiment. Before tracer injection, animals were placed in the PET camera in prone position with their head in the field of view. A transmission scan was performed using a ^{57}Co point source for attenuation and scatter correction. [^{11}C] raclopride was injected (25.75±9.3 MBq) over 1 minute using an automatic injection pump at a speed of 1 mL/min, at the same time the acquisition of the 60 minutes dynamic PET scan was started. During the scan, temperature, heart rate, and blood oxygen saturation were monitored.

Image processing and PET analysis

The 60 min emission list-mode data was used to reconstruct the image in 21 frames (6 x 10s, 4 x 30s, 2 x 60s, 1 x 120s, 1 x 180s, 4 x 300s and 3 x 600s). The frames were iteratively reconstructed using an attenuation-weighted two-dimensional ordered-subset expectation-maximization algorithm (OSEM2; 4 iterations and 16 subsets), and corrected for random coincidences, scatter, decay and attenuation. Final images had a 128 x 128 x 95 matrix with a pixel width of 0.475 mm and slice thickness of 0.796 mm. The PET images were individually co-registered to a [^{11}C]-raclopride brain template [32] (PMOD 3.8; PMOD Technologies LLC, Switzerland) which allowed the use of a predefined volume-of-interest (VOI) map and the reporting of results in Paxinos stereotactic coordinates from the rat brain. After co-registration, time-activity curves (TACs) were generated for nucleus accumbens core and shell (NAc), caudate and putamen (striatum) and cerebellum by applying a predefined mask of VOIs to the dynamic data, as stated elsewhere [32]. Due to limited resolution of the PET scanner used (1.4 mm), only the whole NAc and striatum – and not subsections thereof - have been assessed in order to obtain a better signal-to-noise ratio. The simplified reference tissue model (SRTM) with the cerebellum as a reference region was used to quantify tracer uptake [31,33]. The non-displaceable binding potential (BP_{ND}) was calculated for the chosen areas using the cerebellum as a reference.

Statistical analysis

All reported data are shown as mean ± SEM. Data were analyzed using IBM SPSS 23 software (IBM; Armonk, NY). PET data was analyzed with a one-way analysis of variance (ANOVA) and linear regression was performed to assess effect of the number of trials on attack latency. For all differences, a $p<0.05$ was considered as statistically significant.

Results

Aggressive behavior is altered between groups

Analysis of aggressive behavior by linear regression showed that aggressive animals from cohort 1 had increasingly shorter attack latency until the last trial ($R^2=0.68$, $p<0.001$), with a decrease of 1.7 seconds per exposure. When looking at the first ten trials only, both cohorts show a significant correlation between attack latency and the trial number (Cohort 2: $R^2=0.632$, $p=0.005$; cohort 1: $R^2=0.49$, $p=0.022$ – Figure 3A). However, when observing the remaining trials, the significant correlation is maintained only in cohort 1, although the statistical significance is marginal ($R^2=0.41$, $p=0.046$). In cohort 2, there is no correlation at all between attack latency and trial number for trials 11-38 (figure 3B). Consequently, the average attack latency of the last 10 trials before the PET scan was significantly shorter for cohort 1 than cohort 2 (17.41 ± 3.234 vs. 32.89 ± 4.124; $p=0.008$)

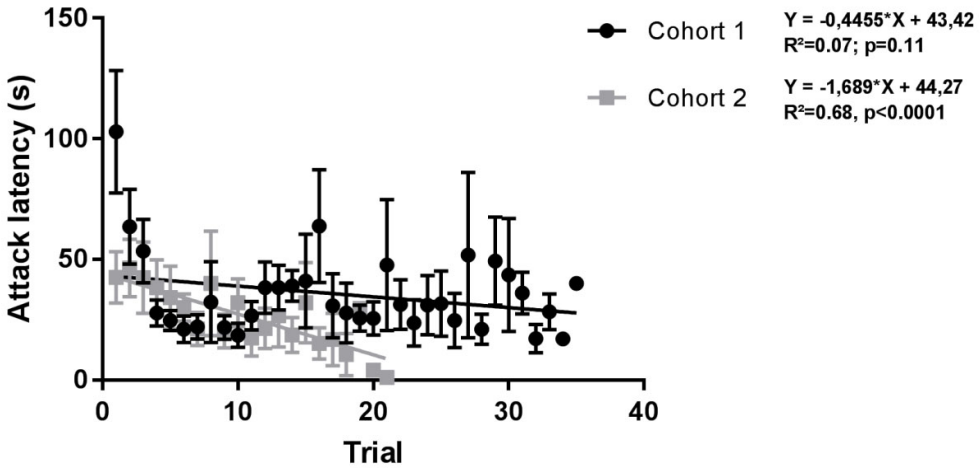

Figure 2: Linear relation of attack latency with trials (time) in both cohorts of animals. Lines represent the linear trend of each group. The equation represents curve slope.

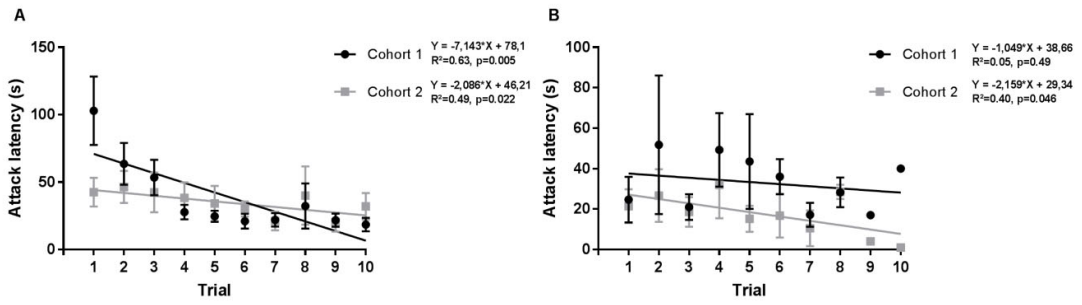

Figure 3: Linear relation of attack latency with the first 10 trials (A) and the remaining trials (B) in both cohorts of aggressive animals. Lines represent the linear trend of each group. Numbers on the X-axis of B are not equivalent to trial numbers. The equation represents curve slope.

Striatal D_2R binding potential is altered by interval from last aggression

[^{11}C]-raclopride PET showed a significant difference in the BP_{ND} in the striatum of aggressive animals from cohort 1 when compared with both controls and aggressive animals for cohort 2 ($F_{(2,25)}$=26.041; p<0.001). There was no significant difference between aggressor animals from cohort 2 and the control group (p>0.05). Repetition of the PET scan in cohort 2 four weeks after the last RSD trial revealed similar [^{11}C]-raclopride binding in striatum as compared to the PET scan made 1 day after the last RSD trial (data not shown).

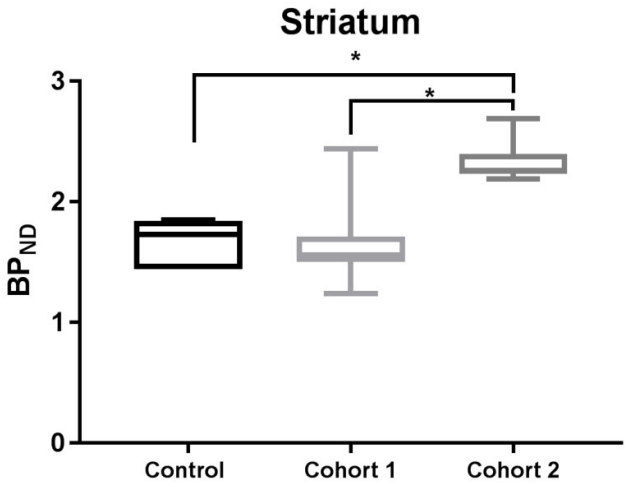

Figure 4: Effect of aggression on [^{11}C]-raclopride binding potential in the striatum. Data expressed as median and interquartile range, whiskers represent the minimum and maximum values. *: p<0.05. N 7-11 per group.

In the nucleus accumbens, there was no significant difference in the binding potential of [^{11}C]-raclopride between groups (all p>0.05). There was also no significant relationship between the uptake of [^{11}C]-raclopride in both brain regions assessed and the average attack latency (all p>0.05).

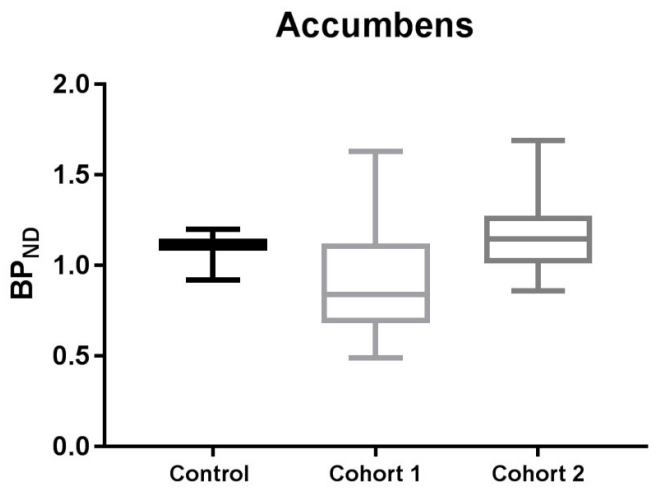

Figure 5: No effect of aggression on the [^{11}C]-raclopride binding potential in the nucleus accumbens. Data expressed as median and interquartile range, whiskers represent the minimum and maximum values. N: 7-11 per group.

