ESCOLA DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM GERONTOLOGIA BIOMÉDICA
DOUTORADO EM GERONTOLOGIA BIOMÉDICA

PAULA KOPSCHINA FELTES

THE TWO SIDES OF THE COIN OF PSYCHOSOCIAL STRESS:
EVALUATION BY POSITRON EMISSION TOMOGRAPHY

Porto Alegre
2018

PAULA KOPSCHINA FELTES

THE TWO SIDES OF THE COIN OF PSYCHOSOCIAL STRESS: EVALUATION BY POSITRON EMISSION TOMOGRAPHY

Tese apresentada ao programa de Pós-Graduação em Gerontologia Biomédica da Pontifícia Universidade Católica do Rio Grande do Sul, como requisito parcial para a obtenção do título de Doutor em Gerontologia Biomédica.

Orientador PUCRS: Prof. Dr. Cristina M. Moriguchi Jeckel
Aspectos biológicos no envelhecimento

Porto Alegre
2018

Ficha Catalográfica

K83t Kopschina Feltes, Paula

The two sides of the coin of psychosocial stress : Evaluation by positron emission tomography / Paula Kopschina Feltes . – 2018.
196 p.
Tese (Doutorado) – Programa de Pós-Graduação em Gerontologia Biomédica, PUCRS.

Orientadora: Profa. Dra. Cristina Maria Moriguchi Jeckel.

1. Psychosocial stress. 2. Positron emission tomography. 3. Depression. 4. Agression. 5. Repeated social defeat. I. Moriguchi Jeckel, Cristina Maria. II. 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

PAULA KOPSCHINA FELTES

THE TWO SIDES OF THE COIN OF PSYCHOSOCIAL STRESS EVALUATION BY POSITRON EMISSION TOMOGRAPHY

Tese apresentada ao programa de Pós-Graduação em Gerontologia Biomédica da Pontifícia Universidade Católica do Rio Grande do Sul, como requisito parcial para a obtenção do título de Doutor em Gerontologia Biomédica.

Aprovada em: _____ de _____ de _____.

BANCA EXAMINADORA:

_____
Prof. Dr. Moisés Evandro Bauer - PUCRS

_____
Prof. Dr. Bartholomeus Johannes Leonardus Eggen - RUG

_____
Prof. Dr. Robert Anton Schoevers - RUG

AGRADECIMENTOS

Gostaria de expressar a minha gratidão a todos do Programa de Pós-Graduação em Gerontologia Biomédica (PUCRS) pelos esforços em coordenar o doutorado com dupla diplomação em colaboração com a Universidade de Groningen (RUG). Meus sinceros agradecimentos a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa de estudos concedida durante o Doutorado.

RESUMO

Sem dúvida a expectativa de vida tem aumentado de forma constante nos últimos 200 anos. Predições para o ano de 2030 apontam que aproximadamente 20% da população terá mais de 65 anos de idade. Neste contexto, é importante compreender fatores que possam afetar a qualidade de vida dos idosos, como doenças neurodegenerativas e distúrbios psiquiátricos como a depressão maior (MDD). A MDD afeta aproximadamente 350 milhões de pessoas em todo o mundo, sendo a doença psiquiátrica mais comumente diagnosticada em idosos e tem como fator de risco o estresse. Durante o envelhecimento há uma redução gradual da capacidade adaptativa ou de recuperação ao estresse (resiliência). Diferenças individuais no processo de envelhecimento podem ser conceituadas como o acúmulo do desgaste diário e estressores maiores de vida, que interagem com a constituição genética e possível presença de adversidades durante a infância e adolescência. Apesar de esforços consideráveis, os mecanismos patofisiológicos completos envolvidos na depressão permanecem desconhecidos. Uma hipótese recente implica a neuroinflamação como importante componente na MDD. Portanto, uma investigação aprofundada em relação às vias biológicas relacionadas ao estresse e à neuroinflamação podem auxiliar no melhor entendimento desta doença. No presente estudo, observamos que a ativação do sistema hipotalâmico-pituitário em resposta ao modelo animal de derrota social é capaz de induzir (neuro)inflamação e sintomas depressivos em roedores. Adicionalmente, demonstramos neste modelo animal que a exposição a eventos adversos durante a adolescência impacta significativamente na resposta comportamental e neuroinflamatória a situações de estresse recorrentes durante a vida adulta. Além disso, observamos que a exposição e a vitória repetidas em confrontos agressivos no modelo de derrota social alteram receptores cerebrais dopaminérgicos, sugerindo um possível efeito de formação de hábito, aumentando comportamentos violentos e agressividade em indivíduos. Nos estudos descritos na presente tese, utilizamos a tomografia por emissão de pósitrons (PET), uma técnica de imagem funcional não invasiva para investigar os mecanismos patofisiológicos envolvidos na MDD e também, em agressividade. Como a mesma metodologia de investigação pode ser aplicada em humanos, o PET é uma ferramenta que pode fornecer informações

importantes relativas a alterações cerebrais tanto em pacientes depressivos quanto agressivos, contribuindo para o diagnóstico e terapia personalizadas.
Palavras-chave: Depressão maior. Tomografia por emissão de pósitrons. Envelhecimento. Inflamação. Glia.

ABSTRACT

Life expectancy has undoubtedly increased over the past 200 years. Predictions for the year of 2030 indicate that approximately 20% of the population will be over 65 years old. In this context, it is important to understand risk factors that may affect the quality of life of the elderly, such as neurodegenerative diseases and psychiatric disorders. Major depressive disorder (MDD) affects approximately 350 million people worldwide, being the most commonly diagnosed psychiatric disease in the elderly. Stress is a known risk factor for MDD development. During aging there is a gradual reduction of the adaptive capacity for stress recovery (resilience). Individual differences in the aging process can be conceptualized as the accumulation of daily stress and also major life stressors, which interact with the genetic constitution and possible presence of adversity during childhood and adolescence. Despite considerable efforts, the complete pathophysiological mechanisms involved in depression remains unknown. A recent hypothesis implies neuroinflammation as an important contributor to MDD. Therefore, an in-depth investigation of the biological interplay between stress and neuroinflammation may be useful in providing a better understanding of the disease. In the present study, it was observed that the activation of the hypothalamic-pituitary system in response to the social defeat animal model is capable of inducing neuro(inflammation) and depressive-like symptoms in rodents. Additionally, we demonstrated in the same animal model that exposure to adverse events during adolescence significantly impacts behavioral and neuroinflammatory response to recurrent stress later in life. Furthermore, repeated exposure to winning confrontations of the residents in the social defeat model may alter dopaminergic brain receptors, suggesting a possible habit-forming effect that increases aggressive and violent behavior in subjects. In the present thesis, we have used positron emission tomography (PET) as a non-invasive functional imaging technique in order to investigate the pathophysiological mechanisms involved in MDD and aggression. Since the same investigational methodology can be applied to humans, PET can provide important information related to brain alterations in depressive and aggressive patients, contributing for personalized diagnosis and therapy.

Key-words: Major depression. Positron Emission Tomography. Aging. Inflammation. Glia.

The two sides of the coin of psychosocial stress: evaluation by positron emission tomography

Paula Kopschina Feltes

The two sides of the coin of psychosocial stress: evaluation by positron emission tomography

Paula Kopschina Feltes

The research reported in this thesis was carried out within the Department of Nuclear Medicine and Molecular Imaging of the University Medical Center Groningen, in collaboration with the Biomedical Gerontology Graduate School at the Pontifical Catholic University of Rio Grande do Sul.

The reported research contained in this thesis was financially supported by a scholarship from the Graduate School of Medical Sciences (Abel Tasman Talent Program) of the University Medical Center Groningen and from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

Printing of this thesis was financially supported by the University Medical Center Groningen, and the research school of Behavioral and Cognitive Neurosciences (BCN).

Cover design and layout: Paula Kopschina Feltes
Image artist: Christos Georghiou
Printing: Ridderprint BV, The Netherlands
ISBN: 978-94-034-0791-3 **(**Hard copy)
ISBN: 978-94-034-0790-6 (electronic version)
Dissertation of University of Groningen, Groningen, The Netherlands
Dissertation of Pontifical Catholic University of Rio Grande do Sul, Rio Grande do Sul, Brazil

university of groningen

PUCRS
Pontifícia Universidade Católica do Rio Grande do Sul

The two sides of the coin of psychosocial stress: evaluation by positron emission tomography

PhD thesis

to obtain the degree of PhD of the
University of Groningen
on the authority of the
Rector Magnificus Prof. E. Sterken
and in accordance with
the decision by the College of Deans.

and

to obtain the degree of Doctor of Biomedical Gerontology of the
Pontifical Catholic University of Rio Grande do Sul
on the authority of the
Rector Magnificus Prof. Evilásio Teixeira
and in the accordance with
the decision by the College of Deans.

Double PhD degree

This thesis will be defended in public on

Wednesday 11 July 2018 at 16:15 hours

by

Paula Kopschina Feltes

born on 8 February of 1989
in Itajaí, Brazil

Supervisors

Prof. R.A.J.O Dierckx

Co-supervisors

Dr. E.F.J. de Vries

Dr. J. Doorduin

Dr. C.M. Moriguchi-Jeckel

Assessment Committee

Prof. R. A. Schoevers

Prof. B. J. L. Eggen

Prof. M.E. Bauer

Paranymphs

Dr. Anniek K.D. Visser

Dr. Luis Eduardo Juárez-Orozco

Table of Contents

GENERAL INTRODUCTION

CHAPTER 1

1. Introduction

"Men ought to know that from the brain and from the brain only arise our pleasures, joys, laughter, and just as well our sorrows, pains, grieves and tears." - Hippocrates

Transient sadness is a natural part of life. However, for some individuals, sad mood is present in a more intense and persistent manner. Many centuries after the Hippocratic view that emotions are derived from our brain only, this paradigm was broken and studies demonstrated that several systems and organs, including the brain, interact and communicate in the realm of emotions and mood disorders. Depression is one of the leading causes of disability worldwide, predicted to cause the biggest economic burden to society by 2030 (1). Despite major research efforts, the complete pathophysiology of depression remains unknown and it is plausible that multiple subtypes exist with different pathophysiological mechanisms (2).

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM), depression consists of several symptoms related to low mood. The DSM-V revision (3) recognizes sad mood and loss of pleasure (anhedonia) as core symptoms of depression. Next to the core symptoms, somatic correlates of a negative mood state are included as symptoms. Somatic symptoms comprise aberrant levels of energy and sleep, as well as mood, weight and food intake fluctuations. Cognitive correlates of a negative mood are also recognized and include subjective impairments of concentration and decision-making, as well as thought patterns of guilt, worthlessness and suicidal ideation.

A number of large population studies generated statistics regarding the prevalence, onset and history of the disease. In the United States of America (USA), the National Comorbidity Survey gave lifetime prevalence estimates of 15-20% and one-month prevalence estimates of 5% for adults (4). In the aged population, depression is the most commonly diagnosed psychiatric condition (5). This is very alarming considering that roughly 20% of the worldwide population will be over 65 years old by the year of 2030 (6). The median age at the onset of major depressive disorder (MDD) is around 20-25 years old (7). Estimates of occurrence range from 50-90%, with increasing odds of recurrence and chronicity with each new episode (8). These findings substantiate that MDD has a very large economic impact to society, mainly due to healthcare costs and loss of productivity (1). Not only the high incidence, disability, mortality (suicide) and the economic burden associated with the disease, but also the high rates of inadequate

treatment of the disorder are a serious concern. Main antidepressant therapies used in the clinical practice modulate monoaminergic neurotransmitter function. This includes the antidepressant classes of selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), tricyclic/tetracyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs) and atypical antidepressants (9). It is estimated that 30-50% of the patients do not respond to treatment with antidepressants (10) due to either lack of efficacy or intolerable side effects (11). Moreover, psychotherapeutic interventions have reported disappointing results (12). The complex and heterogeneous nature of MDD may contribute to these modest results and suggests that other neuromolecular pathways than an imbalance in monoamines targeted by conventional antidepressants are involved. Treatment that works for one specific individual might not work for another, resulting in an attenuated treatment effect. Therefore, unaccounted heterogeneity in symptoms of depression may arrest our knowledge about the aetiology and effective treatments for MDD.

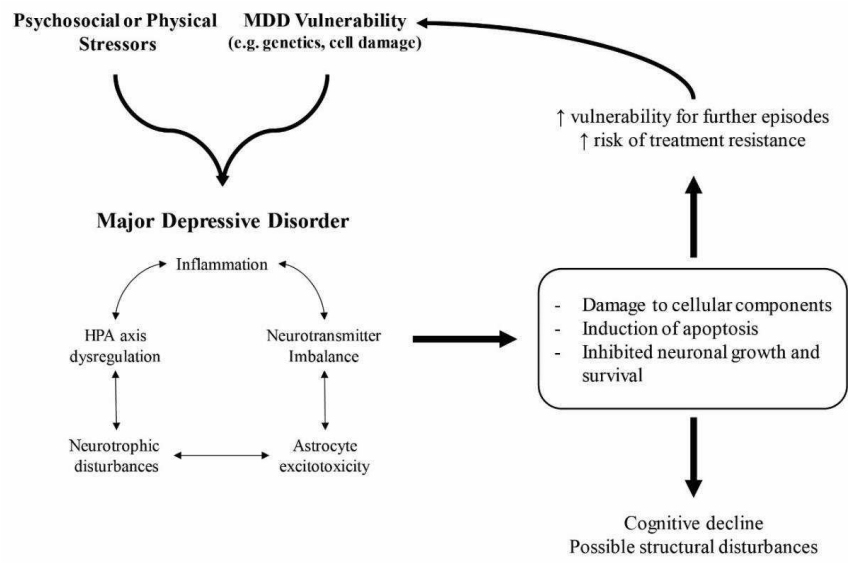


Figure 1: Psychosocial and physical stressors combined with a possible pre-existing predisposition to major depressive disorder (MDD) might induce a first depressive episode. Biochemical processes such as (neuro)inflammation, HPA axis dysfunction, imbalance in neurotransmitter systems, disturbances in neurotrophic factors and astrocyte excitotoxicity interact and may induce cellular damage and apoptosis and inhibit neuronal growth and survival. All these factors might influence treatment response to conventional treatment with antidepressants, increasing the vulnerability of the depressed individuals to further depressive episodes. Moreover, each episode can contribute to cognitive decline and alterations in brain structures. (Adapted from Moylan et al, 2013 (14)).

1.1 Depression as a multifactorial mental disorder

Besides genetic predisposition (35-40% heritable) (13) and dynamic environmental influences, numerous neurobiological mechanisms have been proposed to contribute to the pathogenesis of MDD. Possible pathways include neurotransmitter systems, neurotrophins (14), astrocyte excitotoxicity (2), (neuro)inflammation and HPA axis dysfunction (15) (Figure 1).

1.1.1 Neurotransmitter dysfunction (serotonergic, dopaminergic and noradrenergic systems)

Serotonin (5-HT) is the neurotransmitter most extensively associated with mood disorders such as MDD. 5-HT is produced from tryptophan, an essential amino acid catabolized by indoleamine-2,3-deoxygenase (IDO). Pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) present during an inflammatory state can activate IDO, leading to 5-HT depletion and synthesis of tryptophan catabolites, including kynurenine (KYN) and quinolinic acid (QUIN) in the plasma and in the brain (16). Many antidepressants aim to counteract this effect by increasing synaptic 5-HT levels, for example by inhibiting the neuronal reuptake of the neurotransmitter.

Noradrenergic alterations have been strongly associated with MDD and many antidepressants elevate the synaptic availability of norepinephrine by inhibition of reuptake and/or blockade of presynaptic α-2 adrenoceptors (17). Indeed, post-mortem and functional imaging studies revealed altered density and sensitivity of α-2 adrenoceptors (which modulate noradrenaline release) in the prefrontal cortex of depressed suicidal victims (17; 18).

Diminished levels of dopamine (DA) are also related to MDD. The suggested physiological alterations underlying reduced DA signalling could result from diminished DA release from the presynaptic neurons or impaired signal transduction, either due to changes in receptor number or function and/or altered intracellular signal processing (19). DA is a neurotransmitter involved in motivation, which many MDD patients lack. Interestingly, the increase in 5-HT levels following SSRIs administration might reduce DA neuronal activity. Thus, alterations in dopamine function may partially underpin the resistance towards antidepressants (19). Also, a decreased turnover of homovanillic acid which is the primary metabolite of dopamine has been found in individuals with MDD (20), a finding consistent with depressogenic effects of dopamine depletion in MDD patients (21).

1.1.2 Neurotrophins

Neurotrophins are key mediators of normal neurogenesis and numerous findings support a role of neurotrophins and neurogenesis in MDD. MDD is associated with reduced levels of neurotrophins such as the brain-derived neurotrophic factor (BDNF) (22) and vascular endothelial growth factor (23). Administration of these factors have demonstrated antidepressant effects in animal models and traditional antidepressants were shown to normalize neurotrophin levels in responding patients (24; 25).

1.1.3 Astrocyte excitotoxicity

Astrocytes play a role in synaptic transmission and information processing (26). They express glutamate receptors and therefore, respond to glutamate that spills over from synapses. In turn, this spill over triggers the release of glutamate by glial cells that can modulate neuronal activity, synaptic transmission, plasticity, and also induce excitotoxicity and contribute to neuronal damage and/or dysfunction (27). Glutamate is a major excitatory neurotransmitter in the human brain and its reuptake is critical for regulating concentrations in the synaptic cleft and maintaining normal synaptic activity. Impairment in glutamate transport may thus result in excessive or dysregulated glutamate receptor signalling (28). Under physiological conditions, astrocytes prevent excitotoxicity by maintaining extracellular glutamate levels in the micromolar range via high-affinity glutamate transporters: excitatory amino acid transporter (EAAT) 1 and 2. However, this balance may be disrupted by oxidative stress or (neuro)inflammation leading to necrosis and/or apoptosis through excessive stimulation of glutamate receptors (28).

Furthermore, glutamate transmission via N-methyl-D-aspartate receptors (NMDAR) is crucial for neuronal survival and synaptic plasticity (29). The extrasynaptic NMDAR has been an increasing focus of attention regarding the deleterious effects of glutamate. Extrasynaptic neurotransmission inhibits extracellular signal-regulated kinases. Moreover, extrasynaptic NMDAR activation enhances nitric-oxide production, which is mainly responsible for synaptic damage (29; 30). Ketamine, a NMDAR antagonist has been implicated as a rapid antidepressant, possibly exerting its mechanism of action through the inhibition of extrasynaptic NMDAR 2C and 2D subunits (29).

1.1.4 (Neuro)inflammation and HPA axis dysfunction

Over the last two decades psychiatric research has provided support for the hypothesis that inflammatory processes and brain-immune interactions are involved in the pathogenesis of MDD and may contribute to the serotonergic and noradrenergic dysfunction (31). Inflammation, infection, cell damage or stress might trigger glial cells, in particular microglia cells, to release pro-inflammatory cytokines that may affect the hypothalamic-pituitary-adrenal (HPA) axis and serotonergic and noradrenergic signalling, ultimately leading to MDD and neurodegeneration (15; 32).

The immune and neuroendocrine systems function together in order to restore and maintain physiological homeostasis during inflammation or other harmful stimuli which induce cytokine production. Increased cytokine production may contribute to the development of depression directly via activation of the HPA axis or indirectly through cytokine-induced glucocorticoid (GC) receptor resistance (33). The release of TNF-α and IL-6 increases the production of corticotrophin-releasing hormone, adrenocorticotropic hormone and cortisol by acting directly on hypothalamic and pituitary cells (5). Cytokines might also increase GC receptor resistance through several signalling pathways, including activation of the p38 mitogen-activated protein kinase (MAPK) and by stimulating changes in the expression of GC receptors (33; 34). HPA hyperactivity has been associated with the pathophysiology underlying suicidal behaviour, excessive activity of the noradrenergic system and dysfunction of the serotonergic system (35; 36).

As previously mentioned, pro-inflammatory cytokines increase the activity of IDO and reduce the production of 5-HT through the kynurenine pathway (16; 37; 38), producing KYN and QUIN (39; 40). QUIN exerts agonistic effects on NMDAR leading to excitotoxicity, inhibits glutamate uptake and may cause degeneration of nerve cells and hippocampal cell death. Furthermore, pro-inflammatory cytokines influence neurotransmitter function through disruption of tetrahydrobiopterin (BH4). BH4 is an essential co-factor for the enzymes phenylalanine hydroxylase, tryptophan hydroxylase and tyrosine hydroxylase, which are rate-limiting enzymes for the synthesis of 5-HT, DA and norepinephrine, respectively (41). BH4 is also a co-factor for the enzyme nitric oxide synthase (NOS) that is responsible for the conversion of arginine to nitric oxide (NO) (42). Pro-inflammatory cytokines stimulate the production of NO, increasing the utilization of BH4 and thus decreasing neurotransmitter synthesis (41).

