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. 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. 

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. 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\textsuperscript{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\textsuperscript{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\textsuperscript{16}), environmental effects are the main contributing factors for disease onset, as was shown in several experimental studies with different stressors in humans\textsuperscript{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\textsuperscript{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)\textsuperscript{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, p75NTR 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 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 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)\(^\text{34}\). 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\(^\text{35}\).

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

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic (unpredictable) mild stress - CUMS</td>
<td>Daily use of different stimuli over a long timeframe to induce a recurrent stress response in the animal.</td>
</tr>
<tr>
<td>Forced swim test - FST</td>
<td>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.</td>
</tr>
<tr>
<td>Chronic immobilization</td>
<td>Considered a mild- to moderate stressor, it places the animal in a frame where it cannot move. Test duration varies greatly in literature.</td>
</tr>
<tr>
<td>Social isolation - (SI)</td>
<td>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.</td>
</tr>
<tr>
<td>Repeated social defeat - RSD</td>
<td>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.</td>
</tr>
</tbody>
</table>
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\textsuperscript{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 ($\beta^+$ 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\textsuperscript{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.


37. Real, C. C. et al. Evaluation of exercise-induced modulation of glial activation and