Discussion

In the present study, we have shown changes in D_2-receptor availability in the striatum, but not in the nucleus accumbens, in one cohort of aggressive animals, but not in the other cohort. Additionally, the cohort of aggressors with the increased availability of D_2 receptors showed a shorter attack latency than the other cohort of aggressive animals. These results suggest that changes in D_2 receptor availability could be linked to the aggressiveness of the animals. Changes in DA transmission due to aggression are not surprising. Our results are in agreement with literature showing a relation of DA release with aggressive and violent episodes in people with and without a diagnose of brain disorders [34,35], and also in animal models of aggressive behavior [15,36,37]. The observed increase in DA D_2 receptor availability is in line with the findings of Jupp and colleagues, who showed an increase of D_2/D_3 radiotracer binding in striatum using autoradiography. Nevertheless, they also found an increase in the nucleus accumbens, which was not observed in our study [38]. Increases in D_2 radiotracer binding in the accumbens was also observed by other studies in animal models of aggression [39–41]. Lack of differences in D_2 binding on our experiments may be explained by the limited spatial resolution of the PET camera used, thus making assessment of smaller brain regions such as the accumbens challenging. This generates an additional concern as the core and shell of the nucleus accumbens are shown to have distinct functionalities in the limbic system [42]. Future studies that aim to asses D_2 receptors in such small regions should take the spatial resolution of the assessment tool into account when performing data collection, and using other techniques such as immunohistochemistry might complement possible PET findings.

Animals that went through less aggressive bouts (cohort 1) showed an increased [^{11}C]-raclopride BP_{ND}, meaning that less dopamine is reaching the D_2 receptors in the striatum. The D_2 receptor is associated with inhibition of glutamate signaling in striatal medium spiny neurons (MSN), the main source of GABA [43]. Lack of dopamine reaching the D_2 receptors can lead to a downregulation of MSN activity and a decrease in GABA signaling towards the main limbic structures, such as the frontal cortex, amygdala, and ventral tegmental area. D_2 receptor-mediated signaling has been shown as a suppressing factor of aggression [44]. The PET results may be also explained by the interplay between D_2 receptor availability and DA release. Competitive PET tracers for neurotransmitter receptors are unable to distinguish if changes in BP_{ND} are due to an increased number of receptors, or an increase of endogenous dopamine [45]. In order to confirm this D_2 effect in aggression, it might be of interest for future studies to replicate the RSD protocol using animals with transiently impaired D_2 signaling (e.g. pharmacological intervention with raclopride prior to RSD trial).

In this study, attack latency was the parameter used to discriminate the level of aggression of animals [46]. Changes of dopaminergic receptor availability and/or dopaminergic transmission in different brain areas seem to be associated with the degree of aggression [35,39,41]. We found that the aggressive animals followed an expected pattern of aggression during the RSD, taking fewer seconds to initiate an attack during each session. Initially, the animals showed a gradual decreased in the attack latency with the increasing number of exposures. In both cohorts of animals, this decrease in attack latency was observed until a certain point. In cohort 2, the attack latency remained relatively stable over time from this point onward. It is possible that animals of cohort 2 became habituated to the protocol after a certain period of time, thus explaining why after a certain period of time the attack latency of the animals remains mostly unchanged. To the best of our knowledge, there are no studies regarding the effect that the number of exposures to RSD has on the aggressors, as these animals are not usually the main focus of studies involving this protocol, and usually, are only briefly mentioned in reports. From our results, it is possible to assume that the aggressive behavior initially increases, but stabilizes after a certain point if the animals are overloaded with exposures to RSD. Future studies using the RSD protocol should, therefore, take into consideration the number of trials each animal goes through when planning the study.

However, the fact that animals of cohort 2 showed habituation to the protocol does not explain why the other cohort of aggressors showed a constant decrease in attack latency time until the end. One possibility is that these animals had a decreased burden, having a lower frequency of bouts of RSD during the protocol (14 vs 29 on average over 6 months) over the period of use of these animals. Thus,

animals of cohort 1 had no habituation due to a seemingly long period between trials; or that animals of cohort 2 had so higher a burden that they showed exhaustion towards the end of the protocol.

In conclusion, our results showed a gradual reduction in attack latency over time in both groups at the beginning of the experiment, but this effect was lost after long periods in the cohort with a higher number and higher frequency of exposures. This might be due to habituation or due to exhaustion after several RSD trials. We also found an increase of D_2 tracer binding in the striatum of the more aggressive rats in cohort 1, but not in the less aggressive cohort 2. Additional experiments like immunohistochemistry, microdialysis or liquid chromatography to assess DA itself are needed to discriminate if those results are due to differences in DA release, or due to differences in receptor expression. Nonetheless, these results suggest the participation of D_2 receptor-mediated signaling in aggressive behavior, which can be used as a pharmacological target for diseases associated with aggressive events.

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Chapter 7

Discussion and future perspectives

The main goal of this thesis was to investigate how social stimuli change behavioral, neurotrophic and neuroinflammatory parameters. For this purpose, three kinds of social stimuli were applied: I) social enrichment; II) social isolation; III) social defeat. Together, these factors are able to identify, to a certain extent, how the animal responds to different social cues, and how the brain is modulated by both beneficial and harmful social stimuli. To understand how social stimuli affect the behavior, neurotrophic factors, and neuroinflammatory processes, we investigated several parameters that together modulate – for better or worse – social behavior in animals. Different coping mechanisms against harmful stressors can alter these very outcomes. This chapter will provide an overview of how each of the investigated parameters is related to depressive behavior, and how these findings can be implemented or improved in future studies.

BDNF: just another biomarker?

BDNF has been taken into consideration as a potential biomarker of general brain function for almost as long as its discovery but is rarely used as a diagnostic biomarker or biomarker for therapeutic outcomes. **Chapter 2** of this thesis provides information about how this neurotrophic factor is related to a variety of processes that affect the brain. In this literature review, we showed that there are plenty of studies showing altered concentrations of BDNF in various psychiatric and neurodegenerative diseases. Therefore, it is unlikely that BDNF can present itself as a diagnostic biomarker of one disease, but more as a biomarker of a state of disease of the brain or as a biomarker for the efficacy of therapeutic interventions. BDNF is activity-dependent, meaning that, from protein expression and production to release and binding to the TrkB receptor, it depends mostly on the endogenous and exogenous, beneficial and harmful, stimuli that require constant adapting by the organism. Harmful stimuli such as chronic stress, disease, and neuroinflammation usually decrease BDNF levels, while beneficial stimuli such as physical and cognitive activities and a good social environment enhances its production and release.

In **Chapter 3**, the concept of activity-dependent expression of BDNF is used as a basis to tackle a very fundamental (and often disregarded) question: is what is observed in the serum related to what is observed in the brain? When studying BDNF, animal models show expression of this protein in several cognition- and disease-related brain structures (e.g.: hippocampus, frontal cortex, amygdala) [1,2]. In humans, assessment of brain concentration of BDNF is not yet possible. Therefore, researchers have only observed how this protein behaves in the serum or plasma of a diverse population [3]. However, the few animal studies trying to address the question of whether serum and brain BDNF levels are related more often than not were unable to reach a conclusion [4,5]. In this study we used two

conditions that were shown to modify BDNF levels in serum (in humans) and the brain (in animals): age [6–8] and environment [9,10].

Although age did not have a significant effect on BDNF levels in our study, the environment had an effect not only on the level of BDNF and its precursor proBDNF in the hippocampus but also on anxiety-like behavior, long-term memory, and synaptic plasticity. Interestingly, the effects on BDNF were only found in the brain and were not correlated with serum BDNF. Although translating animal findings to human studies is challenging, these results shed light on how BDNF behaves in the brain after environmental stimuli, and cast a shadow over its use as a serum biomarker of brain functioning. It is possible that this brain/serum discrepancy can be explained by the function of BDNF in each of the regions of interest. In the brain, BDNF is mainly associated with the maintenance of neuronal function by modulating synaptic and dendritic branching, while in the serum, BDNF may have a different, not yet described, function. Peripheral BDNF is produced in several tissues, but there is no clear indication as to what this means on a functional level. BDNF is also stored in platelets and it is hypothesized that upon activity, such as physical exercise [11,12], there is a release of platelet BDNF in the bloodstream, thus influencing, to a certain extent, peripheral BDNF concentration. This hypothesis can lead to two different perspectives: 1) BDNF levels in the brain of animals are loosely related to its levels in the serum, whereas in humans the BDNF levels in the brain and serum are more closely related, as suggested by the literature on serum BDNF in case-control studies [13–15]; 2) There could be an unknown mechanism that maintains peripheral BDNF concentration constant, whether by regulating Bdnf gene expression and production, or by modulating the release of the neurotrophin in the bloodstream. Regardless, these findings question the feasibility of peripheral BDNF as a biomarker for brain function in humans, as it should be taken into account that what is observed in the serum may not reflect what is happening in the brain.