Since the present thesis will mainly focus on the (neuro)inflammatory hypothesis of depression, this topic will be further discussed in **Chapter 2**.

1.2 Stress as a risk factor for MDD development

Stress has been shown to be a major risk factor for developing depression and further sensitization to stress may occur as the disorder progress (43–45). Further investigation of biological pathways related to stress in the depressed population might help to understand the stress-related aetiology of depression (46). The HPA axis is functionally linked to the immune system as GCs (e.g. cortisol) regulate inflammatory responses and increased inflammation is a consequence of stress system activation (47). Exposure to early life stress, for example, is a distal risk factor that is considered a predictor of MDD with an onset in adolescence or early adulthood. Major life events such as loss of a loved one, job loss and divorce often precede depressive episodes later in life (48). This concerns up to 80% of the episodes observed in the general population (49). Virtually anyone will experience major negative life events during life, yet only 20-25% of the population develop depression afterwards (50).

1.3 Vulnerability and resilience to depression

Prolonged stress induces neuroimmune and neuroendocrine responses, and individual differences in these responses likely shape behavioural vulnerability and resilience. In some individuals, overactive unresolved stress responses may increase stress vulnerability and ultimately the development of mood disorders, such as MDD. However, most individuals mount adaptive coping mechanisms (i.e. response in reaction to a stressor) that promotes resilience when facing stress (51). These coping strategies involve reactive or passive strategies. A passive coping involves low aggressiveness, impulsivity and flexibility, with a general tendency to passively accept or introvertly shy away from similar stressful situations. In turn, a reactive coping style is generally characterized by a high level of aggression, impulsivity and other bold/extrovert actions, indicating active attempts to counteract a stressful stimulus (52). These different coping styles have also been associated with distinct patters of the neuroendocrine (re)activity patterns (53).

The HPA axis activation in response to stress increases circulating GCs by promoting their synthesis and release from the adrenal cortex, resulting in widespread physiological, hormonal and neurobiological effects. This circuit may be altered in the chronically stressed brain (51). GCs binds to steroid receptors expressed ubiquitously throughout the brain, altering gene expression and affecting synaptic plasticity, structural remodelling, and ultimately behavioural responses to stress and adaptive coping mechanisms of resilience (54). Moreover, GCs may produce a persistent sensitization of

microglia – maintaining a pro-inflammatory state despite resolution of the inflammatory state - that primes neuroimmune responses to subsequent events (51; 55).

1.4 Animal models of stress-induced depressive-like behaviour

Even though considerable progress has been made in non-invasive human studies of brain structure and function, such studies are still limited in their ability to investigate a causal role in the physiology and molecular biology of the depressed brain (56; 57). This has resulted in a demand for animal models of depression for hypothesis testing and to further understand underlying mechanisms in MDD. However, the choice of which biological correlates to study is not easy, since problems with animal models of human psychiatric disorders include: 1) the difference between the human and animal nervous system; 2) the difficulty in determining analogous behaviours among species (Table 1); and 3) the need of extrapolation of results from animals to humans (58). Such problems most likely reflect a significant difference in aetiology and complexity of depressive behaviour.

Table 1: Comparison of core symptoms of depression in humans with the possible analogous parameters assessable in rodents (59).

Core symptoms in humans	Analogous parameters in rodents
Loss of enjoyment	Anhedonia
Loss of motivation	Passive coping strategies; low locomotor activity
Sleep disturbances	Altered sleep/activity patterns
Anxiety	Anxiety-related behaviour
Hypercortisolism	Hyperactivity of the stress system

Ideally, an animal model should fulfil at least three major criteria: i.e. having face validity, construct validity, and predictive validity, supplemented by a criterion for aetiological validity (60; 61). To summarize these criteria: animal models must resemble the human condition in several aspects, including 1) similarity between the behavioural phenotype and the clinical-symptom profile (face validity); 2) amelioration or attenuation by clinical effective antidepressant treatments and, conversely, absence of changes by clinically ineffective treatment of the human disorder (predictive validity); 3) triggering by events that are known to be important for eliciting the human disorder (aetiological validity); and 4) similar neurobiological underpinnings (construct validity) (61).

An important aspect to consider when selecting an animal model for depression, is that depression is a symptomatic heterogeneous disease. Thus, individual animal models would be expected to simulate endophenotypes or a subset of symptoms, which

are likely defined by the conditions applied. It is essential to use realistic induction conditions (aetiological validity) and to ensure the legitimacy of the underlying pathology (61). In that sense, many depressive-like behaviour animal models have been developed and applied, either based on natural or artificial animal behaviours (58).

In general, stress models with a good aetiological, face and constructive validity have been described (61). From a biological point of view, the social environment should be considered as a source of stress and the processes of fighting for control and losing control are of central importance to the psychosocial situation of the individual. In humans, loss of rank, social status, and/or control are examples of a more general class of loss events, which are increasingly recognized as the specific type of "life events" that are associated with depression (61). Moreover, the animal models should have heuristic value since they investigate the environmental challenges that an animal may meet in its everyday life (i.e. "natural model"). In social settings, this might mean loss of control by social defeat. As described by Koolhaas et al., social defeat is a very special kind of stressor and distinguishes itself from other stress paradigms with respect to the magnitude and the quality of the stress response (62). Moreover, a very interesting feature of this model is that, besides allowing the investigation of physiological, neurobiological and behavioural alterations caused by stress in defeated animals, we can also study these alterations in the winning (i.e. dominant) rat, in particular the brain effects of repetitive victorious confrontations.

Besides the social defeat model, other stress-induced animal models of depression (not used in the present thesis) have been developed. An interesting animal model of stress is the chronic mild stress (CMS) model. It focuses on a core symptom of depression, named anhedonia (in humans) or anhedonia-like behaviour (in animals). The CMS paradigm involves the exposure of animals to a series of mild stressors in an unpredictable manner (isolation or crowded housing, food or water deprivation, disruption of the dark-light cycle, tilting of home cages, dampened bedding, etc.) over a period of several weeks or even months (61). The learned helplessness model is also frequently used, since feelings of helplessness are core symptoms of MDD. It displays good face, construct and predictive validity, however lacking the aetiological validity. The classic design is composed of three groups, one that receives unpredictable, uncontrollable and unavoidable electrical shocks, one that receives controllable electrical shocks and the control group which is not exposed to stress (for a recent review, see (63)). Early-life stress models are based on the observation that negative life events during critical periods

of development may increase the vulnerability for psychiatric conditions later in life, mainly MDD. Maternal separation, for example, is an experimental procedure that is widely used in this context. Previous studies demonstrated that a single or repeated separation of pups from their mother leads to acute or long-term effects on physiology and behaviour. Schmidt et al. recently published a comprehensive review on early-life stress animal models in rodents, questioning the validity of early-life stress paradigms, such as maternal separation, as robust models of depression (59).

2. Nuclear medicine

Nuclear medicine comprises a range of imaging techniques that provide detailed information about a wide range of biological processes at the molecular and cellular level. As opposed to medical imaging techniques, such as computed tomography (CT), X-ray and magnetic resonance imaging (MRI), which provide anatomical images (64), nuclear medicine allows for the in vivo visualization and analysis of the underlying pathology and tissue function (65). In the attempt to complete our knowledge of the pathophysiological mechanisms underlying MDD, nuclear medicine could be a unique tool to be applied in such investigation.

In order to diagnose or characterize disease states with this molecular imaging technology, it is necessary to intravenously administer tracer amounts of a radiolabelled compound (radiotracer, radiopharmaceutical or simply "tracer") with high specificity and affinity for the target of interest. After administration, the radiotracer is distributed throughout the body (65). The distribution is mainly determined by the characteristics of the compound, such as affinity for the desired target (binding potential of a drug) (66), and also by the physiology of the tissue under investigation. The distribution of the radiotracer can be measured with a dedicated camera. A great advantage of nuclear medicine is the availability of radiotracers to image molecular targets involved in specific physiological and pathological processes. Two different nuclear medicine modalities are available: single photon emission computed tomography (SPECT) and positron emission tomography (PET). This thesis focuses on the use of PET for brain imaging.

2.1 Positron emission tomography (PET)

PET is an imaging technology that measures the distribution and concentration of tracers, labelled with positron (i.e. β^+ particle) emitting radioisotopes (e.g. ^{18}F, ^{11}C, ^{15}O and ^{13}N). The emitted positron collides with an electron from the surrounding matter in a process

called annihilation, which results in the generation of two gamma (γ) rays of equal energy (511 keV) and traveling in opposite direction (180°). The PET camera detects the two opposing γ rays through coincidence detection, generating a line of response on which the original decay had occurred (Figure 2).

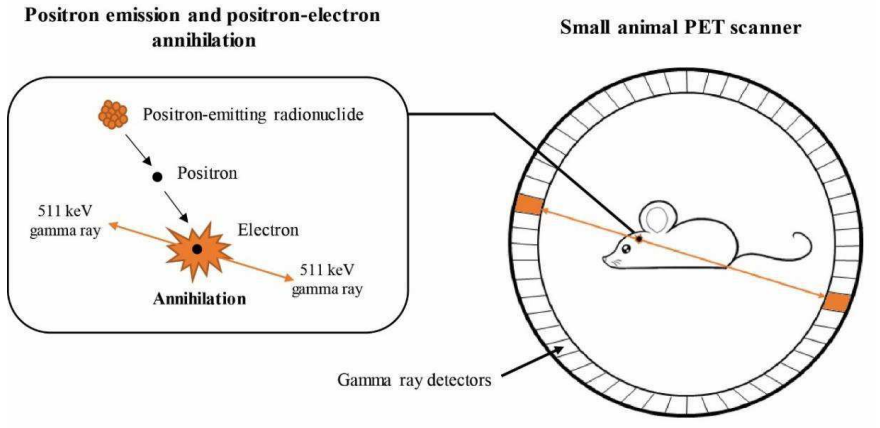


Figure 2: Positrons are emitted by radioisotopes rich in protons. Once emitted, the positron travels a short distance before annihilating with an electron from the surrounding matter. When annihilating, the mass of positron and electron is converted into two gamma rays with an energy of 511 keV each, traveling at an angle of 180°. The gamma rays are detected by coincidence detectors in a PET scanner system (for humans or small animals).

Through the combination of measurement of many coincidences, the system can reconstruct the 3D distribution of the radiotracer as function of time. After the collected data is subjected to physical corrections for dead-time, attenuation, randoms and scatter, the radioactivity concentration in the region of interest can be accurately measured, and the biological processes under investigation can be analysed in a quantitative manner. When combined with anatomical information from another imaging technique, the functional information of PET can be accurately localized and related to specific structures.

2.1.1 PET data quantification methods

Data obtained from PET studies can be evaluated in several different ways. In clinical practice, visual inspection of PET images is the main method for image interpretation (67). Usually, this is performed when a static image is obtained after a certain period of radiotracer uptake in the tissue, considering the observed PET signal in the tissue corresponds to the underlying process of interest. However, a more quantitative approach might be required in cases when the static PET signal does not properly correspond to the

state of interest or when disease progression, treatment response or subtle physiological states are evaluated.

A method to obtain semi-quantitative PET data, frequently used in clinical practice and research, is calculation of the Standardized Uptake Value (SUV). Its main advantage is the simplicity of application, since it requires only the tissue radioactive concentration at a carefully pre-defined time. The radioactivity concentration in tissue is subsequently corrected for the injected dose and some anthropometric characteristic of the subject (generally the body weight or the body surface area), according to Equation 1.

$$SUV = \frac{Measured\ Activity\ Concentration\ [kBq/mL]}{Injected\ dose\ [MBq]/\alpha}$$

$$\text{Where } \alpha = \begin{cases} Body\ Weight\ (kg) \\ Body\ surface\ area\ (m^2) \\ \dots \end{cases}$$

Equation 1: Equation to calculate the SUV from a PET measurement. The measured activity concentration is derived from the PET data, and the injected dose is the amount of radioactivity administered to the patient.

The calculation of the SUV does not require invasive procedures such as arterial blood sampling, which improves patient comfort and enables longitudinal preclinical studies. However, its validity is affected by a number of technical and physiological factors. In fact, the SUV is dependent on, for example, the clearance of the tracer from circulation, metabolism and changes in perfusion and blood flow (67). Due to the aforementioned reasons, SUV is often referred to as a semi-quantitative metric.

In research or when the main purpose of a PET study is to obtain values of parameters that characterize a physiological, biochemical or pharmacokinetic process, a fully quantitative approach is required. In other words, a radiotracer kinetic model, describing the radiotracer under study, is required to translate the measurements of radioactivity into quantitative values of the biological parameter of interest (68). In order to perform the analysis, compartment models are generally used (69). These compartments are not necessarily distinct anatomical compartments, but a convenient way to describe different kinetic "states" of the radiotracer (68). This is especially useful for the analysis of PET data, since the total radioactivity concentration measured from each image voxel is a sum of radiotracer concentrations in different tissues (e.g. brain

parenchyma, vasculature) and physiological states. In this context, different compartments can describe the different specific states in which the radiotracer can be found, such as unbound in plasma, unbound in brain tissue, metabolized, or bound to a specific receptor (70). Moreover, since compartmental modelling provides the most exhaustive description of radiotracer kinetics, it is usually considered the gold standard for PET data analysis (71). The parameters of compartmental models describe changes in radioactive concentrations by first-order differential equations. Exchanges between compartments are described by mass/balance equations, defined by tracer concentrations and kinetic rates constants (K_1, k_2, k_3, etc). Macro-parameters of interest such as metabolic and enzymatic rates, receptor concentrations and others can be obtained from these micro-parameters.

In order to perform the kinetic modelling of the data from the PET study, two datasets are necessary: 1) the time-activity (TAC) curve of the tissue of interest (provided by the PET scanner); 2) the radiotracer concentration in the circulating plasma as function of time (input function), measured in the arterial blood collected at different time points. The radioactivity concentration in blood is the sum of the radioactivity in plasma and the radioactivity associated with the red blood cells. The TAC in tissue corresponds to the radioactivity concentration measured in a specific volume of interest which is the sum of the radioactive concentration in the extra- and intra-cellular compartments, as well as the concentration in the blood pool. In some cases, not all radiotracer in the plasma or tissue is in its original form (e.g. if it is metabolized). Furthermore, not all the radiotracer in the tissue may be involved in the specific process of interest for the study (72). A correct approach to the PET data quantification should account for all these contributions. Correction for radioactive metabolites in plasma is usually performed by assessment of the percentage of intact tracer in the plasma samples.

Different kind of radiotracers are available for brain PET imaging: those that only enter and exit the brain without binding to a target; those that bind to a receptor or a transporter; and those that are metabolized by enzymatic action. Data from such radiotracers need to be analysed with a kinetic model that best suits their kinetic properties.

For radiotracers that only enter and exit the brain without binding, the 1-tissue compartmental model (1TCM) can be used. In this case, there are two compartments, the plasma compartment and the tissue compartment. This model calculates the rate constants K_1 and k_2, which describe the rate of the radiotracer from plasma to tissue, and the rate of

the radiotracer from tissue to plasma, respectively. K_1/k_2 (73) describes the ratio of the radiotracer in the tissue to the arterial plasma concentration at equilibrium and is defined as the distribution volume (V_T).

The 2-tissue compartmental model (2TCM) assumes that the radiotracer goes from arterial plasma to the central non-displaceable compartment, where part of the radiotracer molecules binds to the tissue non-specifically and reaches the equilibrium rapidly and some molecules retain in the tissue fluid as free ligand (Figure 3). The rest of the ligand is transferred from the non-displaceable compartment to the specific compartment, in which it is specifically bound to the target molecule (e.g. a specific receptor). In this case, four parameters, K_1, k_2, k_3 and k_4, are calculated. K_1 and k_2 indicate the rate of the radiotracer from plasma to the non-displaceable compartment, and the rate of the radiotracer from the non-displaceable compartment to plasma, respectively. k_3 reflects the rate of the radiotracer from the non-displaceable compartment to the specific compartment (e.g. binding to a receptor) and k_4 describes the rate of the radiotracer from the specific compartment to the non-displaceable compartment (e.g. release from a receptor). The V_T can be obtained using the equation $K_1/k_2*(1+k_3/k_4)$. Another important macro-parameter derived from the 2TCM is the non-displaceable binding potential BP_{ND}, which is the ratio of k_3 and k_4. BP_{ND} indicates how well the radiotracer binds specifically to the target.

In case radiotracer molecules are trapped in the specific compartment, the k_4 is equal to or approaches zero. 2'-[^{18}F]fluoro-2'-deoxyglucose (^{18}F-FDG), a glucose analogue, is a typical example of an irreversibly bound tracer. The most important pharmacokinetic parameter that can be obtained from an irreversible 2TCM fit is the metabolic rate Ki, calculated as $K\text{i} = K_1*k_3/(k_2+k_3)$ (74).

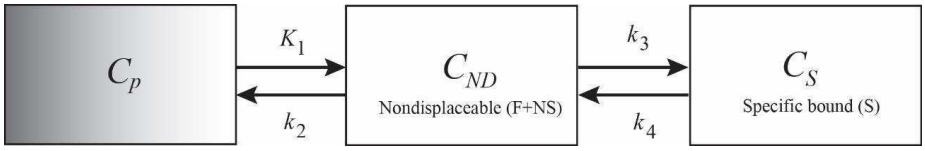


Figure 3: Representation of the 2-tissue compartment model (2TCM). The model assumes that the radiotracer goes from arterial plasma (Cp) to non-displaceable compartment (C_{ND}), where part of the radiotracer molecules binds to the tissue non-specifically (NS) and reach the equilibrium rapidly and some molecules retain in the tissue fluid as free ligand (F). From the non-displaceable compartment, the tracer can go to the specific compartment (C_S), in which the radiotracer is specifically bound to the target molecule. K_1, k_2, k_3 and k_4 represent the exchange rates between compartments.

A disadvantage of performing the full kinetic modelling of the radiotracer in study is that it often requires long image acquisition protocols for accurate pharmacokinetic modelling. Also, data analysis is laborious and time-consuming. Furthermore, obtaining the input function is an invasive method and uncomfortable for patients. In preclinical studies, due to the required amount of arterial blood extracted for constructing an input function, it is often a terminal procedure which precludes longitudinal designs. For that reason, alternatives for the measurement of an arterial input function, including image-derived input functions (75), population-based input functions (76) and the use of reference regions (77), have been developed.

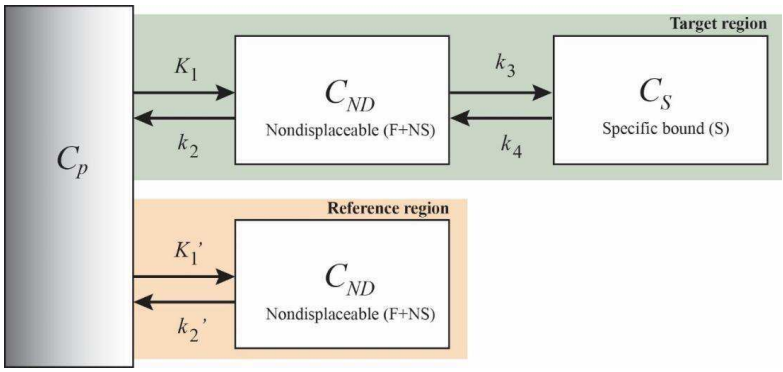


Figure 4: Schematic representation of a reference tissue compartmental model. The method is based on the use of a region devoid of specific binding (reference region, C_{ND}), from which it is possible to infer the receptor binding in the region of interest (target region, C_S). The target and reference region exchange radiotracer with the plasma at a similar rate.

In PET, the use of reference regions is the most popular approach and it is based on the use of a region non-existent specific binding, from which it is possible to infer the receptor binding in the remaining regions of the brain. The method assumes that the non-displaceable distribution volume is the same for both target and reference region and that the K_1 and k_2 in the reference region are equal to those in the target region. Under these assumptions, reference-based models relate the radiotracer kinetics in target region to those in the reference region (Figure 4). The use of this approach is, however, limited. Many receptors are not restricted to particular anatomical regions and therefore no reference region devoid of these receptors can be defined.

3. Thesis aim and outline

As already discussed, depression is multifactorial disease, with high incidences in the general population and high associated disability, mortality and economic burden to

society. Until the present moment, the pathophysiology of depression has not been fully elucidated. As depression hampers the quality of life of nearly half of the patients due to ineffective treatment, it is of utmost importance to elucidate pathophysiological mechanisms underpinning the disorder. Psychosocial and physical stressors capable of inducing (neuro)inflammation seem to be a possible causal role for MDD, in particular in the subgroup of treatment-resistant depressive patients.

In the past, brain alterations related to the disorder could only be obtained post mortem, sadly many times in depressed suicide victims. In this context, PET is an attractive tool for non-invasive in vivo brain imaging that allows to investigate possible alterations in the brain of living depressed patients. With this in mind, this thesis aimed to address the neuroinflammatory hypothesis of depression, using psychosocial stress as a predisposing factor for mood disorders such as depression and aggression, evaluated through PET imaging.

