Social stress: the good, the bad, and the neurotrophic factor
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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 \textit{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. Depression is a multifaceted disorder, with many different factors contributing to its development and progression. 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.

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. 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. 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 \textsuperscript{\text{11}}C\text{PBR-28} scan. In this study, we addressed if treatment with antidepressant and anti-inflammatory compound harmine \textsuperscript{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 \textsuperscript{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 \textsuperscript{\text{11}}C\text{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 \textsuperscript{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. However, the results of our study should be interpreted with care, as there was a very large variance in the $^{[1]}$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. Therefore, researchers investigating cognition in a preclinical setting tend to “bestialize” human behaviors, 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. Several studies have shown an effect of RSD on behavioral outcomes in the short-term (reviewed by Hollis et al.), 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. 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. 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. 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. 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. In humans, aggression has also been associated with altered D2R expression, although there are also reports stating otherwise. 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. In both cohorts, [11C]-raclopride PET imaging was applied to assess D2 receptor availability and compared with non-aggressive control animals. As the timing between the last RSD trial and the [11C]-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 D2 receptor availability, with a two-fold increase in the binding potential of [11C]-raclopride when compared with the other groups.

Striatal D2 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), the dorsal striatum plays a role in habit formation. As binding potential is increased, it means more availability of D2 receptors, which could indicate that less dopamine is bound to this receptor, 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. 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 D2 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 D2 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 D1 receptor availability, as it is already shown that D1 and D2 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.
References