As it is not possible to measure brain BDNF levels in living humans, the need for new techniques that are able to measure BDNF *in vivo* is imperative. PET radioligands for BDNF or tracers that compete with BDNF for its binding to the TrkB receptor could be a potential tool to investigate how this protein behaves in the brain. Small molecules that mimic BDNF and bind as agonists to the receptor TrkB are already on the market, and might have the requirements needed for a good radiotracer candidate (i.e.: strong and specific binding affinity; penetration of the blood-brain barrier; relatively fast clearance from non-target tissues, and systemic clearance that is compatible to tracer binding to its receptor). If a PET technique with such a tracer is successfully developed and can be applied in future studies, it will create a whole new field of opportunities to explore how BDNF can be (or not) used as a biomarker for brain state.

Social stress: a change of paradigm?

Continuing our focus on social stress, the next chapters of this thesis focused more on how negative social stimuli can affect the brain and may lead to stress-dependent depressive-like behavior. In addition, we attempted to modulate the stress response by applying different interventions and we investigated how such negative stimuli would affect depressive and anxiety behaviors, BDNF and neuroinflammatory markers in the brain using PET imaging [16]. Depression is a multifaceted disorder, with many different factors contributing to its development and progression [17]. Therefore, to understand how every system affects depressive behavior is an impossible task. Several hypotheses have been developed to explain the biological aspects of depression. One of the main hypotheses is that unresolved chronic stress can trigger depressive-like behavior by deregulation of the HPA-axis, inducing a modification in the cortisol response (usually increasing cortisol concentration in the blood) and impaired negative feedback of the HPA-axis [18,19].

Taking this paradigm into account, **Chapter 4** applied both neuroendocrine and neuroinflammatory markers in order to better understand how stress-dependent depressive-like behavior is affected in animals that lack a proper HPA-axis response. In this study, we used a repeated social defeat protocol (RSD) as a trigger for stress-induced depressive-like behavior in animals previously adrenalectomized (ADX) and assessed how ADX affects short-term social behavior (1 day after RSD), long-term anxiety-like behavior and neuroinflammatory correlates (14 days after RSD). Animals submitted to ADX showed resilience against stress-dependent impairment of social interaction one day after RSD. This might be explained by the ablation of fight-or-flight response by inhibition of HPA-axis response [20,21]. As anxiety- and depressive-behavior were not observed, we assumed a very specific effect of RSD in social behavior, possibly inducing a fear response towards other animals [22]. By blocking HPA signaling, the fear response is also impaired, and the animal is less reactive to stressors.

As corticosterone measurement is challenging in ADX animals, pharmacological interventions such as antagonists of glucocorticoid and mineralocorticoid receptors (GR and MR, respectively) offer a transient option instead of ADX to measure effects of corticosterone. Other measures for stress can be used to properly address how RSD affects stress response independent of the HPA-axis *in vivo* (e.g.: heart rate recording, temperature measurement) and *ex vivo* (glucocorticoid and mineralocorticoid receptors, adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH)). Lastly, behavioral measurements that are more related with fear behavior (i.e.: passive-avoidance test; unconditional fear stimulus with predator odor – or with the smell of a resident from the RSD protocol)

might be an interesting feature to confirm if the fear response is inhibited in ADX animals submitted to RSD.

In our next study, we decided to modify how the RSD protocol was performed, and change the time between the last RSD trial and the [^{11}C]PBR-28 scan. In this study, we addressed if treatment with antidepressant and anti-inflammatory compound harmine [23–25] would be able to modify the (possible) inflammatory response, behavioral abnormalities and changes in BDNF concentration in the brain of defeated animals. In chapter 4 we applied a barrier in the center of the cage separating intruder and resident, while in **Chapter 5** we placed the intruder in a wire mesh cage, thus providing a more intimidating environment for the intruder, while still avoiding physical contact between resident and intruder [26]. These changes seem to have had a significant short-term effect on the behavior of the intruder, such as more anxiety-like behavior in the open field test and more depressive-like behavior in the sucrose preference test when compared with non-defeated animals. Physiologically, the weight of socially defeated animals also decreased during the RSD protocol, an indication of a state of anhedonia, further confirmed by a lower preference for water with sucrose in the sucrose preference test (SPT). When the same tests were applied at a later time point, however, no effect on the anxiety and depressive-like behavior of the animals submitted to RSD was observed anymore, indicating that the effect of RSD was transient, not lasting until the end of the study protocol. Additionally, we analyzed neuroinflammation by [^{11}C]PBR-28 eleven days after the last RSD trial, and the brain BDNF concentration post-mortem, and no difference between RSD and control were found for these molecular parameters.

Interestingly, RSD has shown a strong data variance in the sucrose preference test one day after RSD. In this regard, it is likely that some animals have shown a tendency to be more sensitive against the stressful stimuli, thus presenting higher anhedonia when compared to the other groups. It is possible that there is a high inter-individual variance in this regard, and future observations are advisable to take this variation into account. Thus, further studies on the matter should consider dividing the animals submitted to RSD into stress-susceptible and resistant subgroups. Once more, the findings of this chapter are related to the ones from chapter 4, as a short- but not long-term effect was observed in the behavioral outcomes for RSD, once again demonstrating that RSD was not as strong as we expected, and its effects disappeared within one week. However, in chapter 5, harmine was able to induce a significant long-term change in the locomotion of the animal, with a lower distance traveled when compared with vehicle animals. We hypothesize that this might be due to the tremorgenic effect of the drug [27], causing a lingering decrease of general locomotion, although there are no other studies showing similar effects. After every injection the animals showed transient tremors, receding after one

hour. However, it is possible that other mechanisms might be playing a role that we failed to assess visually, and therefore might be hampering the locomotion of the animal in a long-term fashion. With the lack of a proper RSD-dependent depressive-like behavior, it is difficult to ascertain the feasibility of harmine as an antidepressant and anti-inflammatory compound. However, the effects on locomotion and weight might point towards a negative effect of harmine when coping with stress, which might be something to consider in future studies using this compound.

Timing matters: neuroinflammatory processes in social stress

The results found in chapters 4 and 5 regarding neuroinflammation were, to a certain extent, surprising, as we hypothesized that the effect of RSD would be stronger than what was observed. We expected a long-term modification in the brain circuitry associated with depressive-like behavior and the neuroinflammatory theory of depression. If the inflammatory process is impaired, or the stressor is repeated over a period of time, it might cause a chronic inflammatory process in the organism, leading to long-term damage to neurons and, consequently, neuronal death.

In this regard, two possibilities arise for the failure to neuroinflammation in our studies: the chronic stress was not strong enough to impose an inflammatory response from microglia; or the timing of PET scan was incorrect, as the microglia may have been less activated than expected, and consequently the inflammatory response was already in its final stage (i.e.: resolution) two weeks after the last social stress trial [28]. However, the results of our study should be interpreted with care, as there was a very large variance in the [^{11}C]PBR28 PET signal in animals submitted to RSD in both studies. This might point towards the presence of stress-sensitive and stress-resilient sub-populations in our study. Mimicking human diseases in animal models in order to better understand the pathophysiology is one of the most difficult challenges of preclinical research. In this respect, depression is a particular challenge in the field of mood and psychiatric disorders. The multimodal pattern of MDD, along with the high variability of symptoms, makes it virtually impossible to study all aspects of the disease in one animal model only [29]. Therefore, researchers investigating cognition in a preclinical setting tend to "bestialize" human behaviors [30], assuming that what is considered threatening or beneficial in humans can be assumed to have the same impact on animals. This might not be the case in the RSD paradigm used [30,31]. Several studies have shown an effect of RSD on behavioral outcomes in the short-term (reviewed by Hollis et al. [32]), but the effects of RSD in the long-term are not very well studied. Thus, some options to improve the stress response to develop depressive-like symptoms include: 1) the use of different stressors (one such stressor could be the previously used long-term social isolation, as it was shown as a promoter of anxiety-like behavior in animals); 2) increase its length, thus increasing the contact with the stressor; 3) animals genetically susceptible to develop depression could decrease

the inter-individual variability at the cost of possibly skewing the results towards one side of the population, disregarding resilient animals; 4) increasing the number of animals in order to take inter-individual variability into consideration in the study. All these options might be needed in order to induce reliable changes in animals for development of depressive behavior.