In **chapter 2**, we review the current knowledge on the (neuro)inflammatory hypothesis of depression. Based on literature findings of preclinical and clinical studies of depressive patients with an elevated inflammatory profile and unresponsiveness to conventional antidepressant therapy, we discuss the usage of non-steroidal anti-inflammatory agents for MDD, as well as the anti-inflammatory properties of some antidepressants.

Since stress seems to be a strong predictor of depression, we used a psychosocial stress rodent model with high ethological validity, namely repeated social defeat (RSD). In **chapter 3**, we aimed to evaluate if RSD was capable of inducing depressive-like behaviour and if these behavioural changes were associated with glial activation and alterations in brain metabolism using ^{11}C-PK11195 PET and ^{18}F-FDG PET, respectively. Furthermore, the persistence of the evaluated parameters was evaluated up to 6 months after the exposure to RSD.

In the pursuit of a more suitable and sensitive radiotracer for detection of glial activation, in **chapter 4** the TSPO ligand ^{11}C-PBR28 was validated and compared to ^{11}C-PK11195 in the neuroinflammatory rodent model of herpes encephalitis (HSE). A full pharmacokinetic modelling was applied, as well as voxel and volume of interest (VOI) analysis for comparison of both radiotracers.

Major stressful life events at young age or adolescence seems to play a crucial role in predisposing individuals for psychiatric disorders, such as MDD, at any point in their life. In **chapter 5**, we tested how a previous exposure to RSD in adolescence affects the neuroendocrine, (neuro)inflammatory, behavioural and brain metabolic response to a second RSD exposure in aged rats of 14 months. The previously validated ^{11}C-PBR28 radiotracer was used to evaluate glial activation and ^{18}F-FDG PET was used for assessment of brain metabolism alterations.

In the RSD paradigm, a resident (dominant) male rat is used to attack and defeat intruder (submissive) rats. Whereas the submissive rats develop depressive-like behaviour, in the resident rats an escalation in the levels of aggressiveness upon exposure to repeated victorious confrontations was observed. In **chapter 6**, we therefore investigated if the dopaminergic D2 receptors, largely associated with the reward system, are altered in the striatal area of the brain and whether changes in the availability of these receptors are related to the behavioural alterations.

References

1. Marcus M, Yasami T, van Ommeren M, Chirsholm D, Saxena S (2012): Depression: A Global Public Health Concern Developed. *65th World Heal Assem*. Geneva, Switzerland, pp 6–8.
2. Wohleb ES (2016): Neuron-microglia interactions in mental health disorders: "For better, and for worse." *Front Immunol*. 7: 1–13.
3. American Psychiatric Association (2013): *Diagnostic and Statistical Manual of Mental Disorders: DSM-5*. Arlington: American Psychiatric Publishing.
4. Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, *et al.* (2003): The Epidemiology of Major Depressive Disorder. *JAMA*. 289: 3095–3105.
5. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctôt KL (2010): A Meta-Analysis of Cytokines in Major Depression. *Biol Psychiatry*. 67: 446–457.
6. Barrientos RM, Frank MG, Watkins LR, Maier SF (2012): Aging-related changes in neuroimmune-endocrine function: Implications for hippocampal-dependent cognition. *Horm Behav*. 62: 219–227.
7. Andrade L, Caraveo-Anduaga, JJ Berglund P, Bijl R, De Graaf R, Vollebergh W, Dragomirecka E, *et al.* (2003): The epidemiology of major depressive episodes: results from the International Consortium of Psychiatric Epidemiology (ICPE) Surveys. *Int J Methods Psychiatr Res*. 12: 3–21.
8. Solomon DA, Keller MB, Leon AC, Mueller TI, Lavori PW, Shea MT, *et al.* (2000): Multiple recurrences of major depressive disorder. *Am J Psychiatry*. 157: 229–233.
9. Holtzheimer PE, Nemeroff CB (2008): Novel Targets for Antidepressant Therapies. *Curr Psychiatry Rep*. 10: 465–473.
10. Bschor T, Ising M, Erbe S, Winkelmann P, Ritter D, Uhr M, Lewitzka U (2012): Impact of citalopram on the HPA system. A study of the combined DEX/CRH test in 30 unipolar depressed patients. *J Psychiatr Res*. 46: 111–117.
11. Rush A, MH T, Wisniewski S, Nierenberg A, Stewart J, Warden D, Niederehe G (2006): Acute and Longer-Term Outcomes in Depressed Outpatients Requiring One or Several

Treatment Steps: A STAR*D Report. *Am J Psychiatry*. 163: 1905–1917.
12. Cuijpers P, Van Straten A, Bohlmeijer E, Hollon SD, Andersson G (2010): The effects of psychotherapy for adult depression are overestimated: A meta-analysis of study quality and effect size. *Psychol Med*. 40: 211–223.
13. Fava M, Kendler KS (2000): Major Depressive Disorder. *Neuron*. 28: 335–341.
14. Moylan S, Maes M, Wray NR, Berk M (2013): The neuroprogressive nature of major depressive disorder: Pathways to disease evolution and resistance, and therapeutic implications. *Mol Psychiatry*. 18: 595–606.
15. Kopschina Feltes P, Doorduin J, Klein HC, Juárez-Orozco LE, Dierckx RA, Moriguchi-Jeckel CM, de Vries EF (2017): Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy. *J Psychopharmacol*. 31: 1149–1165.
16. Myint AM (2012): Kynurenines: from the perspective of major psychiatric disorders. *FEBS J*. 279: 1375–1385.
17. Ordway GA, Schenk J, Stockmeier CA, May W, Klimek V (2003): Elevated agonist binding to α2-adrenoceptors in the locus coeruleus in major depression. *Biol Psychiatry*. 53: 315–323.
18. Escribá P, Ozaita A, García-Sevilla J (2004): Increased mRNA Expression of α2A-Adrenoceptors, Serotonin Receptors and μ-Opioid Receptors in the Brains of Suicide Victims. *Neuropsychopharmacology*. 29: 1512–1521.
19. Dunlop BW, Nemeroff CB (2007): Dunlop & Nemeroff, 2007_the role of dopamine in the pathophysiology of depression. *Arch Gen Psychiatry*. 64: 1–11.
20. Lambert G, Johansson M, Ågren H, Friberg P (2000): Reduced Brain Norepinephrine and Dopamine Release in Treatment-Refractory Depressive Illness. *Arch Gen Psychiatry*. 57: 787–793.
21. Bremner JD, Vythilingam M, Ng CK, Vermetten E, Nazeer A, Oren DA, *et al.* (2003): Regional Brain Metabolic Correlates of α-Methylparatyrosine–Induced Depressive Symptoms. *JAMA*. 289: 3125–3134.
22. Bocchio-Chiavetto L, Bagnardi V, Zanardini R, Molteni R, Gabriela Nielsen M, Placentino A, *et al.* (2010): Serum and plasma BDNF levels in major depression: A replication study and meta-analyses. *World J Biol Psychiatry*. 11: 763–773.
23. Viikki M, Anttila S, Kampman O, Illi A, Huuhka M, Setälä-Soikkeli E, *et al.* (2010): Vascular endothelial growth factor (VEGF) polymorphism is associated with treatment resistant depression. *Neurosci Lett*. 477: 105–108.
24. Hoshaw BA, Malberg JE, Lucki I (2005): Central administration of IGF-I and BDNF leads to long-lasting antidepressant-like effects. *Brain Res*. 1037: 204–208.
25. Allaman I, Fiumelli H, Magistretti PJ, Martin JL (2011): Fluoxetine regulates the expression of neurotrophic/growth factors and glucose metabolism in astrocytes. *Psychopharmacology (Berl)*. 216: 75–84.
26. Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, *et al.* (2001): CXCR4-activated astrocyte glutamate release via TNFa: Amplification by microglia triggers neurotoxicity. *Nat Neurosci*. 4: 702–710.
27. Pascual O, Ben Achour S, Rostaing P, Triller A, Bessis A (2012): Microglia activation triggers astrocyte-mediated modulation of excitatory neurotransmission. *Proc Natl Acad Sci*. 109: E197–E205.
28. Mechawar N, Savitz J (2016): Neuropathology of mood disorders: do we see the stigmata of inflammation? *Transl Psychiatry*. 6: e946.
29. Kim Y-K, Na K-S (2016): Role of glutamate receptors and glial cells in the pathophysiology of treatment-resistant depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 70: 117–126.
30. Molokanova E, Akhtar MW, Sanz-Blasco S, Tu S, Pina-Crespo JC, McKercher SR, Lipton SA (2014): Differential Effects of Synaptic and Extrasynaptic NMDA Receptors on A - Induced Nitric Oxide Production in Cerebrocortical Neurons. *J Neurosci*. 34: 5023–5028.
31. Song C, Wang H (2011): Cytokines mediated inflammation and decreased neurogenesis in animal models of depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 35: 760–

768.
32. Vogelzangs N, Duivis HE, Beekman ATF, Kluft C, Neuteboom J, Hoogendijk W, *et al.* (2012): Association of depressive disorders, depression characteristics and antidepressant medication with inflammation. *Transl Psychiatry*. 2: e79.
33. Anders S, Tanaka M, Kinney DK (2013): Depression as an evolutionary strategy for defense against infection. *Brain Behav Immun*. 31: 9–22.
34. Pace TWW, Hu F, Miller AH (2007): Cytokine-effects on glucocorticoid receptor function: Relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. *Brain Behav Immun*. 21: 9–19.
35. Kim Y, Lee S, Kim S, Shim S, Han S, Choi Sh, Lee B (2008): Differences in cytokines between non-suicidal patients and suicidal patients in major depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 32: 356–361.
36. Steiner J, Bielau H, Brisch R, Danos P, Ullrich O, Mawrin C, *et al.* (2008): Immunological aspects in the neurobiology of suicide: Elevated microglial density in schizophrenia and depression is associated with suicide. *J Psychiatr Res*. 42: 151–157.
37. Dobos N, de Vries EFJ, Kema IP, Patas K, Prins M, Nijholt IM, *et al.* (2012): The role of indoleamine 2,3-dioxygenase in a mouse model of neuroinflammation-induced depression. *J Alzheimers Dis*. 28: 905–915.
38. Myint AM, Bondy B, Baghai TC, Eser D, Nothdurfter C, Schüle C, *et al.* (2013): Tryptophan metabolism and immunogenetics in major depression: A role for interferon-γ gene. *Brain Behav Immun*. 31: 128–133.
39. Guillemin GJ (2012): Quinolinic acid, the inescapable neurotoxin. *FEBS J*. 279: 1356–1365.
40. Zunszain PA, Anacker C, Cattaneo A, Carvalho LA, Pariante CM (2011): Glucocorticoids, cytokines and brain abnormalities in depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 35: 722–729.
41. Miller AH, Haroon E, Raison CL, Felger JC (2013): Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. *Depress Anxiety*. 30: 297–306.
42. Haroon E, Raison CL, Miller AH (2012): Psychoneuroimmunology Meets Neuropsychopharmacology: Translational Implications of the Impact of Inflammation on Behavior. *Neuropsychopharmacology*. 37: 137–162.
43. Post M (1992): Transduction of Psychosocial Stress Into the Neurobiology of Recurrent Affective Disorder. *Am J Psychiatry*. 149: 999–1010.
44. Kendler KS, Thornton LM, Gardner CO (2000): Stressful Life Events and Previous Episodes in the Etiology of Major Depression in Women: An Evaluation of the "Kindling" Hypothesis. *Am J Psychiatry*. 157: 1243–1251.
45. Monroe SM, Harkness KL (2005): Life stress, the "kindling" hypothesis, and the recurrence of depression: Considerations from a life stress perspective. *Psychol Rev*. 112: 417–445.
46. Doolin K, Farrell C, Tozzi L, Harkin A, Frodl T, O'Keane V (2017): Diurnal Hypothalamic-Pituitary-Adrenal Axis Measures and Inflammatory Marker Correlates in Major Depressive Disorder. *Int J Mol Sci*. 18: 1–17.
47. Miura H, Ozaki N, Sawada M, Isobe K, Ohta T, Nagatsu T (2008): A link between stress and depression: Shifts in the balance between the kynurenine and serotonin pathways of tryptophan metabolism and the etiology and pathophysiology of depression. *Stress*. 11: 198–209.
48. Kessler RC (1997): the Effects of Stressful Life Events on Depression. *Annu Rev Psychol*. 48: 191–214.
49. Hammen C (2005): Stress and Depression. *Annu Rev Clin Psychol*. 1: 293–319.
50. de Boer SF, Buwalda B, Koolhaas JM (2017): Untangling the neurobiology of coping styles in rodents: Towards neural mechanisms underlying individual differences in disease susceptibility. *Neurosci Biobehav Rev*. 74: 401–422.
51. Ménard C, Pfau ML, Hodes GE, Russo SJ (2017): Immune and Neuroendocrine Mechanisms of Stress Vulnerability and Resilience. *Neuropsychopharmacology*. 42: 62–80.
52. Coppens CM, de Boer SF, Koolhaas JM (2010): Coping styles and behavioural flexibility: towards underlying mechanisms. *Philos Trans R Soc B Biol Sci*. 365: 4021–4028.
53. Koolhaas JM, de Boer SF, Coppens CM, Buwalda B (2010): Neuroendocrinology of coping

styles: Towards understanding the biology of individual variation. *Front Neuroendocrinol*. 31: 307–321.
54. McEwen B, Gray J, Nasca C (2015): Redefining neuroendocrinology: stress, sex and cognitive and emotional regulation. *J Endocrinol*. 226: T67–T83.
55. Frank MG, Watkins LR, Maier SF (2013): Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. *Brain Behav Immun*. 33: 1–6.
56. Nestler EJ, Hyman SE (2010): Animal models of neuropsychiatric disorders. *Nat Neurosci*. 13: 1161–1169.
57. Hollis F, Kabbaj M (2014): Social defeat as an animal model for depression. *ILAR J*. 55: 221–232.
58. Kalueff A V., Tuohimaa P (2004): Experimental modeling of anxiety and depression. *Acta Neurobiol Exp (Wars)*. 64: 439–448.
59. Schmidt M V., Wang XD, Meijer OC (2011): Early life stress paradigms in rodents: Potential animal models of depression? *Psychopharmacology (Berl)*. 214: 131–140.
60. Willner P (1984): The validity of animal models of depression. *Psychopharmacology (Berl)*. 83: 1–16.
61. Czéh B, Fuchs E, Wiborg O, Simon M (2016): Animal models of major depression and their clinical implications. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 64: 293–310.
62. Koolhaas J, de Ruiter A, Meerlo P, de Boer S (1997): Social stress in rats and mice. *Acta Physiol Scand*. 161: 69–72.
63. Pryce CR, Azzinnari D, Spinelli S, Seifritz E, Tegethoff M, Meinlschmidt G (2011): Helplessness: A systematic translational review of theory and evidence for its relevance to understanding and treating depression. *Pharmacol Ther*. 132: 242–267.
64. Sik A, Ching C, Kuhnast B, Damont A, Roeda D, Tavitian B (2012): Current paradigm of the 18-kDa translocator protein (TSPO) as a molecular target for PET imaging in neuroinflammation and neurodegenerative diseases Medical Imaging : *Insights Imaging*. 3: 111–119.
65. Cohn H (1968): Definition of Nuclear Medicine. *J Nucl Med*. 9: 465.
66. Benadiba M, Luurtsema G, Ana LW, Buchpigel CA, Filho GB (2012): New molecular targets for PET and SPECT imaging in neurodegenerative diseases. *Rev Bras Psiquiatr*. 34: S125–S148.
67. Boellaard R (2009): Standards for PET Image Acquisition and Quantitative Data Analysis. *J Nucl Med*. 50: 11S–20S.
68. Lammertsma AA (2014): Tracer kinetic modelling. In: Dierckx RAJO, de Vries EFJ, van Waarde A, Otte A, Leenders KL, editors. *PET SPECT Neurol*. Springer-Verlag, pp 60–72.
69. Carson RE (2003): Tracer Kinetic Modeling in PET. *Positron Emiss Tomogr*. London: Springer-Verlag, pp 127–159.
70. Morris ED, Endres CJ, Schmidt KC, Christian BT, Muzic RF, Fisher RE (2004): Kinetic Modeling in Positron Emission Tomography. *Emiss Tomogr*. London: Elsevier, pp 499–540.
71. Gunn RN, Gunn SR, Cunningham VJ (2001): Positron Emission Tomography Compartmental Models. *J Cereb Blood Flow Metab*. 21: 635–652.
72. Turkheimer FE, Veronese M, Dunn J (2014): Quantification Methods for PET data Analysis: theory. *Exp Des Pract Data Anal Positron Emiss Tomogr*. London: Published independently, pp 65–76.
73. Innis RB, Cunningham VJ, Delforge J, Fujita M, Gjedde A, Gunn RN, *et al.* (2007): Consensus nomenclature for in vivo imaging of reversibly binding radioligands. *J Cereb Blood Flow Metab*. 27: 1533–1539.
74. Patlak CS, Blasberg RG, Fenstermacher JD (1983): Graphical Evaluation of Blood-to-Brain Transfer Constants from Multiple-Time Uptake Data. *J Cereb blood flow Metab*. 3: 1–7.
75. Mourik JEM, Lubberink M, Schuitemaker A, Tolboom N, van Berckel BNM, Lammertsma AA, Boellaard R (2009): Image-derived input functions for PET brain studies. *Eur J Nucl Med Mol Imaging*. 36: 463–471.
76. Eberl S, Anayat AR, Fulton RR, Hooper PK, Fulham MJ (1997): Evaluation of two population-based input functions for quantitative neurological FDG PET studies. *Eur J Nucl*

Med Mol Imaging. 24: 299–304.
77. Lammertsma AA, Hume SP (1996): Simplified Reference Tissue Model for PET Receptor Studies. *Neuroimage*. 4: 153–158.

Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy

Author(s): Paula Kopschina Feltes, Janine Doorduin, Hans C. Klein, Luis Eduardo Juárez-Orozco, Rudi AJO Dierckx, Cristina M. Moriguchi-Jeckel, Erik FJ de Vries.

CHAPTER 2

as published in the Journal of Psychopharmacology. Kopschina Feltes et al. (2017) *Journal of Psychopharmacology*. 3: 1149-1165.

Abstract

Major Depressive Disorder (MDD) is a highly prevalent and disabling psychiatric disease with rates of non-responsiveness to antidepressants ranging from 30-50%. Historically, the monoamine depletion hypothesis has dominated the view on the pathophysiology of depression. However, the lack of responsiveness to antidepressants and treatment resistance suggests that additional mechanisms might play a role. Evidence has shown that a subgroup of depressive patients may have an underlying immune deregulation that could explain the lack of therapeutic benefit from antidepressants. Stimuli like inflammation, chronic stress and infection can trigger the activation of microglia, the brain's immune cells, to release pro-inflammatory cytokines that can act on two pathways that may lead to MDD and neurodegeneration: (1) activation of the hypothalamic-pituitary adrenal axis, generating an imbalance in the serotonergic and noradrenergic circuits; (2) increased activity of the enzyme indoleamine-2,3-deoxygenase, which catalyzes the catabolism of the serotonin precursor, tryptophan, resulting in depletion of serotonin levels and the production of quinolinic acid, a well-known neurotoxic compound. If this hypothesis is proven true, a subgroup of MDD patients with increased levels of pro-inflammatory cytokines, in particular IL-6, TNF-α and IL-1β, and also hs-CRP, might benefit from an anti-inflammatory intervention. Here we discuss the pre-clinical and clinical studies that have provided support for the benefits of the anti-inflammatory treatment with non-steroidal anti-inflammatory drugs in depressed patients with inflammatory comorbidities and/or an elevated immune profile, as well as evidences for anti-inflammatory properties of standard antidepressants.

Key words

Major depressive disorder, neuroinflammation, microglia, pro-inflammatory cytokines, antidepressants

Introduction

Major depression disorder (MDD) is an important public health issue (1; 2), predicted to be the second leading cause of disability by the year of 2020 behind only ischemic heart disease (3). MDD is the most commonly diagnosed psychiatric disorder in adults over 60 years of age (1). The Diagnostic and Statistical Manual of Mental Disorders (DSM-V) describes that for the diagnosis of MDD, five or more symptoms have to be present during a 2-week period and represent a change from previous functioning; at least one of the symptoms should be either: (i) depressive mood or (ii) loss of interest or pleasure for the major part of the day. The other symptoms that may be present are significant weight loss or weight gain, insomnia or hypersomnia, fatigue or loss of energy, diminished ability to concentrate or indecisiveness, recurrent thoughts of death and suicidal ideation or attempt (4). Not only the high incidence of MDD and the disability associated with the disease, but also the high rate of inadequate treatment of the disorder remains a serious concern (5). It is estimated that 30–50% of the patients do not respond to treatment with antidepressants (6) due to either lack of efficacy or intolerable side effects (7). Another possible reason for the ineffectual treatment of MDD has been the incomplete understanding of the nature of depression (8). The high rate of treatment resistance, together with the high suicide risk in unresponsive patients and the overwhelming economic costs to society constitute the basis of the search for new therapeutic agents (9), aiming to improve the quality of life or even cure these patients. Remission - i.e. (virtual) absence of symptoms - should be the objective of MDD treatment, since it is related to better functioning and a better prognosis than a response without remission (7; 10).