Another point to be taken into consideration is the neuroinflammatory marker that was used in our studies. TSPO as a biomarker for inflammation has been used for a long time in the molecular imaging field, showing promising results for neurodegenerative diseases, where inflammatory processes are usually more pronounced [33,34]. In psychiatric disorders, however, there is still a debate on how reliable TSPO radiotracers can be for neuroinflammatory surveillance and assessment of microglial activation. Consequently, the use of TSPO as an imaging biomarker for neuroinflammation in human and animal studies is under scrutiny [35,36]. For instance, Setiawan and colleagues found in a very large MDD cohort that the volume of distribution (V_t) of the TSPO tracer [^{18}F]-FEPPA in the prefrontal cortex, anterior cingulate cortex, and insular cortex was associated with progression of untreated depressive disorder. However, the same investigators found that this effect is significantly more pronounced in subjects with a long-term, untreated MDD (over 10 years), whereas subjects with shorter-term progression of depression (less than 5 years) show no difference in tracer uptake when compared with control subjects [37]. Other studies with [^{11}C]PBR28 in humans have shown a positive association between increased neuroinflammation in the anterior cingulate cortex and increased progression of perceived depression [38,39]. These results show that, while progression of disease is associated with an increase of TSPO uptake in specific MDD-related brain areas, this effect seems to be observed more in a late-stage of disease (i.e.: chronic MDD), when the brain circuitry is more impaired and damage-associated neuroinflammation is more pronounced.

However, the point where the “allostatic breakdown” of a subject leads to the activation of the neuroinflammatory cascade is not clear yet. It is difficult to draw a conclusion without knowledge of the mechanisms, and inter-individual differences make the challenge even harder. There is a need for novel techniques that show increased sensitivity and specificity in assessing slight neuroinflammatory patterns, or even pro/anti-inflammatory patterns *in vivo*. But in the meantime, studies focusing on PET imaging of TSPO should take advantage of the *in vivo* data collection and try to assess what is the best time point to get the best result out of it, and from there on, focus on development and treatment of inflammation as a part of depressive-like behavior.

To the victor goes the spoils: how dopaminergic neurons react to winning?

Dopamine is a neurotransmitter involved in the functioning of the limbic system, mainly associated with reward [40,41]. In humans, aggression has also been associated with altered D_2R expression [42–44], although there are also reports stating otherwise [45]. In **Chapter 6** we took advantage of the social defeat paradigm to study the behavior of the animals that were trained to be the aggressors (i.e.: residents). In this chapter, we compared two different cohorts of aggressors: one cohort of animals that were used in the studies described in chapters 4 and 5 of this thesis, and a cohort from another independent study using a similar protocol [46]. In both cohorts, [^{11}C]-raclopride PET imaging was applied to assess D_2 receptor availability and compared with non-aggressive control animals. As the timing between the last RSD trial and the [^{11}C]-raclopride PET scan was different between cohorts, the groups were divided between controls (no aggression), cohort 1 (animals used in chapters 4,5) and cohort 2 (animals from another study). There was a significant difference in the behavior of animals, with cohort 2 showing a characteristic decrease in the attack latency over time when compared with cohort 1 and controls. This group also showed a difference in the dorsal striatum D_2 receptor availability, with a two-fold increase in the binding potential of [^{11}C]-raclopride when compared with the other groups.

Striatal D_2 receptors in this region are mainly associated with modulation of medium spiny neurons – the main producers of γ-aminobutyric acid (GABA). While the nucleus accumbens (ventral striatum) drives changes in motivation (i.e.: hedonic state and reward circuitry [47,48]), the dorsal striatum plays a role in habit formation [49,50]. As binding potential is increased, it means more availability of D_2 receptors, which could indicate that less dopamine is bound to this receptor, [51] or overexpression of the receptor. If the decrease in signal is due to a reduced release of dopamine, this might lead to a decreased GABAergic signaling and a consequent lack of inhibition of several limbic system-associated brain regions [51]. It is possible that these disinhibited regions affect the brain in a way that the animals become increasingly more aggressive over time. In the other cohort, the animals may show habituation towards the stressor, explaining the significantly higher attack latency. However, it is difficult to draw further conclusions about innate differences between the cohorts.

As this study was only able to observe D_2 availability after RSD, studies that focus on the development of aggressive behavior, such as the one described in this thesis, would greatly benefit from a baseline PET scan in order to show how D_2 receptors are presented before introducing the animal to the RSD protocol. This way, it would be possible to depict the entire scenario of aggression development. Another interesting topic to observe is the behavior of dopamine D_1 receptor availability, as it is already shown that D_1 and D_2 show distinct functions in the signaling of GABAergic

neurons in the dorsoventral striatum. Lastly, other brain structures are reportedly associated with aggressive behavior and can, unfortunately, not be assessed by [^{11}C]-raclopride PET (e.g.: orbitofrontal cortex [52], hypothalamic structures [53], habenula [54]). All these structures form a complex and intertwined system for modulation of aggressive behavior, making the study of aggressive behavior a very challenging one. Dopamine is one of many players responsible for this behavior, and future studies should take that into account when designing their studies.

Concluding remarks – It is a matter of time

Depression is a major future concern, which will lead to worldwide healthcare and workforce issues. Except in regions of extremely stressful conditions (e.g.: famine, poverty, warzones), modern-day chronic, social stress, has never been so high, which is a major concern for the onset and progression of depression. This thesis has shown that the effect of subchronic and chronic social manipulation – whether positive or negative – affects several brain functions, especially in the short-term. However, when long-term changes were assessed, the parameters analyzed were already normalized. Chronic, positive social stimulation such as environmental enrichment improved several parameters associated with neuronal survival and plasticity and synaptic functioning. On the other hand, chronic social stress, such as the social isolation protocol has shown a tendency to impair these parameters, showing an expected duality between positive and negative stimulation. Interestingly, long protocols such as social isolation and social enrichment were able to induce the modifications needed to assess how BDNF behaves after social stimulation, but there was a discrepancy between what was found in the brain of these animals and what was observed in the serum. Taking all rodent-to-human translation issues into consideration, this lack of interaction between BDNF in the brain and serum will fuel an exciting discussion and should be taken into consideration for future research involving this neurotrophic factor as a relevant peripheral biomarker for brain functioning. For this, PET imaging of TrkB receptors, or BDNF itself, might help us to better understand the behavior of this neurotrophin *in vivo*.

in our studies, subchronic stressors, such as social defeat, induced a short-term but not a long-term effect on the animals' behavior, with no changes in neuroinflammatory and neurotrophic parameters. TSPO imaging showed no effect of RSD in the animals, suggesting that these animals had no neuroinflammation at the point of analysis, and together with the lack of difference in the depressive-like behavioral outcomes and BDNF concentration at the same time point, we can hypothesize that the acute depressive-like behavior shown shortly after RSD was transient, and had normalized within two weeks. It would be interesting to study if, after two weeks, the negative and positive effects of isolation and enrichment, respectively, would still be visible.

Assessment of the other side of social stress yielded some interesting results. [^{11}C]raclopride PET showed a significant change in aggressor animals of one cohort when compared to another aggressive cohort and control animals that did not show any aggressive behavior. This was also observed in the scaling of aggressive behavior, with animals showing higher striatal binding potential also having a lower attack latency. However, the data collected was from animals that underwent several trials of RSD, thus it is not possible to confirm that changes in aggressive behavior were caused by innate lack of dopaminergic binding to D_2 receptors, and other studies using baseline measurements should be done in order to have a reliable starting point and avoiding between-group differences at baseline.

As the prevalence of mood disorders will increase in the coming decades, the need for new and improved methods to diagnose and treat these conditions are of major importance for psychiatrists and neurologists alike, and understanding the mechanisms that affect – or are affected by – such disorders are the first step of this challenge. As the most commonly diagnosed psychiatric disorder, depression is the main example of what we are dealing with a multifaceted disease with many starting points and comorbidities that, in the end, severely affect both individuals and their peers. In order to treat the disorder, first it is needed to understand how these processes are involved in the development of MDD. Only then, it will be possible to strive for individual treatment for this disease.

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Chapter 8

Summary

In modern society, people are living under constant pressure. On the one hand, mild “healthy” stressors can be a motivator, leading to increased individual productivity and creativity. On the other hand, however, constant, excessive stress over a long period of time can impair not only the mind but the overall well-being of a person. The burden of aforementioned societal pressure is shown by the increasing number of stress-associated health issues around the globe. Mood- and psychiatric disorders are just a small part of the wide range of diseases related to stress, according to literature.

One particularly debilitating example of a stress-related health issue is depression. Depression is considered a major global health issue, affecting every cultural, economic and age group. It is very likely that the one reading this thesis knows someone diagnosed with clinical depression, and other two or three that show some symptoms of it. Moreover, these numbers are expected to increase in the future, since society as a whole will likely not change. Estimates for the future are not pleasant, as 15% of the population is projected to develop some symptoms of depression. This would entail a significant burden to healthcare, as patients with depression spend a long time in treatment, and several of these treatments fail or only partially treat the disease. Depression is also a major economic issue, as patients with depression show low motivation and productivity, leading to substantial economic losses. Even more concerning are the comorbidities associated with depressive episodes and, in moderate to severe cases, suicidal tendencies.

Biologically, the brain is the region that is most affected by highly stressful situations. Neurotransmitter signaling, neuroendocrine function, and neuronal signaling are lowered during periods of chronic stress, and if left unchecked, these neurologic changes are amongst the causes of depressive symptoms and the first step towards clinical depression. Alteration of brain-derived neurotrophic factor (BDNF) concentration is one of many biochemical changes associated with depression. It is also observed in other brain conditions. **Chapter 2** shows an overview of the behavior of BDNF in several psychiatric and neurodegenerative disorders. Additionally, we further evaluated the behavior of BDNF both in healthy and pathological conditions, and how BDNF could influence how therapy for these diseases is performed. In this regard we show consistency on BDNF to decrease in diseased states with a few exceptions. Therefore, although this neurotrophin itself is not a specific disease biomarker, BDNF can be associated with a disease pattern – or how (un)healthy the brain is at a specific moment.

In humans, BDNF in the serum has been shown to be decreased in psychiatric and neurodegenerative diseases, and this decrease is mostly correlated with cognitive impairment and behavioral changes. In animal models of disease, similar results have been found in specific brain regions, such as the hippocampus and frontal cortex. However, only a few studies tried to associate peripheral and brain

BDNF, and most of these studies attempted to do so mostly as a secondary goal. In **Chapter 3** we decided to modulate BDNF concentration in rats by using two different factors that are known to have an effect on this neurotrophin: environmental conditions (environmental enrichment, **EE**; impoverished enrichment, **IE**; and standard enrichment, **SE**) and aging (6 months and 17 months, representing middle-aged and elderly in human standards, respectively). After 10 weeks in environmental conditions, we observed that isolated animals show an increased anxiety-like behavior when compared with animals that were submitted to environmental enrichment at both ages. EE animals had increased performance in the novel object recognition test. 2 weeks after behavioral assessment animals were terminated for post-mortem analysis. We found a significant effect of environment, with IE animals showing an overall decreased concentration of mature BDNF (mBDNF); its precursor, proBDNF; and Synaptophysin in the hippocampus when compared with EE, and EE showing significantly higher levels in proBDNF and Synaptophysin, but not BDNF, when compared with SE. Interestingly, none of the significant differences in concentration of mBDNF in the hippocampus were observed in the serum of these animals, showing that mBDNF concentration in the brain is not related to its concentration in the serum. These findings point towards the conclusion that different social environments are able to modify central mBDNF concentration regardless of age, either increasing or decreasing it, depending on the stimuli given, and that cerebral concentration of this neurotrophic is reflected on cognitive performance. However, these changes were not observed in the serum of these animals, which could imply that mBDNF might not be applicable as a serum biomarker for brain changes.

Depression is a multifaceted disorder, with many different factors contributing to its development and progression. One of the main hypotheses is that unresolved chronic stress can trigger depressive-like behavior by deregulation of the HPA-axis, inducing a modification in the cortisol response (usually increasing cortisol concentration in the blood) and impaired negative feedback of the HPA-axis. In **Chapter 4** we used the repeated social defeat (RSD), a model of social stress that is able to induce depressive-like behavior and neuroinflammation in rats, to assess if social stress is modulated by inhibition of stress response (by adrenalectomy - ADX), and how ADX and RSD affect behavior and neuroinflammatory processes. Animals had bilateral **ADX** or **Sham** and submitted to a five-day **RSD** protocol or **control** seven days after surgery. One day after last RSD animals were tested for anxiety, locomotor (open field) and social behavior (social interaction). 1, 7 and 14 days after RSD animals were tested for anhedonic behavior (sucrose preference test). Two weeks after last RSD, animals were scanned for microgliosis using [^{11}C] PBR28. There were no differences in the open field and no surgery, RSD or time effects in the sucrose preference test. Animals under RSD showed lower social interaction in the social interaction test, which was interestingly not observed in animals both adrenalectomized

and defeated. There was no difference in neuroinflammation process between any of the groups. The results show a somewhat expected effect of RSD in the social behavior, but HPA-axis disruption by ADX appears to block this fear-response of the animals towards others, likely by impairment of fight-or-flight response. Interestingly, there were no differences in microgliosis visible on [^{11}C] PBR28, suggesting that, if RSD caused any neuroinflammation, it was already normalized after two weeks. Thus we assume that stronger modulators of depression might be needed in order to induce chronic depressive-like symptoms and neuroinflammation.

In **Chapter 5** we repeated the RSD protocol modifying it to apply a stronger, more reactive interaction between resident and intruder. Additionally, we administered antidepressant and anti-inflammatory alkaloid **harmine**, or **vehicle**, intraperitoneally for 14 days. Animals had their weight observed daily, and locomotion and anxiety-like behavior were assessed by open field 1d and 9d after the last RSD. Anhedonia was measured 1d before, 1d and 10d after last RSD. Memory was assessed 10d after last RSD. Neuroinflammation was assessed 11d after last RSD, and hippocampus and frontal cortex were collected for BDNF concentration analysis. RSD had a significant impact on behavior and anhedonia, as shown by an increased anxiety parameter in the open field, and decreased preference for water with sucrose one day after RSD. There was no effect of harmine on these parameters, but harmine did show a significant lowering effect on weight and locomotor behavior. These effects lasted until the end of the experiment, as shown by the decreased locomotion and lower weight of harmine-treated animals. On the other hand, anxiety-like behavior found right after the last RSD was normalized in the long-term. RSD generated a fluctuation on a short-term (here observed by the behavioral outcome of the first OF and the SPT after RSD), but not a long-term effect, as seen by the lack of difference in uptake of [^{11}C] PBR28 between RSD and control groups, together with unaffected long-term behavioral alterations on anxiety and depressive-like behavior. The changes of RSD or harmine did not alter BDNF concentration in frontal cortex or hippocampus, regions that are key for stress regulation and further brain homeostasis. To better assess the influence of harmine in depression and its effect as an antidepressant, there is a need for further studies using different stressors or longer time RSD protocols in order to induce a chronic stress response in the animals, thus allowing a more efficient analysis of how harmine could act under such conditions.

To understand how RSD works, it is also important to observe the effects this experimental setup has on the aggressors. In **Chapter 6** we evaluated how the dopaminergic – more specifically, the D_2 receptors – are affected by social success, and if it might be related to a pathological increase in aggression (violence). Animals were tested for aggression in order to participate in the RSD experimental setup, and those who failed to show aggressive behavior (average attack latency of 60 seconds after 5 trials) were considered non-aggressive. Animals that were screened as aggressive were

submitted to several trials of RSD. We used [^{11}C] Raclopride to assess D_2 receptors in the striatum (Caudate and Putamen) and nucleus accumbens (core and shell) and scanned at different time points: one day after last RSD (**acute aggressors**) and 14 days after last RSD (**no-acute aggressors**), and compared these animals to **controls**. Animals that had 14 days between last RSD and [^{11}C] Raclopride scan showed higher aggressive behavior, with decreased attack latency over time, when compared with other groups. This effect was also observed in the striatal [^{11}C] Raclopride binding potential, with an almost two-fold increase when compared with the other groups. However, in the nucleus accumbens, there was no difference between groups. The observed increase in D_2 receptor availability in non-acutely exposed animals is in line with the literature. An increase in D_2 radiotracer binding in the accumbens was also observed by other studies in animal models of aggression. Lack of differences in D_2 binding on the experiments may be explained by the heterogeneity of the attack latency found in our results. This can be an explanation for the differences found between the acute and non-acute exposure of the aggressive animals to intruders, since the attack latency of the acute exposed animals did not show an association with the trial number, suggesting that their level of aggression may not be enough to have a significant effect on the binding potential of [^{11}C] raclopride.

In conclusion, social stimuli had an impact in the brain, as shown by differences in behavior, neurotrophin, and synaptic plasticity markers, both in positive and negative social environments. It appears that the longer the stimulation, and the shorter the analysis period, the more pronounced is the behavioral change. It appears that longer stress protocols are needed in order to achieve a chronic state of anxiety- and depressive-like behaviors in an animal model of stress, allied with possibly a shorter interval between stressor and analysis. This was especially true for the social defeat model, as we were able to observe a short-term effect on behavior of animals submitted to social stress, although this effect was normalized in the longer-term. Neuroinflammatory and neurotrophic markers might follow the same pattern, as shown in the literature. Future studies using social defeat protocols, or resident-intruder models, might also need to take into consideration the possibility of intruders not developing depressive-like behavior, while other intruders do. Additionally, it is possible that residents need to be better screened for their aggressive behavior, with possibly other parameters together with attack latency. In our study setup, residents seem to develop a habituation state after being repeatedly introduced to an aggression protocol, as shown by the unchanged attack latency.