Even though information concerning the epidemiology, symptoms and complications of mood disorders are well documented, the etiology and pathophysiology of depression are not completely elucidated (11). The monoamine depletion hypothesis has historically dominated the view on the pathophysiology of depression. It suggests that an imbalance, mainly in serotonergic and noradrenergic neurotransmission is the core of the pathophysiology of depression (12; 13). However, the lack of responsiveness to conventional treatment with antidepressants and high rates of treatment resistance suggests that additional mechanisms might play a role in depression. Over the last 20 years, psychiatric research has provided support for the hypothesis that inflammatory processes and brain–immune interactions are involved in the pathogenesis of MDD and may contribute to the serotonergic and noradrenergic dysfunction (14). Stimuli like inflammation, chronic stress and infection can trigger the activation of microglia, the

brain's immune cells, to release pro-inflammatory cytokines that can act on two pathways that may lead to MDD and neurodegeneration, such as: (1) activation of the hypothalamic–pituitary adrenal axis, generating an imbalance in the serotonergic and noradrenergic circuits; (2) increased activity of the enzyme indoleamine-2,3-deoxygenase (IDO), resulting mainly in depletion of serotonin. Considering that MDD is a very complex and heterogeneous disorder, it is possible that immune deregulation is not present in all depressed patients, but only in specific sub-populations (15). Evidence also shows that lack of therapeutic benefit of antidepressants might be associated with persistent immunological impairment (16).

In this review, we aim to discuss the potential role of anti-inflammatory treatment in MDD. We first address the most relevant immunological mechanisms by which increased levels of pro-inflammatory cytokines may lead to MDD, highlighting the hypothalamic–pituitary–adrenal (HPA) axis hyperactivation and the indoleamine-2,3-dioxygenase (IDO) pathway. Next, we summarize the most recent studies concerning monotherapy with non-steroidal anti-inflammatory drugs (NSAIDs) in MDD patients, discuss the anti-inflammatory effects of standard antidepressant drugs and augmentative strategies with NSAIDs.

The hypothesis of immunological involvement in the pathophysiology of major depressive disorder (MDD)

An exhaustive discussion on all the possible immunological pathways that might play a role in the pathophysiology of depression is out of the scope of this article. Before focusing on the possible anti-inflammatory treatment for depression, however, we would like to review key points and molecular markers that are most relevant for the anti-inflammatory therapeutic strategies further discussed.

Association between pro-inflammatory cytokines and depression: the role of microglia

The hypothesis of a causal relationship between pro-inflammatory cytokines and depression was first described by Smith et al. in 1991, in the macrophage theory of depression. The theory was based on observations that cytokines produced by macrophages, when given to healthy volunteers, induced symptoms of depression and had brain effects that included the activation of the HPA axis (17; 18). Afterwards, Maes et al. corroborated the theory by collecting biochemical evidence for the immunological activation in depressed patients (19; 20). In response to infection or inflammatory

conditions, peripherally produced cytokines can act on the brain and cause behavioral symptoms (21), such as malaise, prostration, fatigue, numbness and anorexia (22). The main elucidated pathways to which pro-inflammatory cytokines can reach the brain include: (1) cytokine passage through leaky regions in the blood–brain barrier (BBB); (2) active transport via saturable transport molecules; (3) activation of endothelial cells and other cell types (including perivascular macrophages) lining the cerebral vasculature (which in turn produce cytokines and other inflammatory mediators); (4) binding to cytokine receptors associated with peripheral afferent nerve fibers (e.g. vagus nerve), delivering cytokine signals to relevant brain regions including the nucleus of the solitary tract and hypothalamus (2; 21). The nuclear factor NF-κB has been identified as an essential mediator at the blood–brain interface that communicates peripheral inflammatory signals to the central nervous system (CNS). Production of inflammatory cytokines can also be induced directly within the brain, via stress or other processes (e.g. vascular insults in late life depression) (2; 23).

In the CNS, microglia cells are the main cellular regulators of the innate immune response to both physiological and pathological conditions (24). They transform from an immunesurveillant into an activated state in response to pathogens and to synaptic and neuronal injury in several neurological disorders. During their activation, microglia change from a ramified to a hyper-ramified (25–27) phenotype and subsequently adopt an amoeboid morphology, a mechanism which has been suggested to help microglia to invade lesions (28). This activation can be acute or chronic, depending on the type of stimulus (inflammation, stress, infection, neuronal injury) and its duration (24). Thus, activation of microglia in stress might be different from microglial activation during inflammation or infection (29). When chronically activated, microglia can produce a wide variety of neurotoxins such as proinflammatory cytokines, free radicals, nitric oxide, chemokines, proteinases and eicosanoids (30) that may cause neuronal dysfunction and aggravate underlying pathologies (31). As such, activated microglia can be a triggering factor for mood disorders (11). Activated microglia have already been found in the brain of stress-induced animal models of depression (32; 33), however the data that would confirm the presence of activated microglia in humans are still limited (34). Evidence for neuroinflammation in MDD could be obtained noninvasively by positron emission tomography (PET) using radioligands that bind to the translocator protein (TSPO), a receptor that is upregulated in the mitochondria of activated microglia cells (35). Recently, the presence of neuroinflammation in depressed patients during a major

depressive episode was demonstrated using PET with the TSPO radioligand [18F]FEPPA (36). The study was conducted on 20 patients in a major depressive episode secondary to MDD that were medication free for at least 6 weeks, and 20 healthy controls. A significant increase in the uptake of the tracer was found in the prefrontal cortex, anterior cingulated cortex and insula, indicating the presence of activated microglia in these brain regions. Moreover, PET tracer uptake (microglia activation) was correlated with the Hamilton Depression Rating Scale (HDRS) score (37) in the anterior cingulated cortex (36). Hannestad et al. (2013) also conducted a study to evaluate the presence of neuroinflammation in patients with mild-to-moderate depression using [11C]PBR28, another TSPO ligand. No difference between patients and controls was found in this study (38). This could be due to the small sample size (n = 10) and the fact that patients with signs of peripheral immune activation (as defined by elevated high sensitive C-reactive protein, hsCRP) were excluded. Further studies with PET imaging should be conducted in order to corroborate or not the presence of activated microglia in MDD in a non-invasive manner. Thus, an increased density of activated microglia was observed post mortem in the anterior midcingulate cortex, dorsolateral prefrontal cortex and mediodorsal thalamus of suicidal patients with affective disorders (39).

More recently, an increased gut permeability or 'leaky gut' theory was described as a possible contributor to the peripheral and central production of pro-inflammatory cytokines by microglia in a subgroup of depressed patients. The investigated subjects were diagnosed with MDD and presented specific symptoms which have been correlated to increased levels of IgM and IgA to lipopolysaccharide (LPS) of enterobacteria in chronic fatigue syndrome (40). The observed symptoms were pain, muscular tension, fatigue, concentration difficulties, failing memory, irritability, stress and irritable bowel, among others. In summary, depressed patients demonstrated elevated serum IgM and IgA levels against LPS of gram-negative enterobacteria, as compared with healthy controls. Increased IgM and IgA levels indicate an increased gut permeability, allowing invasive enterobacteria to cause a systemic and central inflammation (41; 42).

Elevated pro-inflammatory cytokines and hypothalamic–pituitary–adrenal (HPA) dysfunction in major depressive disorder

Numerous studies have indicated that MDD is accompanied by elevated levels of inflammatory biomarkers, such as the proinflammatory cytokines interleukin (IL)-1β, IL-6, IL-18, tumor necrosis factor alpha (TNF-α), interferon-gamma (INF- γ) (1; 26; 27; 43–

53) and the acute phase proteins such as C-reactive protein (CRP) (54; 55). Munzer et al. (2013) even suggested that, besides, for example, stress hormones and psychopathological measures, cytokines may serve as biomarkers for individualized treatment of depression (56). Thus, animal studies have shown that systemic exposure to inflammatory challenges, such as LPS, not only causes a systemic inflammation but also induces a central inflammatory response in the brain, which is reflected by activation of microglia (57).

The pro-inflammatory cytokines produced during activation of microglia might have an effect on central serotonin levels and affect the HPA axis (Figure 1). The immune and neuroendocrine systems act together in order to restore and maintain physiological homeostasis during inflammation and other harmful stimuli that might induce systemic cytokine production (58). Therefore, it has been suggested that abnormalities in the HPA axis might play a key role in the development and recurrence of depression. Increased cytokine production may contribute to the development of depression directly via activation of the HPA axis or indirectly through cytokine-induced glucocorticoid receptor resistance (59). The release of TNF-α and IL-6 increases the production of corticotrophin releasing hormone, adrenocorticotropic hormone and cortisol by acting directly on hypothalamic and pituitary cells (1). Cytokines might also increase glucocorticoid receptor resistance through several signaling pathways, including activation of the p38 mitogen-activated protein kinase (MAPK) and by stimulating changes in the expression of glucocorticoid receptors (59; 60). The high levels of circulating stress hormones in the CNS might affect the neurotransmitter homeostasis, the neuronal growth factor synthesis and ultimately, disturb the functioning of neuronal circuits of the limbic system (61). HPA hyperactivity has been associated with the pathophysiology of suicidal behavior, excessive activity of the noradrenergic system and dysfunction of the serotonergic system (39; 62).

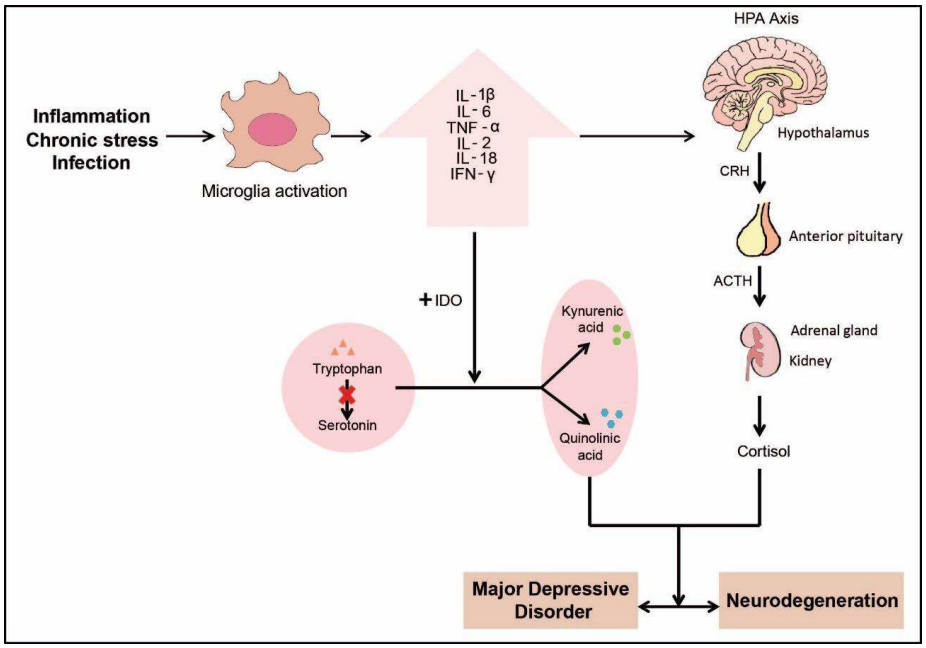


Figure 1: Hypothesis of immune involvement in the pathophysiology of major depressive disorder. Inflammatory, infectious and stressful challenges might trigger the activation of the resident microglia. Activated microglia produce pro-inflammatory cytokines that can contribute to neurodegeneration and depressive disorders through the hyper-activation of the HPA axis and the increase in indoleamine-2,3-dioxygenase (IDO) enzyme activity. Hyper-activation of the HPA axis leads to the increase of corticotrophin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol that disturb neurotransmitter homeostasis (mainly noradrenergic and serotonergic systems) and the neuronal growth factor synthesis. IDO decreases the synthesis of serotonin by switching the balance between the production of serotonin from tryptophan and the production of kynurenic acid (KYN) and quinolinic acid (QUIN). Depletion of serotonin leads to depressive symptoms. QUIN acts as a neurotoxin, gliotoxin, pro-inflammatory mediator and can also alter the integrity of the blood–brain barrier (BBB).

Pro-inflammatory cytokine effects on neurotransmitter metabolism

The link between pro-inflammatory cytokines and decreased serotonergic synthesis has already been extensively explored. It was hypothesized that during inflammation, pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, INF-γ and TNF-α (63–65) increase the activity of IDO and reduce the production of serotonin. IDO catalysis tryptophan (TRP) catabolism through the kynurenine pathway (66–68), producing kynurenic acid (KYN), quinolinic acid (QUIN) and nicotinamide adenine dinucleotide (NAD+) (27; 69). Substantial evidence demonstrates that a pro-inflammatory scenario leads to increased and unbalanced production of tryptophan catabolites (TRYCATs) that play a major role in the development and maintenance of MDD. A recent meta-analysis by Ogawa and colleagues (2014) demonstrated convincing evidence for lowered plasma TRP levels in patients with MDD. The study included MDD patients (n = 744) and healthy controls (n

= 793) and found a highly significant decreased level of TRP in depressed patients vs controls ($p < 0.001$). A secondary analysis using only data of unmedicated MDD patients ($n = 156$) and controls ($n = 203$) demonstrated an even more pronounced difference in TRP levels in unmedicated patients, when compared with controls ($p < 0.001$). These data suggest that psychotropic therapy (antidepressants, antipsychotics and benzodiazepines) reduced the difference in TRP levels between groups (70). Decreased levels of TRP and consequent depletion of serotonin results in the development of depressive symptoms, as proposed by the classic monoamine depletion hypothesis. IDO induction may have evolved as a mechanism for the maintenance of NAD+, which is the final product of the IDO and TRP catabolism pathway. NAD+ is important for the induction of sirtuins, which contribute to many of the processes that are deregulated in depression including neurogenesis, circadian rhythms and mitochondrial regulation (63). Despite the evidence that suggest a role of TRYCATs in depression, one should keep in mind that TRYCATs have also been associated with the psycho-somatic symptoms that accompany depression. Since depression and somatization shared common pathways, it may be difficult to discriminate between these effects (71).

QUIN, a product formed in the TRYCAT pathway, is an endogenous *N*-methyl-D-aspartate (NMDA) receptor agonist, while KYN is an NMDA antagonist. QUIN is a neurotoxin and responsible for the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively). A disrupted balance between KYN and QUIN production is observed in the neurotoxicity associated with several inflammatory brain diseases such as Alzheimer's disease, Parkinson's disease and major psychiatric disorders. Activated microglia and infiltrating macrophages are the major source of QUIN in the brain and it is involved in the deleterious pathophysiological cascade within the CNS (69). An aberrant NMDA receptor stimulation associated with pro-inflammatory cytokines may suppress brain-derived neurotrophic factor translation, neurogenesis, provoke changes in brain volume, along with dendritic atrophy and synaptic loss (72; 73). The atrophy of the hippocampus in patients with MDD has been demonstrated, not only by imaging techniques such as magnetic resonance imaging (MRI) but also in post mortem studies (74). QUIN also increases glutamate release to neurotoxic levels, inducing oxidative and nitrosative stress (O&NS) in MDD (75). O&NS damages lipids, proteins and the DNA, as demonstrated through lipid peroxidation, DNA strand breaks, increased protein carbonyl formation and disruption of mitochondrial function (76; 77). Inflammatory responses are often accompanied by O&NS, as reviewed in detail by Maes et al. (78).

Under normal conditions, the levels of ROS are balanced by an antioxidant defense system. However, when there is an unbalanced condition between oxidants and antioxidants, a state of oxidative stress is achieved. Recently, a meta-analysis confirmed the association between depression and oxidative stress, measured mainly in plasma or serum of depressed patients and healthy controls (79). It is also known that lower levels of antioxidants, such as co-enzyme Q10, glutathione, ascorbic acid, vitamin E, zinc and polyunsaturated fatty acids are regularly detected in the blood of depressed patients (75; 80), supporting the notion of an oxidative-stress state in this population.

Other potential harmful effects of inflammatory cytokines on neurotransmitter function are due to the disruption of tetrahydrobiopterin (BH4). BH4 is an essential enzyme co-factor for phenylalanine hydroxylase, tryptophan hydroxylase and tyrosine hydroxylase which are rate-limiting enzymes for the synthesis of serotonin, dopamine and norepinephrine, respectively (81). Moreover, BH4 is also an enzyme co-factor for the conversion of arginine to nitric oxide (NO) through nitric oxide synthase (NOS) (82). Inflammatory cytokines stimulate the production of NO, increasing the utilization of BH4 and thus decreasing neurotransmitter synthesis (81).

TNF-α is a specific pro-inflammatory cytokine that has received attention as a potential modulator of the serotonin transporter (SERT or 5-HTT) and consequently 5-HT uptake and brain availability. The first study to demonstrate in vitro the capacity of TNF-α to increase the expression of SERT in mouse brain cell lines was conducted in 2006 (83). Afterwards, another study showed that prolonged in vitro treatment with TNF-α enhances SERT expression and activity in both glial and neuronal cells, suggesting that the p38 MAPK pathway could be involved (84). Therefore, it was hypothesized that under conditions of chronic inflammation, increased levels of pro-inflammatory cytokines such as TNF-α would enhance SERT-mediated 5-HT uptake and significantly impact the available extracellular 5-HT. Since astrocytes rapidly degrade 5-HT following uptake, enhanced astrocyte uptake might affect the turnover rate of this neurotransmitter, resulting in decreased total brain 5-HT (84). In a proof-of-concept study conducted by Cavanagh et al. (2010), six patients with rheumatoid arthritis were treated with adalimumab (a TNF-α inhibitor) and tested the hypothesis that TNF-α blockade would alter SERT activity in the brain of the patients, through single photon emission tomography (SPECT). In addition, depressive severity was evaluated through the HDRS. SPECT scans were conducted 14 days before the start of the treatment and repeated 4 days after the last treatment. There was a significant decrease in SERT density ($p = 0.03$),

with five of the patients exhibiting a 20% decrease. Depressive scores improved in all subjects. This represents one of the first in vivo studies suggesting the link between TNF-α blockade and SERT modulation (85).

All the aforementioned pathways have detrimental effects in clinical depression and additionally play a role in chronic depression. Pro-inflammatory cytokines, TRYCATs and O&NS together may contribute to a state called neuroprogression, related to neurodegeneration, reduced neurogenesis, neural plasticity and apoptosis (86).

Major depressive disorder as a comorbidity to pro-inflammatory medical conditions: circumstantial evidence

Several inflammatory diseases have also been associated with higher risks of development of depression and this might provide further clues for our understanding of the underlying mechanism of MDD. Patients with a myocardial infarction (MI), for example, have a prevalence of depressive disorder that is about three times higher than in the general population (87). MI triggers an inflammatory cascade that leads to increased pro-inflammatory cytokines in plasma. These cytokines can be transported across the blood–brain barrier and promote the activation of microglia (88). Conventional antidepressants generally have limited effect in MI patients (87), probably due to the presence of neuroinflammation as a result of the chronic elevated immune profile. Autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis are also associated with a higher prevalence of depression. The association might be explained by two hypotheses: (1) chronic stress derived from long-term use of corticosteroids impairs corticosteroid-receptor signaling, therefore, the severe clinical condition and the inadequate adaptation to stress cause persistent hyper-secretion of stress hormones; (2) persistent elevation of pro-inflammatory cytokines due to the chronic inflammation leading to neuroinflammation through the aforementioned pathways (89). Obesity has also been linked to the development of depression via the elevated inflammatory profile associated with the disorder (90). This relation might be partially explained by the fact that adipocytes in the white adipose tissue secrete cytokines, mainly IL-6 and TNF-α, that are referred to as adipocytokines (91). The secretion of the pro-inflammatory markers might lead to an immune activation and be a risk factor for the development of MDD. In fact, this theory has been supported by a meta-analysis conducted by Luppino et al. (2010) showing a clear bidirectional association between depression and obesity: obese people have a 55% increased risk of developing

depression over time, while depressed people had a 58% increased risk of becoming obese. Depression's causal role in obesity might be due to neuroendocrine disturbances, through a long-term activation of the HPA axis and release of cortisol, along with an unhealthy lifestyle (92).

In summary, there are circumstantial evidences that links (neuro)inflammation to MDD, in particular: (1) microglia activation that occurs in a number of neuropsychiatric conditions (22); (2) pro-inflammatory conditions like obesity, MI and autoimmune diseases that are often accompanied by depression (93); (3) presence of neuroinflammation during a major depressive episode in MDD patients visualized through PET imaging (36); (4) significant microgliosis in depressed patients that committed suicide (39); (5) elevated profile of pro-inflammatory cytokines in the blood of depressed patients as compared with controls (94); (6) development of "depressive-like behavior" in rodents systemically exposed to inflammatory conditions, exhibiting elevated levels of activated microglia (33; 95).

Anti-inflammatory treatment for major depressive disorder with nonsteroidal anti-inflammatory drugs

Cyclooxygenases in neuroinflammation: pre-clinical studies

As previously discussed, MDD appears to be associated with elevation of pro-inflammatory cytokines both in peripheral blood and the brain, at least in a subpopulation of the patients. These pro-inflammatory cytokines can trigger an inflammatory cascade in the brain, which includes the induction of cyclooxygenases (COXs) that are key enzymes in the production of prostaglandins (96). Based on this observation, one could hypothesize that treatments targeting the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) could have a beneficial effect in the subgroup of depressed patients with elevated levels of pro-inflammatory cytokines. Indeed, elevated COX-2 messenger ribonucleic acid (mRNA) expression was found for the first time in peripheral blood of patients with recurrent depressive disorder by Gałecki et al. (2012) (97). Both COX isoforms catalyze the same reactions: oxidation of arachidonic acid (AA) to yield prostaglandin G2 (PGG2), followed by a peroxidase reaction which converts PGG2 to prostaglandin H2 (PGH2). In these reactions, reactive oxygen species are also produced that can cause severe cell damage. PGH2 is transformed into PGE2, PGF2α, PGD2, PGI2 and TXB2 by specific terminal synthases (98). PGE2 is the main prostaglandin implicated in the inflammatory response, pain, fever and autonomic functions (99). Furthermore,

COX-1 and COX-2 are both expressed in the brain. COX-2 is detected in synaptic dendrites and excitatory terminals, mainly in cortex, hippocampus and amygdala, whereas COX-1 is expressed by microglia and perivascular cells (100).

COX-1 has been shown to support the inflammatory process and facilitate pro-inflammatory upregulation of prostaglandins in animal models of neuroinflammation (98). Indeed, Choi et al. (2008) demonstrated that mice deficient of COX-1 showed less neuron degeneration, less microglia activation and lower expression of pro-inflammatory cytokines and PGE2 after exposure to LPS via lateral ventricle injection than wild-type mice. Likewise, inhibition of COX-1 with SC-560 (COX-1 selective inhibitor) showed similar effects as the genetic deletion of COX-1 (101).

In contrast to COX-1, COX-2 can have either a neurotoxic or anti-inflammatory role depending on inflammatory stimuli. Results of pre-clinical studies, mainly with celecoxib (a COX-2 selective inhibitor) treatment are contradictory. In a model of chronic unpredictable stress in rats, celecoxib treatment was administered for 21 days. The depressive behavior in the stressed rats was reversed by the NSAID and PGE2 concentrations decreased relative to untreated controls (102). Another well-known rat model of depression, olfactory bulbectomy (OBX), was used to evaluate the antidepressant effect of celecoxib treatment for 14 days. Behavioral alterations of OBX rats were reversed by the drug, whereas pro-inflammatory cytokines IL-1β and TNF-α levels in the pre-frontal cortex and hypothalamus decreased, probably by reduction of systemic PGE2 synthesis (103). Also, the hypothesis that aging contributes to behavioral impairment and increases in the pro-inflammatory markers in the hippocampus was tested by Casolini et al (2002), using rats aged 12- and 24-months old. Chronic treatment with celecoxib for 4 months reduced the levels of IL-1β, TNF-α and PGE2 in the hippocampus, and lower corticosterone levels in the 12-month-old rats (beginning of the aging process). This experiment also demonstrated a possibility for improvement of cognitive impairment and the inflammatory state at the beginning of the aging process (104). However, COX-2 might have also a neuroprotective function in response to an inflammatory challenge. Genetic deletion of COX-2 enhanced the vulnerability towards an LPS challenge, resulting in increased neuronal damage in the hippocampus, increased activation of scavenger receptor A mRNA (specific marker for phagocytic microglia) and increased the expression of TNF-α, IL-6 and IL-1β, as compared with wild-type mice. Furthermore, inhibition of COX-2 by chronic administration of celecoxib for 6 weeks caused an

increase in IL-1β levels in the brain of wild-type mice exposed to LPS, as compared with nontreated LPS-exposed mice (105).

Table 1. Summary of results obtained in pre-clinical studies applying NSAID treatment in (neuro)inflammation models. The table presents the animal model used, number of subjects (*n*), duration of treatment, NSAID (selectivity), type of treatment (preventive or curative) and final outcome (beneficial/not beneficial).

Authors	Animal model	*N*	Duration	NSAID (selectivity)	Type of treatment	Outcome
Aid et al., 2008 (105)	LPS stereotactic injection in the brain of mice	Not described	42 days	Celecoxib (COX-2 selective)	Preventive	Not beneficial
Blais et al., 2005 (99)	LPS i.p. injection in mice	86	30 min	SC-560 (COX-1 selective); NS-398 (COX-2 selective); ketorolac and indomethacin (COX non-selective)	Preventive	Not beneficial for all treatments
Casolini et al., 2002 (104)	Aging rats (12 and 18 months)	60	4 months	Celecoxib (COX-2 selective)	Preventive	Beneficial
Choi et al., 2008 (101)	LPS stereotactic injection in the brain of mice	Not described	7 days	SC-560 (COX-1 selective)	Preventive	Beneficial
Guo et al., 2009 (102)	Chronic unpredictable stress in rats	70	21 days	Celecoxib (COX-2 selective)	Curative	Beneficial
Myint et al., 2007 (103)	Olfactory bulbectomized model of depression in rats	32	14 days	Celecoxib (COX-2 selective)	Curative	Beneficial
Scali et al., 2003 (106)	Quisqualic acid injection into the nucleus basalis in the brain of rats	Not described	7 days	Rofecoxib (COX-2 selective)	Curative	Beneficial
Kurhe et al., 2014 (107)	High fat diet; obesity in mice	36	28 days	Celecoxib (COX-2 selective)	Curative	Beneficial

COX, cyclooxygenase; i.p., intraperitoneal injection; LPS, lipopolysaccharide; NSAID, non-steroidal anti-inflammatory drug; NS-398, COX-2 selective inhibitor; SC-560, COX-1 selective inhibitor.

Taken together, these data suggest that the enzyme COX-1 mainly has a pro-inflammatory role in the brain, whereas COX-2 could be involved in both pro- and anti-inflammatory responses. Interestingly, curative treatment with COX-2 selective inhibitors in (neuro)inflammatory animal models have shown mostly beneficial outcomes by decreasing inflammatory markers in the brain and reversing behavioral alterations, suggesting that there might be a possible application for patients with depression and elevated pro-inflammatory profile (data summarized in Table 1). Also, attenuating the pro-inflammatory role of COX-1 seems to be a good strategy to avoid activation of microglia and the support for the neuroinflammatory process. Further animal studies with selective COX-1 inhibitors still need to be conducted in order to obtain a better understanding of their role in neuroinflammation and putative therapeutic implications.

NSAID monotherapy for major depressive disorder: clinical studies

NSAIDs demonstrated promising results in clinical trials for depression, mainly involving patients with inflammatory disease comorbidities. In patients with osteoarthritis, depression is 2–3 times more prevalent than in age-matched controls (108). In a study including pooled data from five randomized, multicenter, double-blind, placebo-controlled trials on 1497 patients with osteoarthritis, subjects were screened for MDD with the standard patient health questionnaire-9 (PHQ-9) and were divided into three treatment groups: ibuprofen/naproxen (non-selective COX inhibitors), placebo or celecoxib, administered for a duration of 6 weeks. Both groups using NSAIDs (ibuprofen/naproxen or celecoxib) showed a trend towards a reduction in depressive symptoms in patients with osteoarthritis, based on the PHQ-9 scores (108). A possible limitation of this study that might have affected the results was the celecoxib dosage. The recommended therapeutic dose is 400 mg/day, while the patients included in this study received only 200 mg/day.

A study that evaluated the efficacy of anti-inflammatory treatment for depressive symptoms alleviation not linked to inflammatory comorbidities demonstrated that it might not have any beneficial effect. An investigation of the therapeutic benefits of COX inhibitors in late-life depression was performed in 2528 participants over 70-years old with or without significant depressive symptoms, which were screened and randomized to receive celecoxib, naproxen or placebo for 12 months. Only 449 patients were considered depressed at baseline according to their score on the Geriatric Depression Scale (GDS). After the treatment with either drug, the GDS score was not reduced (109). Even though the sample size of this study was big and the treatment period was long, a critical measure of the inflammatory markers was not performed. Thus, it is conceivable that some of the patients included in this study might not have an elevated immune profile and therefore, would not have any benefit from the therapy with NSAIDs.

An epidemiological study called ‘The Health in Men Study’ published two papers regarding the usage of aspirin in older men (aged 69–87 years old) as prevention for the development of depression. The first study evaluated 5556 patients, of which 4461 (89.9%) had a cardiovascular disease. A 5-year follow up revealed that aspirin did not reduce the odds of developing depression in late life. One possible explanation is that aspirin might lead to greater medical complications due to bleeding, increasing the risk of small cerebrovascular lesions that contribute to a higher incidence of depression (110). The second study evaluated a sample of 3687 patients to access the relationship of high

plasma homocysteine (tHcy), which is associated with higher risk of cardiovascular events, and the onset of depression. The study confirmed that high tHcy is associated with an increased risk of depression, with an odds ratio (OR) of 1.80 (95% confidence interval (CI) = 1.39–2.35) and that the usage of aspirin is associated with a decrease in the risk of depression among these patients, with an OR of 0.60 (95% CI = 0.20–1.79) (111). Another study in 345 female subjects evaluated the risk of developing depression in relation to the usage of aspirin during a 10-year follow-up. Estimated rates of MDD were 1.7 (95% CI = 0.4–6.9) per 1000 person-years for subjects using aspirin and 12.2 (95% CI = 7.9–19.0) per 1.000 person-years for the non-users. This study suggests that exposure to aspirin seems to be associated with a reduced risk of developing MDD (112).

As summarized in Table 2, available data from a limited number of studies suggest that curative treatment of MDD with NSAIDs can have a beneficial effect on the relief of depressive symptoms, whereas data on the preventive treatment with COX inhibitors are still inconclusive. A meta-analysis conducted by Kohler et al. (2014) demonstrated that monotherapy treatment with celecoxib had borderline significance in the relief of depressive symptoms (113). Future clinical randomized clinical trials using NSAID treatment monotherapy should be better structured, including only patients with elevated levels of inflammatory markers in the blood or cerebral spinal fluid (CSF) in combination with questionnaires for scoring depressive symptoms. NSAID monotherapy should not be encouraged in the absence of inflammation and neither used as replacement of conventional antidepressants. Moreover, a better description of adverse effects in the studies is paramount. An advantage of anti-inflammatory treatment strategies is that definitive readouts of target engagement are available. Since the majority of anti-inflammatory therapies have an effect on inflammatory markers, it can be easily determined through blood/plasma samples if the treatment is indeed acting on the desired target (114) and terminated as soon as the inflammation is resolved. Complementary diagnostic tools such as brain imaging of inflammatory biomarkers (e.g. TSPO) through PET would be desirable, since the relationship between central and peripheral inflammation in MDD is still understudied. Only when the effects on both depressive scores and inflammatory markers are known, a clear conclusion can be reached about the link between inflammation reduction and depression alleviation. Treatment with NSAIDs in a subgroup of patients with low depressive symptoms and immune deregulation/inflammatory comorbidities (113) seems to be the best approach for future research. Nevertheless, indiscriminate use of anti-inflammatory treatments for MDD

patients without inflammation might be harmful, since inflammatory cytokines play a pivotal role in learning and memory, as well as in neural integrity, neurogenesis and synaptic pruning (115). Thus, a maximum safe treatment length is yet to be established.

Table 2. Résumé of the outcome of clinical studies relating the usage of non-steroidal anti-inflammatory drug (NSAID) and depression. The table shows the number of subjects (*n*), type of subjects, duration of treatment/prevention, NSAID (selectivity), type of treatment (preventive/curative) and final outcome (beneficial/not beneficial).

Authors	*N*	Subjects	Duration	NSAID (selectivity)	Type of treatment	Outcome
Almeida et al., 2010 (110)	5556	Aged men (69-87) with cardiovascular disease	5 years	Aspirin (COX non-selective)	Preventive	Not beneficial
Almeida et al., 2012 (111)	3687	Aged men (69-87) with high levels of plasma homocysteine	5 years	Aspirin (COX non-selective)	Preventive	Beneficial
Fields et al., 2012 (109)	2312	Depressive (449) Healthy (2079) Aged 70+	12 months	Celecoxib (COX-2 selective); naproxen (COX non-selective)	Preventive	Not beneficial for both treatments
Pasco et al., 2010 (112)	345	22 MDD and 323 controls	10 years	Aspirin (COX non-selective)	Preventive	Beneficial
Chen et al., 2010 (116)	1 case report	Depressed patient	5 years	Celecoxib (COX-2 selective)	Curative	Beneficial
Iyengar et al., 2013 (108)	1497	Osteoarthritis patients	6 weeks	Celecoxib (COX-2 selective); ibuprofen or naproxen (COX non-selective)	Curative	Beneficial for all treatments

COX, cyclooxygenase; MDD, major depressive disorder; NSAID, non-steroidal anti-inflammatory drug.

Side effects of non-steroidal anti-inflammatory drugs

Considering the possible beneficial aspects of NSAID treatment for depression, one should be aware of the possible side effects. In a review by Funk and FitzGerald (2007), COX-2 inhibition was associated with an increased susceptibility to thrombosis, hypertension and atherosclerosis due to a thrombotic effect by inhibition of prostacyclin derived from endothelial COX-2 (117; 118). A meta-analysis conducted by Kearney et al. (2006) extracted data from randomized controlled trials regarding the risk of vascular events associated with the usage of selective COX-2 inhibitors vs placebo, and vs traditional NSAIDs (n = 145,373). In the comparison between selective COX-2 inhibitors vs placebo, a 42% higher incidence of vascular events occurred in COX-2 users as compared with placebo. A twofold increase in MI was also observed. When evaluating stroke incidence, no difference between groups was found. Importantly, 121 trials were long-term trials (mean of 139 weeks) while 112 were short-term trials (mean of 11 weeks). Two thirds of vascular events occurred in nine long-term trials; therefore, the

hazards of cardiovascular events emerged after a year to 18 months of selective COX-2 inhibitors' chronic usage. With regard to selective COX-2 inhibitors vs traditional NSAIDs, no significant difference was found regarding the risk of vascular events (119). The increased risk of vascular events might be due to an increased Th1 immune response (pro-inflammatory) when selective COX-2 inhibitors are chronically used. This hypothesis was based on the atherosclerotic plaques scenario, where selective COX-2 inhibitors might provoke macrophage accumulation at the inflamed arterial endothelial site. This response leads to increased production of pro-atherogenic cytokines, attracting lymphocytes and macrophages that will exacerbate the inflammation, increase plaque instability and vulnerability to rupture, embolization and consequent MI (120). Still, this immunological modulation is a slow process, occurring after 12 months of chronic therapy (119; 120). This possible mixed anti-inflammatory and pro-inflammatory effect of COX-2 deserves consideration before chronic usage recommendation in clinical practice (121).

Additionally, NSAIDs are well recognized for causing peptic ulceration and ulcer complications. Cohort studies have estimated that the total risk of hospitalization for gastrointestinal complications associated with NSAID use are between 1.3 and 2.2 events per 1000 patients. Protective strategies as co-prescription of a protective drug such as misoprostol or a proton pump inhibitor can be applied to reduce those events (122). Selective COX-2 inhibitors present an advantage in this matter as the incidence of clinically significant ulcers were reduced by 54% and ulcer complications by 57%, as compared with nonselective NSAIDs (123).

In summary, if the inflammatory hypothesis for MDD is confirmed, the implication would be that anti-inflammatory strategies might hold promise for the treatment of depressed patients with chronically elevated inflammatory biomarkers or inflammatory comorbidities. The effectiveness of the treatment must be followed by measurements of peripheral inflammatory biomarkers associated with depressive scores and the obtained data should be used to determine the required treatment length. Nonetheless, NSAIDs are far from being a panacea for depression and might benefit only a subgroup of depressed patients.

The anti-inflammatory effect of antidepressants

Effect of antidepressants on cytokine levels

Although antidepressants have been used in therapy for depression for more than 50 years, the mechanism of action of most of these drugs still remains a mystery. Antidepressant drugs usually act on the monoaminergic systems, although they can have different mechanisms of action. Some antidepressant drugs were also found to elicit anti-inflammatory and neuroprotective effects, which might be partly due to their influence on cytokine production (124). The influence on the cytokine production might be related to antidepressant action on cyclic adenosyl monophosphate (cAMP), serotonin metabolism, the HPA axis or through a direct action on neurogenesis (125).

The silencing of over-activated glia by antidepressants could stop neuroinflammation and may therefore be beneficial not only for MDD, but also for other CNS diseases. The effects of antidepressant drugs on cytokine levels have been investigated in vitro in cell cultures. A recently published study by Obuchowicz et al. (2014) evaluated the anti-inflammatory effects of imipramine, a tricyclic antidepressant (TCA), and fluoxetine, a selective serotonin reuptake inhibitor (SSRI), on IL-6, IL-1β and TNF-α secretion by primary mixed glial cultures stimulated with LPS. Even though both drugs were able to decrease the levels of the proinflammatory cytokines, only imipramine prevented morphological changes and activation of microglia (124). In another study, Xia et al. (1996) tested the inhibitory effect of the antidepressants imipramine, clomipramine (TCAs) and citalopram (SSRI) on the release of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α by stimulated human lymphocytes and monocytes. All antidepressants exerted inhibitory effects on cytokine release and also increased the levels of cAMP (126). In another in vitro study, clomipramine, sertraline (SSRI) and trazodone (heterocyclic antidepressant) were able to decrease IFN-γ levels and increase the production of the anti-inflammatory cytokine IL-10 (127). Using the same methodology, Maes et al. (2005) found that fluoxetine (SSRI) also has immunomodulatory effects on the secretion of cytokines, such as TNF-α and IFN-γ (128). Nonetheless, conflicting results have been published by Munzer et al. (2013), who tested the effect of escitalopram (an SSRI), citalopram and mirtrazapine (serotonin and noradrenaline reuptake inhibitors, SNRIs) on the secretion levels of cytokines IL-1β, IL-2, IL-4, IL-6, IL-17, IL-22 and TNF-α in stimulated whole blood of 15 depressed patients' ex vivo. A T cell (OKT3) and a B cell (5C3) stimulant was used in the whole blood to induce cytokine production in vitro. Curiously, citalopram increased the levels of IL-1β, IL-6, TNF-α, and IL-22,

whereas mirtrazapine increased IL-1β, TNF-α and IL-22 and escitalopram decreased IL-17. The differences in cytokine production levels might be due to distinctive therapeutic effects between the drugs (56).

Pre-clinical studies have yielded interesting results for antidepressants that decrease cytokine levels, mainly TCAs and SSRIs. Alboni et al. (2013) demonstrated promising effects with imipramine and fluoxetine. Both imipramine and fluoxetine were able to reduce the levels of IFN-γ, IL-6 and increase the expression of the anti-inflammatory cytokine IL-4 in the hypothalamus of healthy male Sprague-Dawley rats after a 28-day treatment (129). Moreover, imipramine was also able to decrease mRNA levels for IL-6 in brain microglia in a rat model of social defeat, which was accompanied by a reversal in social avoidance behavior (130). In a model of MI, rats were either treated with saline or escitalopram for 2 weeks. Plasma levels of IL-1β, TNF-α and PGE2 were significantly decreased after treatment (131).

Several studies have explored the anti-inflammatory effect of antidepressants in clinical studies, as summarized in Table 3. The class of antidepressants that was mostly tested was the SSRI, followed by TCA and SNRI. Most studies showed anti-inflammatory effects of antidepressants. For instance, Brunoni et al. (2014) recently published the results of a trial with 103 depressed patients treated with sertraline for 6 weeks. The levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ were found to be significantly decreased after the treatment (132). 'The Netherlands Study of Depression and Anxiety' evaluated IL-6 and TNF-α levels in depressed patients and found that SSRIs were able to decrease IL-6 levels, but not TNF-α (15). The effect on IL-6 is in agreement with other studies published (125; 133–136). Summarizing, SSRIs have an effect on cytokine levels, mainly on IL-6, TNF-α and IL-1β. On the other hand, when evaluating the effects of venlafaxine (SNRI) on TNF-α and IL-1β in 12 MDD patients after 8 weeks of treatment, the HDRS score decreased at least 50% compared with baseline, whereas no decrease was observed in pro-inflammatory cytokine levels (137). This might be due to two factors: the length of the study was too short to observe any effects on cytokine levels, or because of the known pro-inflammatory effect of norepinephrine on innate immune cells (138). However, the small number of patients completing the trial (n = 12) might be a major limitation of the study. The authors mentioned that the power analysis performed revealed that an n = 12 would be sufficient to detect an antidepressant-induced effect on pro-inflammatory cytokines assuming the agent would act as an SSRI as reported by Leo et al. (2006) (139). SSRIs and SNRIs have different mechanisms of action, and therefore the same

antidepressant- induced effect on pro-inflammatory cytokines should not be expected. Also, the sample size of the study of Leo et al. (2006) was of 46 MDD patients and 46 age-matched healthy controls. Therefore, the obtained data by Piletz et al. (2009) (137) should be interpreted with care. The limited duration of the treatment with antidepressant drugs applied and the small number of subjects evaluated seem to be general limitations of the studies published until now.