Chapter 9

Nederlandse samenvatting

In de hedendaagse maatschappij neemt de druk voor mensen steeds verder toe. Aan de ene kant kunnen gematigde “gezonde” stress factoren een goede motivator zijn voor het verhogen van individuele productiviteit en creativiteit,maar aan de andere kant kan constante, overmatige stress over een lange tijdsperiode schadelijke gevolgen hebben; niet alleen op de geest, maar ook op de lichamelijke gezondheid van een individu. De ernst van de toename van de druk blijkt ook uit de wereldwijde verhoging van stress-geassocieerde gezondheidsproblemen. Volgens de huidige onderzoeken zijn stemming en psychiatrische stoornissen zelfs maar een klein deel van het brede scala aan stress gerelateerde ziekten.

Van deze stress gerelateerde ziekten, wordt depressie als een van de grootste problemen voor de mondiale volksgezondheid beschouwd. Het komt voor in iedere cultuur, economische status, en alle leeftijden. Het is dan ook erg aannemelijk dat diegene die deze thesis nu leest persoonlijk iemand kent met klinisch gediagnosticeerde depressie, en waarschijnlijk nog enkele anderen die hier symptomen van vertonen. De verwachting is dat de kans op depressie in de toekomst verder zal toenemen, aangezien de gemeenschap als een geheel niet snel zal veranderen. Voorspellingen voor de toekomst zijn zorgwekkend. Er wordt ingeschat dat over … jaren 15% van de bevolking een klinische depressie of symptomen ervan zal ontwikkelen. Dit betekent een grote druk op de gezondheidszorg, omdat patiënten met depressie vaak een lange behandelingstijd nodig hebben en verscheidene therapieën niet zullen werken of maar ten dele de klachten verhelpen. De economie lijdt hier ook onder, doordat patiënten met depressie een lage motivatie en productiviteit hebben, leidind tot substantiële economische schade. Een nog groter probleem zijn de bijkomende klachten die het gevolg kunnen zijn van depressieve episodes, waaronder in de ernstige gevallen suïcidale neigingen, met in het uiterste geval zelfs suïcidepogingen als gevolg.

Vanuit biologisch oogpunt, zijn de hersenen het meest gevoelige orgaan met betrekking tot stressvolle situaties. Bij chronische stress zijn de neurotransmitter signalering, neuro-endocrine functies en neuronale signalering verlaagt en indien dit niet wordt hersteld, veroorzaken deze veranderingen depressieve symptomen en later mogelijk ook een klinische depressie. Veranderingen in de concentratie van de “brain-derived neurotrophic factor” (BDNF) is een van de vele neurobiochemische veranderingen, geassocieerd met depressie. Het wordt vaak ook geobserveerd bij andere hersenaandoeningen. In hoofdstuk 2 wordt een overzicht weergegeven van hoe de BDNF concentratie zich gedraagt in zowel gezonde als pathologische setting en hoe BDNF invloed zou kunnen hebben op de behandeling van deze ziekten. Hierbij werd aangetoond dat de BDNF concentratie in bijna alle onderzochte neurologische aandoeningen afneemt als gevolg van ziekte. Hoewel deze neurotrofine op zichzelf geen specifieke ziekte biomarker is, kan BDNF wel geassocieerd zijn met een bepaald ziekte patroon – of inzicht geven over hoe (on)gezond de hersenen op een bepaald moment zijn.

Bij mensen blijkt de BDNF concentratie in het serum verlaagd te zijn in verscheidene psychiatrische en neurodegeneratieve ziekten, en deze afname hangt sterk samen met gedragsveranderingen en een verlaging van cognitieve functies. In diermodellen zijn bijpassende bevindingen gedaan in specifieke hersenregio's, zoals de hippocampus en de frontale cortex. Er zijn echter slechts weinig onderzoeken waarbij geprobeerd werd een associatie te vinden tussen de perifere en hersen BDNF-concentratie, en in deze onderzoeken, was dit meestal slechts een secundaire doelstelling. In hoofdstuk 3 besloten we om bij ratten de BDNF concentratie te moduleren door gebruik te maken van twee verschillende factoren die er om bekend staan dat zij een effect te hebben op deze neurotrofine: omgevingsfactoren (environmental enrichment, EE; impoverished enrichment, IE; en standard enrichment, SE) en leeftijd (6 maanden en 17 maanden, welke in humane begrippen, mensen van middelbare leeftijd en bejaarden vertegenwoordigen). Na 10 weken met verschillende omgevingsomstandigheden, observeerden we dat dieren in isolement vaker angst-achtig gedrag vertoonde in vergelijking tot EE dieren van dezelfde leeftijd. EE dieren presteerden beter in de nieuwe object herkenningstest. 2 weken na de gedragsexperimenten werden de dieren geëuthanaseerd voor post-mortem analyses. Hierbij vonden we een significant effect van de omgeving, waarbij IE dieren gemiddeld genomen een verlaagde concentratie vertoonden van zowel matuur BDNF (mBDNF);zijn precursor (proBDNF); en synaptophysine in de hippocampus in vergelijking met dieren in de EE groep, en EE had een significante hogere concentraties van proBDNF en Synaptofysine, maar geen BDNF, in vergelijking met SE. Merkwaardig genoeg, werden deze significante verschillen in concentratie van mBDNF in de hippocampus niet waargenomen in het serum van deze dieren, wat laat zien dat de concentratie mBDNF in de hersenen niet gerelateerd is aan de concentratie in het serum. Deze bevindingen illustreren dat een verschil in sociale omgeving de centrale mBDNF concentratie kan beïnvloeden, ongeacht de leeftijd, en dat de concentratie van dit neurotrofine invloed had op cognitieve prestaties. Er werden echter geen veranderingen waargenomen in het serum van deze dieren, en dit impliceert dat mBDNF als een serum biomarker mogelijk niet geschikt is om veranderingen in de hersenen aan te tonen.

Depressie is een veelzijdige stoornis. Vele verschillende factoren dragen bij aan de ontwikkeling en progressie ervan. Een van de hoofd hypotheses hierbij is dat verwaarloosde chronische stress depressie-achtig gedrag kan veroorzaken door de deregulatie van de HPA-as, het induceren van een verandering in de cortisol spiegels (gebruikelijk een verhoging van de cortisol concentratie in het bloed) en aantasting van negatieve feedback op de HPA-as. In hoofdstuk 4, stelden we ratten bloot aan repeated social defeat (RSD), een model voor sociale stress dat depressie-achtig gedrag en bijbehorende neuro-inflammatie veroorzaakt. Hierbij analyseerden wij of inhibitie van de stress reactie (door adrenalectomie – ADX) sociale stress vermindert, en wat voor invloed ADX en RSD hebben op

gedrag en neuro-inflammatie. Dieren ondergingen bilaterale ADX of een schijnoperatie., Zeven dagen nadien werden de dieren gedurende vijf dagen blootgesteld aan een RSD protocol of een controleprotocol. Eén dag na de laatste RSD dag, werden de dieren getest voor angst, locomotor (open veld) en sociaal gedrag (sociale interactie). 1, 7 en 14 dagen na RSD werden de dieren getest voor anhedonisch gedrag (suiker voorkeurstest). Twee weken na de laatste RSD, werden de dieren gescand voor microgliose met [11C]PBR28. Er werd geen verschil gevonden tussen de open veld test, en geen operatie, RSD of tijds effecten in de suiker voorkeurstest. Dieren onder RSD hadden een lagere sociale interactie in de sociale interactie test. Dit werd interessant genoeg niet geobserveerd in de dieren die naast de RSD ADX hadden ondergaan. Er was geen verschil in neuro-inflammatie tussen de groepen. De resultaten lieten een effect van RSD op sociaal gedrag zien dat in lijn was met de verwachtingen, maar de HPA-as verstoring door ADX blijkt deze angst-response ten opzichte van andere dieren te blokkeren, hoogstwaarschijnlijk door aantasting van de vecht-of-vlucht response. Er waren geen verschillen in microgliose zichtbaar op de [11C]PBR28 scans, wat suggereert dat enig effect veroorzaakt door RSD twee weken later te niet gedaan was. Wij veronderstellen dat sterkere modulatoren van depressie noodzakelijk zijn voor het induceren van chronische depressie-achtige klachten en neuro-inflammatie.

In hoofdstuk 5 herhaalden we het RSD protocol, met aanpassingen om een sterkere, meer reactieve interactie tussen de resident en de binnendringer te bewerkstelligen. Daarnaast hebben we ook antidepressieve en anti-inflammatoire alkaloid harmine, of vehicle, intraperitoneaal toegediend voor 14 dagen. Het gewicht van de dieren werd dagelijks gecheckt, en de locomotie en angst-achtig gedrag werd geanalyseerd met behulp van een open veld test op 1 en 9 dagen na de laatste RSD. Anhedonie werd gemeten 1 dag voor, en zowel 1 dag als 10 dagen na de laatste RSD. Geheugen werd geanalyseerd 10 dagen na de laatste RSD. Neuro-inflammatie werd 11 dagen na de laatste RSD beoordeeld middels histologie, en de hippocampus en frontale cortex werden geëxtraheerd voor BDNF concentratie bepaling.