Table 3. Summary of clinical studies that assessed the anti-inflammatory effect of antidepressants on cytokines in MDD. The table describes the type and number of subjects investigated, antidepressant used, duration of treatment, cytokines assessed and final outcome of the study.

Author	Type subjects	Antidepressant(s)	Duration	Cytokines assessed	Outcome
Brunoni et al., 2014 (132)	103 unipolar depressive patients	Sertraline (SSRI)	6 weeks	IL-2, IL-4, IL-6, IL-10, IL-17, INF-γ, TNF-α	↓IL-2, ↓IL-4, ↓IL-6, ↓IL-10, ↓IL-17, ↓INF-γ
Basterzi et al., 2005 (133)	23 MDD and 23 controls	Not specified (SSRI)	6 weeks	IL-6	↓IL-6
Eller et al., 2008 (140)	100 MDD and 45 controls	Escitalopram (SSRI)	12 weeks	sIL-2R, IL-8, TNF-α	↓sIL-2R
Eller et al., 2009 (141)	28 MDD and 45 controls	Escitalopram + Bupropion (SSRI + atypical AD)	6 weeks	sIL-2R, IL-8, TNF-α	↑IL-8
Hernandez et al., 2008 (142)	31 MDD and 22 controls	Fluoxetine, paroxetine, setraline (SSRI)	52 weeks	IFN-γ, IL-1β, IL-2, IL-4, IL-10, IL-13	↑IFN-γ, ↑IL-1β, ↓IL-2, ↓IL-4, ↓IL-10, ↓IL-13
Lanquillon et al., 2000 (143)	24 MDD and 15 controls	Amitriptyline (TCA)	6 weeks	IL-6, TNF-α	↓IL-6, ↓TNF-α
Piletz et al., 2009 (137)	22 MDD and 17 controls	Venlafaxine (SNRI)	8 weeks	IL-1β, TNF-α	No significant change
Sluzewska et al., 1995 (135)	22 MDD and 11 controls	Fluoxetine (SSRI)	8 weeks	IL-6	↓IL-6
Taraz et al., 2013 (125)	50 MDD patients	Sertraline (SSRI)	12 weeks	IL-6, TNF-α, IL-10	↓IL-6, ↓TNF-α, ↑IL-10
Tousolis et al., 2009 (144)	250 with HF (154 with MDD)	Not specified (SSRI and SNRI/TCA)	6 months	IL-6, TNF-α	SNRI/TCA: ↓TNF-α
Tuglu et al., 2003 (145)	26 MDD and 17 controls	Sertraline, fluoxetine, citalopram, fluvoxamine, paroxetine (SSRI)	6 weeks	TNF-α	↓TNF-α
Vogelzangs et al., 2012 (15)	1132 current depression; 789 remitted depression; 494 controls	Not specified (SSRI, TCA and SNRI)	8 years	IL-6, TNF-α	SSRI: ↓IL-6
Yoshimura et al., 2009 (136)	51 MDD and 30 controls	Paroxetine, sertraline, fluvoxamine (SSRI); milnacipran (SNRI)	8 weeks	IL-6, TNF-α	↓IL-6

AD, atypical antidepressant; HF, heart failure; IFN, interferon; IL, interleukin; MDD, major depressive disorder; sIL-2R, soluble interleukin-2 receptor; SNRI, serotonin and noradrenaline reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TNF, tumor necrosis factor.

Even though promising results were obtained in vitro and in pre-clinical studies with TCAs, limited clinical trials have been performed with this class of antidepressant. Tousoulis et al. (2009) conducted a clinical study in 154 patients that suffered from heart failure and developed MDD. A total of 120 patients received an SSRI and 34 patients were treated with a TCA/SNRI. The ones that were treated with TCA/SNRI had lower

levels of TNF-α and CRP compared with the SSRI group (144). The underlying mechanism of action that can explain how TCAs affect pro-inflammatory cytokines is largely unknown. It is hypothesized that antidepressants (mainly SSRIs and TCA) decrease pro-inflammatory cytokines levels through the cAMP pathway (Figure 2). 5-HT (5-hydroxytryptamine or serotonin) increases intracellular cAMP levels via G protein-coupled serotonin receptors that can stimulate adenylyl cyclase, which results in inhibition of the protein kinase A pathway and a reduction in the expression of cytokines (126). For imipramine in particular, the effect might be explained by the down-regulation of microglial activation (130).

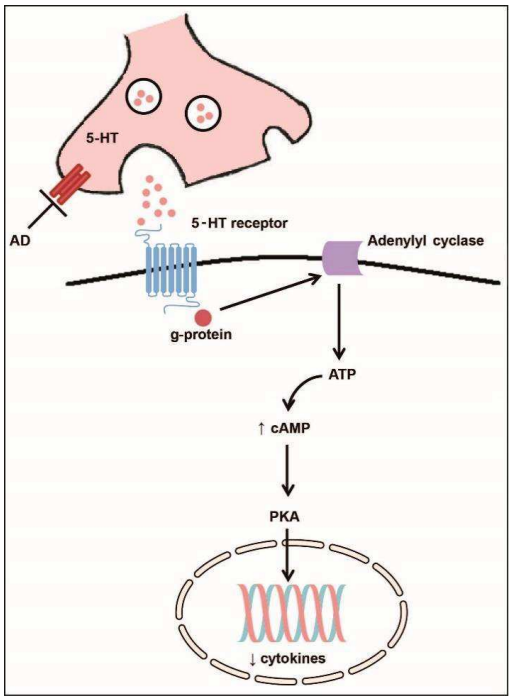


Figure 2. Possible anti-inflammatory mechanism of action of antidepressants. Antidepressants that increase the levels of serotonin (i.e. SSRIs) might exert their anti-inflammatory effects by cAMP-mediated pathways. 5-HT increases intracellular cAMP levels via 5-HT receptors linked to G protein-mediated stimulation of adenylyl cyclase, leading to a reduction in the expression of cytokines via inhibition of the protein kinase A (PKA) pathway.

Possible biomarkers for major depressive disorder: can we predict response to therapy?

As already suggested, pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, could be useful biomarkers for investigating the presence or level of inflammation during MDD screening. Moreover, studies have demonstrated that the levels of pre-treatment biomarkers can be useful predictors of treatment response.

Lanquillon et al. (2000), performed a study in 24 MDD patients and 15 controls, using a 6-week treatment protocol with amitriptyline (TCA) and evaluating immunological parameters such as IL-6 and TNF-α in whole blood. After the treatment period, patients were classified as responders and non-responders according to HAM-D scale. Patients who responded to treatment had significantly lower levels of IL-6 than controls ($p < 0.05$) or non-responders ($p < 0.05$) on baseline measurements. Moreover, regardless of treatment outcome, IL-6 levels in both responders and non-responders returned to control levels after 6 weeks of treatment. TNF-α levels were elevated in responders and non-responders at baseline, as compared with controls ($p < 0.01$). After treatment, the decrease in TNF-α levels was only significant in the responder group ($p < 0.05$). The data suggest that IL-6 levels can predict response to therapy, whereas TNF-α paralleled the clinical response (143). Another study conducted by O'Brien et al. (2007) investigated the differences in the levels of IL-6, IL-8, IL-10, soluble IL-6 receptor (which may act as an agonist of IL-6) and TNF-α between depressive patients who were SSRI-treatment resistant, former SSRI-treatment-resistant patients that are now euthymic due to therapy change and healthy controls. The depressed patients who were SSRI non-responders had marked activation of pro-inflammatory cytokines with high levels of IL-6 and TNF-α. Currently euthymic patients with a prior history of SSRI resistance had pro-inflammatory cytokine levels similar to healthy subjects (146). Eller et al. (2008) also found that higher levels of TNF-α are predictive for nonresponse in depressed patients (140). Yoshimura et al. (2009) found a correlation between high baseline levels of IL-6 with refractory depression (136). Therefore, it seems that suppression of pro-inflammatory cytokines may be necessary for clinical recovery from depression and that reduction in inflammation might not happen in depressed patients who fail to respond to treatment.

Increased pro-inflammatory cytokines are known to induce acute phase (inflammatory) response in the liver, increasing the levels of CRP (2) and lower albumin and zinc in depression (19; 147), suggesting that these could be useful biomarkers of depression. An interesting study conducted by Raison et al. (2013) tested if infliximab, a monoclonal antibody directed against TNF-α, would improve the mood of treatment-resistant depressive patients, since this proinflammatory cytokine has been associated with depression and poor treatment response. The study randomized 60 medically healthy adults with treatment-resistant major depression, to either receive three infusions of infliximab (5 mg/kg) or three saline infusions at baseline, week 2 and 6. Clinical

assessments of depressive scores were made through HAM-D, the Clinical Global Impression – Severity scale, and inflammatory status (hs-CRP, and TNF and its soluble receptors I and II), which were conducted at baseline and weeks 1, 2, 3, 4, 6, 8, 10 and 12. At the end of the trial (week 12), neither differences in HAM-D score between groups, nor significant interactions between treatment and time were found. A significant effect of time on decreased the HAM-D score was observed in both groups ($p < 0.05$). However, when analyzing baseline hs-CRP values to predict response to infliximab, a plasma hs-CRP concentration greater than 5mg/L was found to be the point at which infliximab-treated patients exhibited a greater decrease in HAM-D score than placebo patients (148). The effect size of participants with hs-CRP levels higher than 5mg/L was 0.41, which is in line with the efficacy of antidepressants against placebo in most studies (149).

When selecting immunological biomarkers, one should take into consideration classical markers that already have standardized assays for clinical application, such as CRP (17), albumin and zinc. Pro- and anti-inflammatory cytokines should be determined when possible, even though the assays are not standardized yet and are more restricted to research trials. Ideally, the biomarkers should be measured through peripheral and central (CSF) samples.

Side effects of antidepressants

Despite all the aforementioned benefits of antidepressants, the treatment is not free from risks and side effects. In a study to assess self-discontinuation of antidepressants, 313 patients were interviewed to investigate the reason for stopping the treatment. Side effects were reported by 20% of the patients as the main cause of discontinuation (150). Premature self-discontinuation is associated with relapse and incomplete response to the treatment. The key side effects associated with antidepressant use vary by class of the antidepressant and might vary also by medication within each class (151). Even though TCAs are very effective in treating depression, they also act on other receptor systems, including histaminic, cholinergic, adrenergic and postsynaptic serotonin receptors, leading to significant and sometimes intolerable side effects. Moreover, TCAs have a narrow therapeutic index and at higher doses, they might cause seizures and death due to slowing of intraventricular conduction, leading to complete heart block or ventricular re-entry arrhythmias (152). SSRIs, on the other hand, are better tolerated and significantly less toxic in an overdose than TCAs. The most common side effects related to SSRIs are gastrointestinal like nausea, activation syndrome, sexual dysfunction, body weight gain,

insomnia and serotonin syndrome (153). Antidepressants that also act on the noradrenergic system (i.e. SNRIs) are associated with significantly greater increase in blood pressure and heart rate than SSRIs (151) and also with gastrointestinal effects, activation syndrome, insomnia and sexual dysfunction (153). In order to decrease self-discontinuation, it is important to choose an efficient antidepressant with the profile of side effects that will not hamper the quality of life of the patient.

Augmentative anti-inflammatory strategies

Non-steroidal anti-inflammatory drugs

For those patients who fail to respond to the initial antidepressant therapy, alternative treatment approaches are switching medication, augmentation or combination therapies (154). Augmentation strategies involve the usage of agents that are non-standard antidepressants to enhance the therapeutic effect of a known antidepressant (155). Augmentation with COX-2 inhibitors such as celecoxib has been studied in clinical trials with promising results. For instance, a double-blind, randomized, placebo-controlled study was conducted in 40 patients, using celecoxib + reboxetine (a norepinephrine reuptake inhibitor) or placebo + reboxetine, as treatment for 6 weeks. A decrease of 55% in the HDRS score was observed after treatment with celecoxib + reboxetine, compared with a 33% decrease after treatment with reboxetine + placebo. Interestingly, 45% of the patients in the celecoxib group showed complete remission after 6 weeks, compared with 20% in the placebo group (156). Abbasi et al. (2012) conducted a clinical trial assessing the cytokine profile and depressive symptoms in a group of patients treated with sertraline + celecoxib and in a group treated with sertraline + placebo; 40 patients were randomly assigned to each group and the HDRS score as well as the levels of IL-6 were measured at baseline and after 6 weeks of treatment. At the end of week 6, the HDRS score was reduced by 96% in the celecoxib group compared with a 50% reduction in the placebo group. Also, the IL-6 levels in serum were significantly more reduced in the celecoxib group compared with the placebo group (157). Celecoxib was also successfully used in a 6-week combination treatment with another SSRI, fluoxetine, in 40 depressive patients. The combination of celecoxib with fluoxetine was able to reduce the HDRS score with 90%, while placebo + fluoxetine reduced only 50% (155).

Since the add-on strategy with NSAIDs, mainly celecoxib, could be a potentially effective augmentation, a meta-analysis of the data from randomized clinical trials was performed by three authors. Faridhosseini et al. (2014) analyzed data from four

randomized clinical trials that used celecoxib as augmentation therapy for unipolar depression (n = 160 patients). Celecoxib or placebo was used in combination with sertraline, reboxetine or fluoxetine and the effect on HDRS score was evaluated. The pooled OR of treatment response of celecoxib vs placebo was 6.6, 95% CI = 2.5–17, $p <$ 0.0001; pooled OR of remission response was 6.6, 95% CI = 2.7–15.9, $p < 0.0001$; pooled OR in means of HDRS score decrease at week 6 was 3.43, 95% CI = 1.9–4.9, $p < 0.0001$. In summary, celecoxib in a daily dose of 400 mg/day as an add-on therapy to antidepressants is effective in MDD. No adverse effects attributable to celecoxib were observed (158). Kohler et al. (2014) included 10 randomized clinical trials, from which only 4 were add-on therapy with NSAID (i.e. celecoxib) or placebo with an SSRI or SNRI. A significant improvement in depressive symptoms was observed when compared with placebo in four trials (n = 132); standardized mean difference (SMD) was −0.82, 95% CI = −1.17 to −0.46, $p < 0.001$). In addition, add-on treatment improved remission with OR 7.89, 95% CI = 2.94–21.17, $p < 0.001$ and response (three trials, n = 92 patients) with OR 6.59, 95% CI = 2.24–19.42, $p < 0.001$). No evidence of increased gastrointestinal or cardiovascular adverse effects was reported; the length of trials ranged from 6–8 weeks (159). Rosenblat et al. (2016) preformed a meta-analysis on data from randomized clinical trials focused on bipolar depression. Only two studies used NSAIDs (aspirin or celecoxib) and compared the efficacy with placebo (n = 53). Pooled effects size revealed an SMD of 0.02, 95% CI = −0.52–0.56, $p > 0.05$. Add-on treatment strategy had no significant difference in depressive symptoms (HDRS) or young mania rating scale (YMRS). This result might be attributable to the low level of add-on NSAID studies in bipolar disorder and therefore, it might be underpowered and less robust than other meta-analyses (160).

Based on these data, it seems that adjuvant treatment with COX-2 inhibitors might be a good strategy to improve responsiveness of unipolar depressive patients. Even though the clinical studies performed until now focused mainly on the reduction of depressive symptoms as the outcome parameter, effects of treatment on immunological biomarkers (e.g. cytokines, CRP) should be an outcome parameter as well, especially when SSRIs and NSAID combination treatment is applied. More clinical studies with larger samples size and improved study design should be conducted in order to have more substantial evidence for the additional efficacy of add-on NSAID treatment.

Moreover, when assessing the adverse effects of add-on NSAID treatment in the reported trials, no relevant adverse effects were found. This might be attributable to the short treatment period that ranged from 6–8 weeks. This period might be too short to

identify any adverse effects. Recently, a study was performed on a Korean population to investigate the risk of intracranial hemorrhage among patients treated either with the combination of antidepressants and NSAIDs (n = 2,072,613) or antidepressants alone (n = 2,072,613), during a 30-day follow up after the first antidepressant prescription. An increased risk of intracranial hemorrhage was found when combined therapy was used (hazard ratio 1.6, 95% CI = 1.32–1.85). The risk of intracranial hemorrhage was greater in men than women (2.6, 95% CI =1.93–3.42 vs 1.2, CI 95% = 0.89–1.57). No significant difference was found between antidepressant classes (161). Even though the study provides alarming data, further research is required to replicate the data in different ethnic groups. No explanation was given concerning the higher risks in men than women, or if the risk increases with aging. Another study by Bak et al. (2002) (162) was conducted to investigate association of SSRI usage with ischemic stroke or intracerebral hemorrhage, since previous published data demonstrated that SSRI attenuated platelet activation and decreased the risk of thromboembolism formation (163). The results suggest that SSRIs are not a risk factor for intracerebral hemorrhage and are probably not associated with decreased risk of ischemic stroke. In a secondary analysis, an investigation of current associations of SSRI and NSAIDs, only five events of intracerebral hemorrhage were described among 659 total intracerebral hemorrhage events (162). Nevertheless, in primary psychiatric care, caution must be taken when prescribing antidepressants for current NSAID users with other associated risk factors (e.g. anti-platelet aggregation therapy).

Non-pharmacological anti-inflammatory strategies

Non-pharmacologic augmentation strategies such as exercise and mind–body therapies (MBTs) have demonstrated anti-inflammatory effects in several diseases, such as heart failure (164) and depression (165). MBTs include meditation, yoga, progressive relaxation and Tai Chi (166). These practices have been described as able to regulate emotional and affective response to stress and therefore, influence the immune system. A meta-analysis conducted by Morgan et al. (2014) demonstrated a significant effect of MBTs in decreasing CRP as compared with controls (167). Evidence also suggests that regular endurance exercise decreases inflammatory markers, mainly CRP (168). Although MBTs and exercise are non-pharmacological augmentative strategies, both are able to decrease stress levels and modulate immunity, increasing the quality of life of MDD patients.

Besides MBTs other lifestyle changes, such as dietary interventions, could have a beneficial effect on depression as well. For example, nutraceuticals like zinc and omega-3 fatty acids were shown to have beneficial effects on depression (169). These nutrients may exert their effect by interaction with inflammatory pathways (170).

Concluding remarks

Several theories are available to explain the pathophysiology of MDD, mainly focusing on the involvement of environmental factors associated with genetic and biochemical components (171). Inflammatory deregulation as an etiological factor appears to be a plausible hypothesis to explain why 30–50% of the depressive patients do not respond to conventional therapy. The presence of continuous stimuli that increase the levels of pro-inflammatory cytokines in the brain can cause neurotransmitter imbalance, neuroinflammation and neurodegeneration. Intervention in this process could be an important aim to prevent further damage in the CNS. The levels of inflammatory biomarkers, mainly IL-1β, IL-6, TNF-α and hs-CRP in the serum of depressed patients could be useful biomarkers, along with evaluation of depressive scores. This assessment would be important to guide physicians in patients' classification (e.g. depression with elevated immune profile), selection of the best treatment to be applied and as an indicator of possible treatment resistance to conventional antidepressant drugs.

Concerning treatment, SSRIs were shown to have an anti-inflammatory effect in clinical trials, as they proved potent agents able to decrease the levels of pro-inflammatory cytokines and to diminish depressive symptoms in a subset of patients. Another benefit regarding the usage of SSRI is their higher tolerability in patients. More studies need to be conducted to further elucidate the anti-inflammatory mechanism action of SSRIs and other classes of antidepressants, such as TCAs. Imipramine in particular, shows an interesting effect in vitro and in pre-clinical studies; besides decreasing the pro-inflammatory cytokines, it prevented morphological changes and activation of microglia, thus limiting neuroinflammation. This effect might be useful to prevent further neurodegeneration associated with chronic activation of microglia not only in MDD, but in other CNS diseases associated with neuroinflammation. In general, the best results seem to be obtained with the combination of antidepressants with NSAIDs when initial antidepressant therapy fails (i.e. augmentative therapy). NSAIDs alone have shown best antidepressant results when administered as curative treatment to patients with an inflammatory comorbidity.