RSD had een significante invloed op gedrag en anhedonie, zoals waarneembaar is door een verhoogde angst parameter in het open veld, en verlaagde voorkeur voor water met sucrose 1 dag na RSD. Er was geen effect van harmine op deze parameters, maar harmine had een significante invloed op het gewicht en locomotor gedrag. Deze effecten duurden tot het einde van het experiment. De effecten gevonden direct na de laatste RSD normaliseerden echter op de lange termijn. RSD veroorzaakte een fluctuatie op de korte termijn (hier geobserveerd door de gedragsuitkomst van de eerste OF en de SPT na RSD), maar geen lange termijn effecten, wat geïllustreerd werd door het gebrek aan verschillen in

opname van [11C]PBR28 tussen de RSD en controle groepen, samen met de onaangetaste lange termijn gedragsveranderingen op angst- en depressie-achtig gedrag. De veranderingen van RSD of harmine resulteerde niet in veranderingen van de BDNF concentratie in de frontale cortex of hippocampus, regionen die een hoofdrol spelen in de stress regulatie en verdere hersenhomeostatis. Voor een betere uiteenzetting van de invloed van harmine in depressie en zijn werking als een antidepressivum, zijn vervolg studies nodig, die gebruik maken van andere stressoren of langere durende RSD protocollen voor het induceren van een chronische stress response in de proefdieren, om meer efficiënte analyse mogelijk te maken van hoe harmine werkt onder zulke omstandigheden.

Om te begrijpen hoe RSD werkt, is het ook belangrijk om de effecten van dit experiment te observeren op de andere partij: de agressoren. In hoofdstuk 6 evalueerden we hoe de dopaminerge – meer specifiek, de D2 receptoren – beïnvloed worden door deze situatie van "sociaal succes", en of het gerelateerd is aan een toename van agressie (geweld). Dieren werden getest voor agressie om mee te kunnen doen aan het RSD experiment, en diegene die geen agressief gedrag lieten zien (gedefinieerd als een gemiddelde aanval latentie van 60 seconden na 5 pogingen) werden als niet-agressief beschouwd. Dieren die als agressief werden beschouwd ondergingen meerdere experimenten met RSD. We gebruikten [11C]Raclopride voor het onderzoeken van de D2 receptoren in de striatum (caudatus en putamen) en nucleus accumbens (kern en buitenkant) en scanden op verschillende tijdpunten: 1 dag na laatste RSD (acute agressoren) en 14 dagen na laatste RSD (niet-acute agressoren), en vergeleken deze dieren met controles. Dieren met 14 dagen tussen de laatste RSD en [11C]Raclopride scan vertoonden meer agressief gedrag, met verlaging van de aanvalslatentie gedurende de tijd, in vergelijking met andere groepen. Deze effecten kwamen ook overeen met de striatale [11C]Raclopride binding potentiaal, met een bijna tweevoudige verhoging in vergelijking met de andere groepen. Echter, in de nucleus accumbens, werd geen verschil waargenomen tussen de groepen. De geobserveerde verhoging in de beschikbaarheid van D2 receptoren in niet-acute blootgestelde dieren komt overeen met de literatuur. Het uitblijven van verschillen in D2 binding in de experimenten kan verklaard worden door de heterogeniteit van de aanvalslatentie in onze onderzoeksresultaten. Dit kan een verklaring zijn voor de verschillen die gevonden werden gevonden tussen de acute en niet-acute blootstelling van agressieve dieren aan de binnendringers. Dat er voor de aanvalslatentie van de acute blootgestelde dieren geen associatie werd gevonden met de hoeveelheid experimenten waarin deze dieren meededen, suggereert dat hun agressiviteit wellicht niet genoeg was om een significant verschil in de binding potentieel van [11C]raclopride te kunnen waarnemen.

Concluderend: sociale stimuli hadden een impact op de hersenen, zoals we hebben laten zien door verschillen in gedrag, neurotrofine en synaptische plasticiteit markers, zowel in de verrijkte als in de

verarmde sociale omgeving. Het blijkt dat hoe langer de stimulatie, en hoe korter de analyse periode, hoe meer uitgesproken de gedragsverandering. Het lijkt er op dat langere stress protocollen noodzakelijk zijn om een chronische staat van angst- en depressieachtige gedrag in een diermodel van stress te kunnen waarnemen, met wellicht een kortere interval tussen stressoren en analyses. Dit was vooral van toepassing op het RSD experiment, aangezien we korte termijn effecten op het gedrag van dieren konden waarnemen, terwijl dit effect op lange termijn afwezig was. Neuro-inflammatoire en neurotrofe markers volgen wellicht hetzelfde patroon, zoals aangetoond is in voorgaande onderzoeken. Toekomstige onderzoeken die gebruik maken van RSD protocollen, of het resident-binnendringer model, dienen rekening te houden met de mogelijkheid dat niet alle binnendringers depressie-achtig gedrag zullen ontwikkelen. Daarnaast is het mogelijk dat residenten beter gescreend moeten worden op agressief gedrag, met mogelijk andere parameters naast de nu gebruikte aanvalslatentie. In onze onderzoeksopstelling, vertoonden residenten habituatie na herhaalde blootstelling aan het agressie protocol, zoals te zien is aan de onveranderde aanvalslatentie.

Acknowledgments

"Life before death. Strength before weakness. Journey before destination."

In his *magnum opus*, Brandon Sanderson made these very small sentences the foundation of a whole new world. Three sentences. Nine words. An immeasurable meaning, at least, for me.

But, truth be told, this is my PhD thesis, and these are my acknowledgments, so this is the moment I can write whatever I want. 'Such a conceited and arrogant kid' one might think, and in a way, one might be right. What is academia, if not a bunch of arrogant people trying to convince the reader that their work is a piece of art and the other one is not? That one's research is the tallest tree, the one that will get the most of the sunlight? This might hurt some people. The truth, it hurts…

…And Brandon Sanderson has nothing to do with it. Back to the topic. I will not try and explain what those words mean (I am not THAT conceited). But I will try to show why these very words should be here. Why they deserve a place in this book.

Life before death: that is the easy one. It is hard to die before living, and living is tougher than dying. Well, life must start somehow. And for that, I would like to thank the Big Bang for giving the starting point (Mom, this is the closest I can ever be of thanking God, so consider it a victory!). I would also like to thank my family. I will not bother with names. There are so many people, blood-related or not, that fits in this description perfectly. So consider this a collective hug, so that fewer people will be worried that "he forgot him or her, the dog and the cat". You all know how much you mean to me (dogs and cats included), especially when away from home for two years. With that being said, let us move on.

I would like to thank my four moms (yes, you read it right!). First, the one that gave me birth and carefully tended to me when I was just a toddler, listened to me and gave me tips on life when I was a teenager. The one and only Mom. Probably you have no idea how much you mean to me, so I will say it. Nara, I love you.

Mom number 2, Márcia. Thank you for being there whenever I needed. Thank you for the tips, for the scolds, for the love you gave me when I needed most. Thank you for your attention and support. Mom number 3, Rejane. You are probably the kindest person I have ever met. For being there always when I was alone, and also when I was not. For giving me an adoptive brother. Please, never change, because if you do the world will become more miserable. Thank you, I love you.

Mom number 4, and by far the most important. Dad. What you did, what you sacrificed for me and my brother is immeasurable, and it is impossible for us to ever repay what you gave us. I saw you working from 7 A.M. until 10-11 P.M. and start all over again the next day. I saw you working tirelessly without having a single vacation day for years and years. And you were always there, like a standing stone, solid on the ground. Or like an island being hit by the waves, unwavering, even when hit by the worst storms. Always there. A safe haven for us whenever we needed. You encouraged me to become a better person, criticized me when I was on my way to not being one, but most of all, you are always there when I just need to talk, even the most trivial things ever.

To call you a hero would be overestimating heroes. You are better. You are my father. And for that, thank you. I love you.

Strength before weakness*:* in an epic fantasy setting like the one from the book I borrowed the sentence of, it fits almost perfectly: if you are strong, help those that are in need. Simple as that. But this is a PhD thesis and I see no fantasy castles nearby (this is The Netherlands after all…). So I had to come up with something more based on… reality.

Science usually follows this romantic circle of being curious with something, then experiment on it, explain what happened, lay the foundations for future possibilities, become curious again. Rinse and repeat (better rinse three times with PBS for five minutes under gentle agitation). This is science. But it is virtually impossible to know everything about everything and all steps to achieve flawlessness. Thus, to have someone that can support you with something you lack, and on the other hand give support to those that need help, especially in things you excel at.