Even though considerable lines of research support the immunological hypothesis of depression, the main treatment targets have remained limited to the monoaminergic system. This is mainly due to the fact that the evidence for successful treatment of MDD with anti-inflammatories is still insufficient to adapt treatment guidelines for the subgroup of depressed patients with increased cytokine levels. In the present review, we have summarized the evidence for the benefits of anti-inflammatory treatment for MDD, either as single treatment or as augmentative therapy, and highlighted the anti-inflammatory effect that some antidepressants exert by themselves. However, before anti-inflammatory therapies for MDD can be applied in regular patient care, prospective studies on the efficacy of the anti-inflammatory treatment that combine the evaluation of depressive symptoms with quantification of inflammatory biomarkers are much needed. With substantial proof of efficacy, these alternative approaches can be applied in patient-tailored therapy-selection strategies, thus striving for an improved quality of life, especially for depressive patients with an immunological deregulation profile and treatment resistance to conventional therapy.

Declaration of conflicting interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctôt KL (2010): A Meta-Analysis of Cytokines in Major Depression. *Biol Psychiatry*. 67: 446–457.
2. Miller AH, Maletic V, Raison CL (2009): Inflammation and Its Discontents: The Role of Cytokines in the Pathophysiology of Major Depression. *Biol Psychiatry*. 65: 732–741.
3. Rush AJ, Fava M, Wisniewski SR, Lavori PW, Trivedi MH, Sackeim H, *et al.* (2004): Sequenced treatment alternatives to relieve depression (STAR*D): rationale and design. *Control Clin Trials*. 25: 119–142.
4. American Psychiatric Association (2013): *Diagnostic and Statistical Manual of Mental Disorders: DSM-5*. Arlington: American Psychiatric Publishing.
5. Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, *et al.* (2003): The Epidemiology of Major Depressive Disorder. *JAMA*. 289: 3095–3105.
6. Bschor T, Ising M, Erbe S, Winkelmann P, Ritter D, Uhr M, Lewitzka U (2012): Impact of citalopram on the HPA system. A study of the combined DEX/CRH test in 30 unipolar depressed patients. *J Psychiatr Res*. 46: 111–117.

7. Rush A, MH T, Wisniewski S, Nierenberg A, Stewart J, Warden D, Niederehe G (2006): Acute and Longer-Term Outcomes in Depressed Outpatients Requiring One or Several Treatment Steps: A STAR*D Report. *Am J Psychiatry*. 163: 1905–1917.
8. Friedman A (2014): Jump-starting natural resilience reverses stress susceptibility. *Science*. 346: 555–555.
9. Brunello N, Alboni S, Capone G, Benatti C, Blom JMC, Tascedda F, *et al.* (2006): Acetylsalicylic acid accelerates the antidepressant effect of fluoxetine in the chronic escape deficit model of depression. *Int Clin Psychopharmacol*. 21: 219–225.
10. Trivedi MH, Rush A, Wisniewski SR, Nierenberg AA, Warden D, Ritz L, *et al.* (2006): Evaluation of Outcomes With Citalopram for Depression Using Measurement-Based Care in STAR*D: Implications for Clinical Practice. *Am J Psychiatry*. 163: 28–40.
11. Rosenblat JD, Cha DS, Mansur RB, McIntyre RS (2014): Inflamed moods: A review of the interactions between inflammation and mood disorders. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 53: 23–34.
12. Massart R, Mongeau R, Lanfumey L (2012): Beyond the monoaminergic hypothesis: neuroplasticity and epigenetic changes in a transgenic mouse model of depression. *Philos Trans R Soc B Biol Sci*. 367: 2485–2494.
13. Prins J, Olivier B, Korte SM (2011): Triple reuptake inhibitors for treating subtypes of major depressive disorder: the monoamine hypothesis revisited. *Expert Opin Investig Drugs*. 20: 1107–1130.
14. Song C, Wang H (2011): Cytokines mediated inflammation and decreased neurogenesis in animal models of depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 35: 760–768.
15. Vogelzangs N, Duivis HE, Beekman ATF, Kluft C, Neuteboom J, Hoogendijk W, *et al.* (2012): Association of depressive disorders, depression characteristics and antidepressant medication with inflammation. *Transl Psychiatry*. 2: e79.
16. Carvalho L, Torre J, Papadopoulos A, Poon L, Juruena M, Markopoulou K, *et al.* (2013): Lack of clinical therapeutic benefit of antidepressants is associated overall activation of the inflammatory system. *J Affect Disord*. 148: 136–140.
17. Dantzer R, O'Connor JC, Lawson M a, Kelley KW (2011): Inflammation-associated depression: From serotonin to kynurenine. *Psychoneuroendocrinology*. 36: 426–436.
18. Smith RS (1991): The macrophage theory of depression. *Med Hypotheses*. 35: 298–306.
19. Maes M (1995): Evidence for an immune response in major depression: A review and hypothesis. *Prog Neuropsychopharmacol Biol Psychiatry*. 19: 11–38.
20. Maes M, Bosmans E, De Jongh R, Kenis G, Vandoolaeghe E, Neels H (1997): Increased serum IL-6 and IL-1 receptor antagonist concentrations in major depression and treatment resistant depression. *Cytokine*. 9: 853–858.
21. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008): From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci*. 9: 46–56.
22. Frick LR, Williams K, Pittenger C (2013): Microglial Dysregulation in Psychiatric Disease. *Clin Dev Immunol*. 2013: 1–10.
23. Nadjar A, Bluthé R-M, May MJ, Dantzer R, Parnet P (2005): Inactivation of the Cerebral NFκB Pathway Inhibits Interleukin-1β-Induced Sickness Behavior and c-Fos Expression in Various Brain Nuclei. *Neuropsychopharmacology*. 30: 1492–1499.
24. Czeh M, Gressens P, Kaindl AM (2011): The yin and yang of microglia. *Dev Neurosci*. 33: 199–209.
25. Pace T, Mletzko T, Alagbe O, Musselman D, Nemeroff C, Miller A, Heim C (2006): Increased Stress-Induced Inflammatory Responses in Male Patients With Major Depression and Increased Early Life Stress. *Am J Psychiatry*. 163: 1630–1633.
26. Rawdin B, Mellon S, Dhabhar F, Epel E, Puterman E, Su Y, *et al.* (2013): Dysregulated relationship of inflammation and oxidative stress in major depression. *Brain Behav Immun*. 31: 143–152.
27. Zunszain PA, Anacker C, Cattaneo A, Carvalho LA, Pariante CM (2011): Glucocorticoids, cytokines and brain abnormalities in depression. *Prog Neuro-Psychopharmacology Biol*

Psychiatry. 35: 722–729.
28. Raivich G (2005): Like cops on the beat: the active role of resting microglia. *Trends Neurosci*. 28: 571–573.
29. Sugama S, Takenouchi T, Fujita M, Conti B, Hashimoto M (2009): Differential microglial activation between acute stress and lipopolysaccharide treatment. *J Neuroimmunol*. 207: 24–31.
30. Venneti S, Loprestil B, Wiley C (2013): Molecular imaging of microglia / macrophages in the brain. *Glia*. 61: 10–23.
31. Venneti S, Lopresti BJ, Wiley CA (2006): The peripheral benzodiazepine receptor (Translocator protein 18kDa) in microglia: From pathology to imaging. *Prog Neurobiol*. 80: 308–322.
32. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, *et al.* (2011): β-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat. *J Neurosci*. 31: 6277–6288.
33. Wohleb ES, Fenn AM, Pacenta AM, Powell ND, John F, Godbout JP (2012): Peripheral innate immune challenge exaggerated microglia activation, increased the number of inflammatory CNS macrophages, and prolonged social withdrawal in socially defeated mice. *Psychoneuroendocrinology*. 37: 1491–1505.
34. Kreisel T, Frank MG, Licht T, Reshef R, Ben-Menachem-Zidon O, Baratta M V, *et al.* (2014): Dynamic microglial alterations underlie stress-induced depressive-like behavior and suppressed neurogenesis. *Mol Psychiatry*. 19: 699–709.
35. Doorduin J, de Vries EF, Dierckx RA, Klein H (2008): PET Imaging of the Peripheral Benzodiazepine Receptor: Monitoring Disease Progression and Therapy Response in Neurodegenerative Disorders. *Curr Pharm Des*. 14: 3297–3315.
36. Setiawan E, Wilson AA, Mizrahi R, Rusjan PM, Miler L, Rajkowska G, *et al.* (2015): Role of Translocator Protein Density, a Marker of Neuroinflammation, in the Brain During Major Depressive Episodes. *JAMA Psychiatry*. 72: E1–E8.
37. Hamilton M (1960): A rating scale for depression. *J Neurol Neurosurg Psychiatry*. 23: 56–62.
38. Hannestad J, DellaGioia N, Gallezot J, Lim K, Nabulsi N, Esterlis I, *et al.* (2013): The neuroinflammation marker translocator protein is not elevated in individuals with mild-to-moderate depression: A [11C]PBR28 PET study. *Brain Behav Immun*. 33: 131–138.
39. Steiner J, Bielau H, Brisch R, Danos P, Ullrich O, Mawrin C, *et al.* (2008): Immunological aspects in the neurobiology of suicide: Elevated microglial density in schizophrenia and depression is associated with suicide. *J Psychiatr Res*. 42: 151–157.
40. Maes M, Mihaylova I, Leunis J-C (2007): Increased serum IgA and IgM against LPS of enterobacteria in chronic fatigue syndrome (CFS): Indication for the involvement of gram-negative enterobacteria in the etiology of CFS and for the presence of an increased gut–intestinal permeability. *J Affect Disord*. 99: 237–240.
41. Maes M, Kubera M, Leunis J-C (2008): The gut-brain barrier in major depression: intestinal mucosal dysfunction with an increased translocation of LPS from gram negative enterobacteria (leaky gut) plays a role in the inflammatory pathophysiology of depression. *Neuro Endocrinol Lett*. 29: 117–124.
42. Slyepchenko A, Maes M, Jacka FN, Köhler CA, Barichello T, McIntyre RS, *et al.* (2017): Gut Microbiota, Bacterial Translocation, and Interactions with Diet: Pathophysiological Links between Major Depressive Disorder and Non-Communicable Medical Comorbidities. *Psychother Psychosom*. 86: 31–46.
43. Alboni S, Montanari C, Benatti C, Blom JMC, Simone ML, Brunello N, *et al.* (2011): Constitutive and LPS-regulated expression of interleukin-18 receptor beta variants in the mouse brain. *Brain Behav Immun*. 25: 483–93.
44. Hasler G (2010): Pathophysiology of depression: do we have any solid evidence of interest to clinicians? *World Psychiatry*. 9: 155–161.
45. Krogh J, Benros ME, Jørgensen MB, Vesterager L, Elfving B, Nordentoft M (2014): The association between depressive symptoms, cognitive function, and inflammation in major depression. *Brain Behav Immun*. 35: 70–76.
46. Loftis JM, Huckans M, Morasco BJ (2010): Neuroimmune mechanisms of cytokine-induced depression: Current theories and novel treatment strategies. *Neurobiol Dis*. 37: 519–533.

47. Maes M, Leonard BE, Myint AM, Kubera M, Verkerk R (2011): The new "5-HT" hypothesis of depression: cell-mediated immune activation induces indoleamine 2,3-dioxygenase, which leads to lower plasma tryptophan and an increased synthesis of detrimental tryptophan catabolites (TRYCATs), both of which contribute to the onset of depression. *Prog Neuropsychopharmacol Biol Psychiatry*. 35: 702–721.
48. Messay B, Lim A, Marsland AL (2012): Current understanding of the bi-directional relationship of major depression with inflammation. *Biol Mood Anxiety Disord*. 2: 1–4.
49. Najjar S, Pearlman DM, Alper K, Najjar A, Devinsky O (2013): Neuroinflammation and psychiatric illness. *J Neuroinflammation*. 10: 1-24.
50. Pace TWW, Miller AH (2009): Cytokines and glucocorticoid receptor signaling. Relevance to major depression. *Ann N Y Acad Sci*. 1179: 86–105.
51. Raison CL, Miller AH (2011): Is Depression an Inflammatory Disorder? *Curr Psychiatry Rep*. 13: 467–475.
52. Steiner J, Walter M, Gos T, Guillemin GJ, Bernstein H-G, Sarnyai Z, *et al.* (2011): Severe depression is associated with increased microglial quinolinic acid in subregions of the anterior cingulate gyrus: Evidence for an immune-modulated glutamatergic neurotransmission? *J Neuroinflammation*. 8: 1-9.
53. Sukoff Rizzo SJ, Neal SJ, Hughes Z a, Beyna M, Rosenzweig-Lipson S, Moss SJ, Brandon NJ (2012): Evidence for sustained elevation of IL-6 in the CNS as a key contributor of depressive-like phenotypes. *Transl Psychiatry*. 2: e199.
54. Jokela M, Virtanen M, Batty GD, Kivimäki M (2016): Inflammation and Specific Symptoms of Depression. *JAMA Psychiatry*. 73: 87-88.
55. Valkanova V, Ebmeier KP, Allan CL (2013): CRP, IL-6 and depression: A systematic review and meta-analysis of longitudinal studies. *J Affect Disord*. 150: 736–744.
56. Munzer A, Sack U, Mergl R, Schönherr J, Petersein C, Bartsch S, *et al.* (2013): Impact of Antidepressants on Cytokine Production of Depressed Patients in Vitro. *Toxins (Basel)*. 5: 2227–2240.
57. Qin L, Wu X, Block ML, Liu Y, Breese GR, Knapp DJ, *et al.* (2010): Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration. *Glia*. 55: 453–462.
58. Beishuizen A, Thijs LG (2003): Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J Endotoxin Res*. 9: 3–24.
59. Anders S, Tanaka M, Kinney DK (2013): Depression as an evolutionary strategy for defense against infection. *Brain Behav Immun*. 31: 9–22.
60. Pace TWW, Hu F, Miller AH (2007): Cytokine-effects on glucocorticoid receptor function: Relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. *Brain Behav Immun*. 21: 9–19.
61. Guloksuz S, Rutten BPF, Arts B, van Os J, Kenis G (2014): The Immune System and Electroconvulsive Therapy for Depression. *J ECT*. 30: 132–137.
62. Kim Y, Lee S, Kim S, Shim S, Han S, Choi Sh, Lee B (2008): Differences in cytokines between non-suicidal patients and suicidal patients in major depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 32: 356–361.
63. Anderson G, Kubera M, Duda W, Lasoń W, Berk M, Maes M (2013): Increased IL-6 trans-signaling in depression: focus on the tryptophan catabolite pathway, melatonin and neuroprogression. *Pharmacol reports*. 65: 1647–1654.
64. Kim H, Chen L, Lim G, Sung B, Wang S, Mccabe MF (2012): Brain indoleamine 2,3-dioxygenase contributes to the comorbidity of pain and depression. *J Clin Invest*. 122: 2940–2954.
65. Myint A, Kim Y (2003): Cytokine–serotonin interaction through IDO: a neurodegeneration hypothesis of depression. *Med Hypotheses*. 61: 519–525.
66. Dobos N, de Vries EFJ, Kema IP, Patas K, Prins M, Nijholt IM, *et al.* (2012): The role of indoleamine 2,3-dioxygenase in a mouse model of neuroinflammation-induced depression. *J Alzheimers Dis*. 28: 905–915.
67. Myint A-M, Schwarz MJ, Müller N (2012): The role of the kynurenine metabolism in major depression. *J Neural Transm*. 119: 245–251.
68. Myint AM, Bondy B, Baghai TC, Eser D, Nothdurfter C, Schüle C, *et al.* (2013): Tryptophan

metabolism and immunogenetics in major depression: A role for interferon-γ gene. *Brain Behav Immun*. 31: 128–133.
69. Guillemin GJ (2012): Quinolinic acid, the inescapable neurotoxin. *FEBS J*. 279: 1356–1365.
70. Ogawa S, Fujii T, Koga N, Hori H, Teraishi T, Hattori K, *et al.* (2014): Plasma L-Tryptophan Concentration in Major Depressive Disorder. *J Clin Psychiatry*. 75: e906–e915.
71. Maes M, Rief W (2012): Diagnostic classifications in depression and somatization should include biomarkers, such as disorders in the tryptophan catabolite (TRYCAT) pathway. *Psychiatry Res*. 196: 243–249.
72. Savitz J, Drevets WC, Smith CM, Victor T a, Wurfel BE, Bellgowan PS, *et al.* (2015): Putative Neuroprotective and Neurotoxic Kynurenine Pathway Metabolites Are Associated with Hippocampal and Amygdalar Volumes in Subjects with Major Depressive Disorder. *Neuropsychopharmacology*. 40: 463–471.
73. Swardfager W, Rosenblat JD, Benlamri M, McIntyre RS (2016): Mapping inflammation onto mood: Inflammatory mediators of anhedonia. *Neurosci Biobehav Rev*. 64: 148–166.
74. Stockmeier C, Mahajan GJ, Konick LC, Overholser JC, Jurjus GJ, Meltzer HY, *et al.* (2004): Cellular changes in the postmortem hippocampus in major depression. *Biol Psychiatry*. 56: 640–650.
75. Slyepchenko A, Maes M, Köhler CA, Anderson G, Quevedo J, Alves GS, *et al.* (2016): T helper 17 cells may drive neuroprogression in major depressive disorder: Proposal of an integrative model. *Neurosci Biobehav Rev*. 64: 83–100.
76. Bryleva EY, Brundin L (2017): Kynurenine pathway metabolites and suicidality. *Neuropharmacology*. 112: 324–330.
77. Morris G, Berk M, Klein H, Walder K, Galecki P, Maes M (2017): Nitrosative Stress, Hypernitrosylation, and Autoimmune Responses to Nitrosylated Proteins: New Pathways in Neuroprogressive Disorders Including Depression and Chronic Fatigue Syndrome. *Mol Neurobiol*. 54: 4271–4291.
78. Maes M (2011): Depression is an inflammatory disease, but cell-mediated immune activation is the key component of depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 35: 664–675.
79. Palta P, Samuel LJ, Miller ER, Szanton SL (2014): Depression and oxidative stress: results from a meta-analysis of observational studies. *Psychosom Med*. 76: 12–19.
80. Maes M, Galecki P, Chang YS, Berk M (2011): A review on the oxidative and nitrosative stress (O&NS) pathways in major depression and their possible contribution to the (neuro)degenerative processes in that illness. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 35: 676–692.
81. Miller AH, Haroon E, Raison CL, Felger JC (2013): Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. *Depress Anxiety*. 30: 297–306.
82. Haroon E, Raison CL, Miller AH (2012): Psychoneuroimmunology Meets Neuropsychopharmacology: Translational Implications of the Impact of Inflammation on Behavior. *Neuropsychopharmacology*. 37: 137–162.
83. Zhu C-B, Blakely RD, Hewlett WA (2006): The Proinflammatory Cytokines Interleukin-1beta and Tumor Necrosis Factor-Alpha Activate Serotonin Transporters. *Neuropsychopharmacology*. 31: 2121–2131.
84. Malynn S, Campos-Torres A, Moynagh P, Haase J (2013): The Pro-inflammatory Cytokine TNF-α Regulates the Activity and Expression of the Serotonin Transporter (SERT) in Astrocytes. *Neurochem Res*. 38: 694–704.
85. Cavanagh J, Paterson C, McLean J, Pimlott S, McDonald M, Patterson J, *et al.* (2010): Tumour necrosis factor blockade mediates altered serotonin transporter availability in rheumatoid arthritis: a clinical, proof-of-concept study. *Ann Rheum Dis*. 69: 1251–1252.
86. Maes M, Berk M, Goehler L, Song C, Anderson G, Gałecki P, Leonard B (2012): Depression and sickness behavior are Janus-faced responses to shared inflammatory pathways. *BMC Med*. 10: 1-19.
87. Liu H, Luiten P, Eisel U, Dejongste M, Schoemaker R (2013): Depression after myocardial infarction: TNF-α-induced alterations of the blood–brain barrier and its putative therapeutic implications. *Neurosci Biobehav Rev*. 37: 561–572.