Strength before weakness. Help those in need; be helped whenever in need.

The problem of a Ph.D.² is that I need to thank the double amount of people. So let us go in order. From Brazil, I have many people to thank for their scientific support and help. Kelem, Márcio, Daiane, Juliano, Betânia, thank you for being around helping me whenever I needed help. Thank you for letting me help you with your amazing projects. Believe me when I say that this book would not be the same without your help. You guys are awesome, and I wish you the best in the future.

From The Netherlands: first I need to thank the biocrew: Rodrigo, Anna, Luíza. You guys were amazing in teaching me things about PET and rats (ok, ok, and fishes as well…) that I had absolutely no idea. For helping me whenever I had to split myself in three to finish a day of work. For just saying "don't worry, we are here if you need", and I could be sure that you actually meant it, and you would be there whatever happens. For pointing out what I did wrong, for praising what I did right. I could keep writing about it for a long time, but I think I can summarize it. You guys are the best. Thank you for everything.

I also need to add Paula somewhere around here, since the foundation of three projects of mine came from her hard work (and she would hit me if I did not place her here). You are an amazing researcher, and I am proud of having you as a friend as well. For that and much more, thank you.

Lara, Laura, Nynke, and the professors from Neuroscience and Cell Biology departments that allowed and helped me in performing many of the molecular analysis, thank you very much for your support and for your teachings.

From the staff of the CDP: Michel, Daryll, Ar, Gerward, Miriam, Catriene, Minke. Your skill and knowledge of animal behavior, physiology and the support in surgeries, and how to deal with the bureaucracy of working with small animals were invaluable. For taking care of my animals and for being there if needed, thank you very much.

Jurgen, thank you for all the training and support you gave me for the use of PET. This thesis would take at least one year more to be finished if not for you. Also, for managing and maintaining the

PET camera and all its intricacies, I will not be the first nor the last to say: thank you very much for your help.

As they say in the corridors of the department: no tracer no fun. So a big, very big thank you for the staff that helps to produce the tracers. That includes Bram, the cyclotron master; Chantal, Gina, and Janet, for producing the tracers that we use. Rolf, thank you for all the help you gave in C-Lab and quality control. Inês, Khaled, Gonçalo, Lara, Verena, thank you for helping with whatever questions I had in a chemistry laboratory (and for the laughs). You are all amazing, and I wish you the best.

David, thank you very much for all the help you gave me: from designing experiments together when I had absolutely no idea on how to make a PET study and all the support with the PET data analysis and maintenance of the system. Thank you for all the teachings on PET data analysis and statistics. Without you, this thesis would not happen. So thank you for everything.

***Journey before destination*:** This one is very easy to figure out, but very hard to put it into words. Every story comes to an end eventually and there is a moment where you look behind you and realize everything that happened up to this point. A teacher I had at the beginning of my PhD once said that we are what we make of our memories and that those memories shape our future selves (interestingly I forgot half of the saying, so that might actually sound somewhat hypocritical, but anyway…). Imagine this part of life as climbing up a mountain (definitely not in The Netherlands): you start at the base of it, and steadily climb up, initially stumbling over every step, but as you build enough experience in climbing, you actually start to enjoy it. And at the end of four years of climbing, you reach the top and look down, observe the landscape, what you went through to reach this moment.

Breathtaking.

Well, in the previous part I thanked everyone that helped me in the climbing, giving me the experience needed to perform it smoothly and safely. But now, this part is about all the people that allowed me to enjoy this climbing. The landscape. The mountain. The people, so many. It is difficult to find a starting point. But I need to start somehow.

A big, very big thank you to my brothers. Rafael, Aleksander, Miguel, Leandro, Felipe. The first one by blood, the other ones by life. We had amazing moments and I cannot thank you all enough. It seems that whatever I write here will eventually lack something, so I will simply say: thank you for everything. You are the best. Thank you for everything.

Thank you again for Kelem, for being such a great friend. I really hope you have a great time now in this new step of your life. Be always this precise and meticulous person, and you will go far in whatever path you wish to take, and I am proud of calling you a big, big friend.

Márcio, Fabiano, thank you for being amazing friends, for all the talks about politics, ethics and whatever comes to mind at 8, 9 P.M. For being always there whenever we just wanted to talk, without prejudice, without criticizing one another, just three friends discussing unfriendly matters, friendly matters, or completely irrelevant and useless matters. I could not seem to get enough of that. You guys are among the smartest people I ever met, and I hope we will be able to reunite ourselves again and have a nice “coffee with ethics” once again. My brothers, thank you.

Débora (Dérba), Natália, Stefani, Paula (Bittencourt). For all the talking, all the stupidity and all the time spent together, you are basically my sisters. Annoying sisters, from time to time, but in a good way (well… maybe not always *insert evil laugh here*). Thank you for everything.

Luiza, firstly, thank you for opening the doors for me to the Netherlands. I'll never be able to repay this, but I'll do my best. Now, that being said, thank you for being such an amazing person, always there to help and supportive in many ways. I hope you get the best in life because you deserve that and much, much more. You are amazing. Thank you from the bottom of my heart.

Débora (Débs), Gui. I wish you all the best in the coming years because you deserve it. Thank you for being such amazing people and for the laughter, memes, games, and whatever stupid talk or YouTube video comes up in our heads.

We could make a religion out of it! Thank you for everything.

Gabriel, Vivian (or, as we know, the Marmitts). We know each other for a relatively short period of time, but it seems that we are friends for years. All the weekends spent together made me feel like we were basically a Brazilian family stranded in the middle of The Netherlands. Those were great days, and I sincerely hope some more gatherings will come by, even if I am a tad bit away from you guys. Thank you.

A big thank you to Anna, who helped me not only in my experiments, but also in helping me make sense of data and kinetic modeling, and also for all the talking. Oh, and the occasional chocolate! Always helpful! Thank you.

To everyone at the office during my time here, it was a pleasure and an honor to share the place with such amazing people, both not-so-new and new ones. I expect lots of exciting researches on whatever field you are, or plan to be in the future, and hope that you guys can achieve all the things you want in any field you'd like to be. Thank you for many laughs, many (un)scientific discussions, fun and sad moments. Those are the ones that make us who we are, so I hope they are as precious to you as they were to me.

For the people that are not from the office, but still amazing friends I got from my work, a big thank you. From the people that are not even in the hospital, but are part of my life in The Netherlands, thank you.

To the people of the football (on both sides of the Atlantic), thank you. Those moments were lots of fun, and I hope I improved a bit on the game (likely not, but I'll keep playing anyway).

A los bandidos, muchas gracias por todo!

Last, but far from least, my paranymphs.

Rodrigo, I think it's safe to say that you're the most charismatic person I ever met in my whole life. Also, you are an outstanding researcher. Without you, this thesis would not be done. I could easily place your name on the cover of this thesis and no one that saw us working would be able to disagree. I lost the count of how many times you helped me, taught me, and supported me.

Kars, you are one of the kindest people I've known, even though you try very hard to hide that fact. You are the kind of annoying brother that is always trying to… well… annoy others, but is the first one to come in defense of those you like, doing everything you possibly can to help. Don't ever change, be always this honest person and this amazing researcher.

I am proud of working with both of you, but I am even prouder of calling you my friends.

Thank you for everything.

I guess that's it. This seems to be the end of a fun, tough journey. Well… since this counts for two PhDs, I assume journeys would be more appropriate. But as much of an end this book is, it is also another step towards another beginning. But, as Robert Jordan also wrote once, a long time ago:

"There are neither beginnings nor endings to the Wheel of Time"

Thank you

About the author

Bruno Lima Giacobbo was born in Porto Alegre, in the State of Rio Grande do Sul, Brazil, in 1988. Bruno pursued his Bachelor in Biological Sciences at the Rio Grande do Sul State Pontifical Catholic University (PUCRS) in 2006, obtaining his B.Sc. diploma in 2011. In 2008, Bruno entered the Laboratory of Biology and Nervous System Development under the supervision of professor Elke Bromberg by the Science Ministry's Scientific Initiation program. In 2012, Bruno entered the Master's program in Molecular and Cellular Biology, once more under tutelage of Prof. Bromberg, obtaining a graduate scholarship issued by the Brazilian government.

Bruno initiated his studies as a Ph.D. in Molecular and Cellular Biology in the beginning of 2015, a few months after obtaining his Ms.C. diploma at the same program. During his Ph.D. Bruno entered in contact with Professor Erik de Vries about the possibility of finishing the last part of his studies in Groningen's University Medical Center (UMCG) Department of Nuclear Medicine and Molecular Imaging. Thanks to the Abel Tasman Talent Program (ATTP), Bruno was offered a position as a Ph.D. student under the tutelage of Professors E. De Vries and Rudi Dierckx, and supervision of Janine Doorduin, in order to complete his double Ph.D. degrees in Molecular and Cellular Biology and Nuclear Medicine and Molecular Imaging.

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