88. Rana I, Stebbing M, Kompa A, Kelly DJ, Krum H, Badoer E (2010): Microglia activation in the hypothalamic PVN following myocardial infarction. *Brain Res*. 1326: 96–104.
89. Postal M, Appenzeller S (2015): The importance of cytokines and autoantibodies in depression. *Autoimmun Rev*. 14: 30–35.
90. Pan A, Sun Q, Czernichow S, Kivimaki M, Okereke OI, Lucas M, *et al.* (2012): Bidirectional association between depression and obesity in middle-aged and older women. *Int J Obes*. 36: 595–602.
91. Shelton RC, Miller AH (2010): Eating ourselves to death (and despair): The contribution of adiposity and inflammation to depression. *Prog Neurobiol*. 91: 275–299.
92. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BWJH, Zitman F (2010): Overweight, Obesity, and Depression. *Arch Gen Psychiatry*. 67: 220–229.
93. Raison CL (2014): Inflammatory Depression: A Trifecta of Trouble. *J Clin Psychiatry*. 75: 663–664.
94. Howren MB, Lamkin DM, Suls J (2009): Associations of Depression With C-Reactive Protein, IL-1, and IL-6: A Meta-Analysis. *Psychosom Med*. 71: 171–186.
95. Wohleb ES, McKim DB, Shea DT, Powell ND, Tarr AJ, Sheridan JF, Godbout JP (2014): Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain. *Biol Psychiatry*. 75: 970–981.
96. Harden LM, Kent S, Pittman QJ, Roth J (2015): Fever and sickness behavior: Friend or foe? *Brain Behav Immun*. 50: 322–333.
97. Gałecki P, Gałecka E, Maes M, Chamielec M, Orzechowska A, Bobińska K, *et al.* (2012): The expression of genes encoding for COX-2, MPO, iNOS, and sPLA2-IIA in patients with recurrent depressive disorder. *J Affect Disord*. 138: 360–366.
98. Aïd S, Bosetti F (2011): Targeting cyclooxygenases-1 and -2 in neuroinflammation: Therapeutic implications. *Biochimie*. 93: 46–51.
99. Blais V, Turrin NP, Rivest S (2005): Cyclooxygenase 2 (COX-2) inhibition increases the inflammatory response in the brain during systemic immune stimuli. *J Neurochem*. 95: 1563–1574.
100. Maes M (2012): Targeting cyclooxygenase-2 in depression is not a viable therapeutic approach and may even aggravate the pathophysiology underpinning depression. *Metab Brain Dis*. 27: 405–413.
101. Choi S, Langenbach R, Bosetti F (2008): Genetic deletion or pharmacological inhibition of cyclooxygenase-1 attenuate lipopolysaccharide-induced inflammatory response and brain injury. *FASEB J*. 22: 1491–1501.
102. Guo J-Y, Li C-Y, Ruan Y-P, Sun M, Qi X-L, Zhao B-S, Luo F (2009): Chronic treatment with celecoxib reverses chronic unpredictable stress-induced depressive-like behavior via reducing cyclooxygenase-2 expression in rat brain. *Eur J Pharmacol*. 612: 54–60.
103. Myint AM, Steinbusch HWM, Goeghegan L, Luchtman D, Kim YK, Leonard BE (2007): Effect of the COX-2 inhibitor celecoxib on behavioural and immune changes in an olfactory bulbectomised rat model of depression. *Neuroimmunomodulation*. 14: 65–71.
104. Casolini P, Catalani A, Zuena AR, Angelucci L (2002): Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and behavioral impairments in the rat. *J Neurosci Res*. 68: 337–343.
105. Aid S, Langenbach R, Bosetti F (2008): Neuroinflammatory response to lipopolysaccharide is exacerbated in mice genetically deficient in cyclooxygenase-2. *J Neuroinflammation*. 5: 1–14.
106. Scali C, Giovannini M., Prosperi C, Bellucci A, Pepeu G, Casamenti F (2003): The selective cyclooxygenase-2 inhibitor rofecoxib suppresses brain inflammation and protects cholinergic neurons from excitotoxic degeneration in vivo. *Neuroscience*. 117: 909–919.
107. Kurhe Y, Mahesh R, Gupta D (2014): Effect of a Selective Cyclooxygenase Type 2 Inhibitor Celecoxib on Depression Associated with Obesity in Mice: An Approach Using Behavioral Tests. *Neurochem Res*. 39: 1395–1402.
108. Iyengar RL, Gandhi S, Aneja A, Thorpe K, Razzouk L, Greenberg J, *et al.* (2013): NSAIDs Are Associated with Lower Depression Scores in Patients with Osteoarthritis. *Am J Med*. 126: 1017.e11-1017.e18.

109. Fields C, Drye L, Vaidya V, Lyketsos CG (2012): Celecoxib or naproxen treatment does not benefit depressive symptoms in persons aged 70 and over: findings from a randomized controlled trial. *Am J Geriatr Psychiatry*. 20: 505–513.
110. Almeida OP, Alfonso H, Jamrozik K, Hankey GJ, Flicker L (2010): Aspirin use, depression and cognitive impairment in later life: the health in men study. *JAGS*. 58: 990–992.
111. Almeida OP, Flicker L, Yeap BB, Alfonso H, McCaul K, Hankey GJ (2012): Aspirin decreases the risk of depression in older men with high plasma homocysteine. *Transl Psychiatry*. 2: e151.
112. Pasco J, Jacka FN, Williams LJ, Henry MJ, Nicholson GC, Kotowicz M, Berk M (2010): Clinical Implications of the Cytokine Hypothesis of Depression: The Association between Use of Statins and Aspirin and the Risk of Major Depression. *Psychother Psychosom*. 79: 323–325.
113. Köhler O, Benros ME, Nordentoft M, Farkouh ME, Iyengar RL, Mors O, Krogh J (2014): Effect of Anti-inflammatory Treatment on Depression, Depressive Symptoms, and Adverse Effects. *JAMA Psychiatry*. 71: 1381-1391.
114. Miller A, Raison C (2015): Are anti-inflammatory therapies viable treatments for psychiatric disorders? Where the rubber meets the road. *JAMA Psychiatry*. 72: 527–528.
115. Yirmiya R, Goshen I (2011): Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain Behav Immun*. 25: 181–213.
116. Chen C-Y, Tzeng N-S, Chen Y-C (2010): Maintenance therapy of celecoxib for major depression with mimicking neuropsychological dysfunction. *Gen Hosp Psychiatry*. 32: 647.e7-647.e9.
117. Funk C, FitzGerald G (2007): COX-2 inhibitors and cardiovascular risk. *J Cardiovasc Pharmacol*. 50: 470–479.
118. Schjerning Olsen A, Fosbøl EL, Lindhardsen J, Andersson C, Folke F, Nielsen MB, *et al.* (2013): Cause-Specific Cardiovascular Risk Associated with Nonsteroidal Anti-Inflammatory Drugs among Myocardial Infarction Patients - A Nationwide Study. (U. Thiem, editor) *PLoS One*. 8: e54309.
119. Kearney PM, Baigent C, Godwin J, Halls H, Emberson J, Patrono C (2006): Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *BMJ*. 332: 1302–1308.
120. Padol IT, Hunt RH (2010): Association of myocardial infarctions with COX-2 inhibition may be related to immunomodulation towards a Th1 response resulting in atheromatous plaque instability: an evidence-based interpretation. *Rheumatology*. 49: 837–843.
121. Scher JU, Pillinger MH (2009): The anti-inflammatory effects of prostaglandins. *J Investig Med*. 57: 703–708.
122. Hawkey C (2003): Non-steroidal anti-inflammatory drugs: overall risks and management. Complementary roles for COX-2 inhibitors and proton pump inhibitors. *Gut*. 52: 600–608.
123. Bombardier C, Laine L, Reicin A, Shapiro D, Burgo-Vargas R, Davis B, *et al.* (2000): Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. *N Engl J Med*. 343: 1520–1528.
124. Obuchowicz E, Bielecka AM, Paul-Samojedny M, Pudełko A, Kowalski J (2014): Imipramine and fluoxetine inhibit LPS-induced activation and affect morphology of microglial cells in the rat glial culture. *Pharmacol Reports*. 66: 34–43.
125. Taraz M, Khatami M, Dashti-Khavidaki S, Akhonzadeh S, Noorbala A, Ghaeli P, Taraz S (2013): Sertraline decreases serum level of interleukin-6 (IL-6) in hemodialysis patients with depression: Results of a randomized double-blind, placebo-controlled clinical trial. *Int Immunopharmacol*. 17: 917–923.
126. Xia Z (1996): Tricyclic antidepressants inhibit IL-6, IL-1beta and TNF-alfa release in human blood monocytes and IL-2 and interferon- T in T cells. *Immunopharmacology*. 34: 27–37.
127. Maes M, Song C, Lin AH, Bonaccorso S, Kenis G, De Jongh R, *et al.* (1999): Negative immunoregulatory effects of antidepressants inhibition of interferon-gamma and stimulation of interleukin-10 secretion. *Neuropsychopharmacology*. 20: 370–379.
128. Maes M, Kenis G, Kubera M, De Baets M, Steinbusch H, Bosmans E (2005): The negative immunoregulatory effects of fluoxetine in relation to the cAMP-dependent PKA pathway.

Int Immunopharmacol. 5: 609–618.
129. Alboni S, Benatti C, Montanari C, Tascedda F, Brunello N (2013): Chronic antidepressant treatments resulted in altered expression of genes involved in inflammation in the rat hypothalamus. *Eur J Pharmacol*. 721: 158–167.
130. Ramirez K, Shea DT, McKim DB, Reader BF, Sheridan JF (2015): Imipramine attenuates neuroinflammatory signaling and reverses stress-induced social avoidance. *Brain Behav Immun*. 46: 212–220.
131. Bah TM, Benderdour M, Kaloustian S, Karam R, Rousseau G, Godbout R (2011): Escitalopram reduces circulating pro-inflammatory cytokines and improves depressive behavior without affecting sleep in a rat model of post-cardiac infarct depression. *Behav Brain Res*. 225: 243–251.
132. Brunoni AR, Machado-Vieira R, Zarate C a., Valiengo L, Vieira EL, Bense??or IM, *et al.* (2014): Cytokines plasma levels during antidepressant treatment with sertraline and transcranial direct current stimulation (tDCS): Results from a factorial, randomized, controlled trial. *Psychopharmacology (Berl)*. 231: 1315–1323.
133. Basterzi AD, Aydemir Ç, Kisa C, Aksaray S, Tuzer V, Yazici K, Goka E (2005): IL-6 levels decrease with SSRI treatment in patients with major depression. *Hum Psychopharmacol*. 20: 473–476.
134. Hannestad J, DellaGioia N, Bloch M (2011): The Effect of Antidepressant Medication Treatment on Serum Levels of Inflammatory Cytokines: A Meta-Analysis. *Neuropsychopharmacology*. 36: 2452–2459.
135. Sluzewska A, Rybakowski JK, Laciak M (1995): Interleukin-6 Serum Levels in Depressed Patients before and after Treatment with Fluoxetine. *Ann New York Acad Sci*. 762: 474–476.
136. Yoshimura R, Hori H, Ikenouchi-Sugita A, Umene-Nakano W, Ueda N, Nakamura J (2009): Higher plasma interleukin-6 (IL-6) level is associated with SSRI- or SNRI-refractory depression. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 33: 722–726.
137. Piletz JE, Halaris A, Iqbal O, Hoppensteadt D, Fareed J, Zhu H, *et al.* (2009): Pro-inflammatory biomakers in depression: Treatment with venlafaxine. *World J Biol Psychiatry*. 10: 313–323.
138. Thayer JF, Sternberg EM (2010): Neural aspects of immunomodulation: Focus on the vagus nerve. *Brain Behav Immun*. 24: 1223–1228.
139. Leo R, Di Lorenzo G, Tesauro M, Razzini C, Forleo GB, Chiricolo G, *et al.* (2006): Association Between Enhanced Soluble CD40 Ligand and Proinflammatory and Prothrombotic States in Major Depressive Disorder. *J Clin Psychiatry*. 67: 1760–1766.
140. Eller T, Vasar V, Shlik J, Maron E (2008): Pro-inflammatory cytokines and treatment response to escitalopram in major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry*. 32: 445–450.
141. Eller T, Vasar V, Shlik J, Maron E (2009): Effects of bupropion augmentation on pro-inflammatory cytokines in escitalopram-resistant patients with major depressive disorder. *J Psychopharmacol*. 23: 854–858.
142. Hernández ME, Mendieta D, Martínez-Fong D, Loría F, Moreno J, Estrada I, *et al.* (2008): Variations in circulating cytokine levels during 52 week course of treatment with SSRI for major depressive disorder. *Eur Neuropsychopharmacol*. 18: 917–924.
143. Lanquillon S, Krieg J-C, Bening-Abu-Shach U, Vedder H (2000): Cytokine Production and Treatment Response in Major Depressive Disorder. *Neuropsychopharmacology*. 22: 370–379.
144. Tousoulis D, Drolias A, Antoniades C, Vasiliadou C, Marinou K, Latsios G, *et al.* (2009): Antidepressive treatment as a modulator of inflammatory process in patients with heart failure: Effects on proinflammatory cytokines and acute phase protein levels. *Int J Cardiol*. 134: 238–243.
145. Tuglu C, Hakan Kara S, Caliyurt O, Vardar E, Abay E (2003): Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder. *Psychopharmacology (Berl)*. 170: 429–433.
146. O'Brien SM, Scully P, Fitzgerald P, Scott L V, Dinan TG (2007): Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. *J*

Psychiatr Res. 41: 326–331.
147. Roomruangwong C, Kanchanatawan B, Sirivichayakul S, Mahieu B, Nowak G, Maes M (2016): Lower Serum Zinc and Higher CRP Strongly Predict Prenatal Depression and Physio-somatic Symptoms, Which All Together Predict Postnatal Depressive Symptoms. *Mol Neurobiol*. 6: 1–13.
148. Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, *et al.* (2013): A Randomized Controlled Trial of the Tumor Necrosis Factor Antagonist Infliximab for Treatment-Resistant Depression. *JAMA Psychiatry*. 70: 31-41.
149. Raison CL (2016): The Promise and Limitations of Anti-Inflammatory Agents for the Treatment of Major Depressive Disorder. *Curr Top Behav Neurosci*. 287–302.
150. Samples H, Mojtabai R (2015): Antidepressant Self-Discontinuation: Results From the Collaborative Psychiatric Epidemiology Surveys. *Psychiatr Serv*. 66: 455–462.
151. Kemp DE (2014): Managing the side effects associated with commonly used treatments for bipolar depression. *J Affect Disord*. 169: S34–S44.
152. Ferguson JM (2001): SSRI Antidepressant Medications: Adverse Effects and Tolerability. *Prim Care Companion J Clin Psychiatry*. 3: 22–27.
153. Miskovic M (2015): Comparison of Tolerance of Venlafaxine, Paroxetine and Amitriptyline in Depression Therapy. *Med Arch*. 69: 107–109.
154. Mendlewicz J (2008): Towards achieving remission in the treatment of depression. *Dialogues Clin Neurosci*. 10: 371–375.
155. Akhondzadeh S, Jafari S, Raisi F, Nasehi AA, Ghoreishi A, Salehi B, *et al.* (2009): Clinical trial of adjunctive celecoxib treatment in patients with major depression: a double blind and placebo controlled trial. *Depress Anxiety*. 26: 607–611.
156. Müller N, Schwarz MJ, Dehning S, Douhe A, Cerovecki A, Goldstein-Müller B, *et al.* (2006): The cyclooxygenase-2 inhibitor celecoxib has therapeutic effects in major depression: results of a double-blind, randomized, placebo controlled, add-on pilot study to reboxetine. *Mol Psychiatry*. 11: 680–684.
157. Abbasi S-H, Hosseini F, Modabbernia A, Ashrafi M, Akhondzadeh S (2012): Effect of celecoxib add-on treatment on symptoms and serum IL-6 concentrations in patients with major depressive disorder: randomized double-blind placebo-controlled study. *J Affect Disord*. 141: 308–314.
158. Faridhosseini F, Sadeghi R, Farid L, Pourgholami M (2014): Celecoxib: a new augmentation strategy for depressive mood episodes. A systematic review and meta-analysis of randomized placebo-controlled trials. *Hum Psychopharmacol Clin Exp*. 29: 216–223.
159. Köhler O, Benros ME, Nordentoft M, Farkouh ME, Iyengar RL, Mors O, Krogh J (2014): Effect of Anti-inflammatory Treatment on Depression, Depressive Symptoms, and Adverse Effects. *JAMA Psychiatry*. 71: 1381-1391.
160. Rosenblat JD, Kakar R, Berk M, Kessing L V., Vinberg M, Baune BT, *et al.* (2016): Anti-inflammatory agents in the treatment of bipolar depression: A systematic review and meta-analysis. *Bipolar Disord*. 18: 89–101.
161. Shin J-Y, Park M-J, Lee SH, Choi S-H, Kim M-H, Choi N-K, *et al.* (2015): Risk of intracranial haemorrhage in antidepressant users with concurrent use of non-steroidal anti-inflammatory drugs: nationwide propensity score matched study. *BMJ*. 351: 1-7.
162. Bak S, Tsiropoulos I, Kjaersgaard JO, Andersen M, Mellerup E, Hallas J, *et al.* (2002): Selective Serotonin Reuptake Inhibitors and the Risk of Stroke: A Population-Based Case-Control Study. *Stroke*. 33: 1465–1473.
163. Chu C-S, Chou P-H, Lin C-H, Cheng C, Tsai C-J, Lan T-H, *et al.* (2016): Use of Selective Serotonin Reuptake Inhibitors and Risks of Stroke in Patients with Obsessive Compulsive Disorder: A Population-Based Study. *PLoS One*. 11: e0162239.
164. Pullen PR, Nagamia SH, Mehta PK, Thompson WR, Benardot D, Hammoud R, *et al.* (2008): Effects of Yoga on Inflammation and Exercise Capacity in Patients With Chronic Heart Failure. *J Card Fail*. 14: 407–413.
165. Lavretsky H, Alstein LL, Olmstead RE, Ercoli LM, Riparetti-Brown M, St. Cyr N, Irwin MR (2011): Complementary Use of Tai Chi Chih Augments Escitalopram Treatment of Geriatric Depression: A Randomized Controlled Trial. *Am J Geriatr Psychiatry*. 19: 839–850.

166. Muehsam D, Lutgendorf S, Mills PJ, Rickhi B, Chevalier G, Bat N, *et al.* (2017): The embodied mind: A review on functional genomic and neurological correlates of mind-body therapies. *Neurosci Biobehav Rev*. 73: 165–181.
167. Morgan N, Irwin MR, Chung M, Wang C (2014): The Effects of Mind-Body Therapies on the Immune System: Meta-Analysis. *PLoS One*. 9: e100903.
168. Haaland D a, Sabljic TF, Baribeau D a, Mukovozov IM, Hart LE (2008): Is Regular Exercise a Friend or Foe of the Aging Immune System? A Systematic Review. *Clin J Sport Med*. 18: 539–548.
169. Sarris J, Murphy J, Mischoulon D, Papakostas GI, Fava M, Berk M, Ng CH (2016): Adjunctive Nutraceuticals for Depression: A Systematic Review and Meta-Analyses. *Am J Psychiatry*. 173: 575–587.
170. Johnson RW (2015): Feeding the beast: Can microglia in the senescent brain be regulated by diet? *Brain Behav Immun*. 43: 1–8.
171. Maciel IS, Silva RBM, Morrone FB, Calixto JB, Campos MM (2013): Synergistic effects of celecoxib and bupropion in a model of chronic inflammation-related depression in mice. *PLoS One*. 8: e77227.

Repeated social defeat induces transient glial activation and brain hypometabolism: a PET imaging study

Author(s): Paula Kopschina Feltes, Erik FJ de Vries, Luis Eduardo Juarez-Orozco, Ewelina Kurtys, Rudi AJO Dierckx, Cristina Maria Moriguchi-Jeckel, Janine Doorduin.

CHAPTER 3

as published in the Journal of Cerebral Blood Flow & Metabolism. Kopschina Feltes et al. (2017) *Journal of Cerebral Blood Flow and Metabolism*. E-pub ahead of print. DOI: 10.1177/0271678X17747189.

Abstract

Psychosocial stress is a risk factor for the development of depression. Recent evidence suggests that glial activation could contribute to the development of depressive-like behaviour. This study aimed to evaluate *in vivo* whether repeated social defeat (RSD) induces short- and long-term inflammatory and metabolic alterations in the brain through positron emission tomography (PET). Male Wistar rats (n=40) were exposed to RSD by dominant Long-Evans rats on 5 consecutive days. Behavioural and biochemical alterations were assessed at baseline, day 5/6 and day 24/25 after the RSD protocol. Glial activation (^{11}C-PK11195 PET) and changes in brain metabolism (^{18}F-FDG PET) were evaluated on day 6, 11 and 25 (short-term), and at 3 and 6 months (long-term). Defeated rats showed transient depressive- and anxiety-like behaviour, increased corticosterone and brain IL-1β levels, as well as glial activation and brain hypometabolism in the first month after RSD. During the 3- and 6-month follow-up, no between-group differences in any investigated parameter were found. Therefore, non-invasive PET imaging demonstrated that RSD induces transient glial activation and reduces brain glucose metabolism in rats. These imaging findings were associated with stress-induced behavioural changes and support the hypothesis that neuroinflammation could be a contributing factor in the development of depression.

Keywords: brain metabolism, depression, neuroinflammation, PET imaging, repeated social defeat.

Introduction

Major depressive disorder (MDD) is a highly prevalent mental disorder affecting approximately 350 million people worldwide (1). Although MDD patients can benefit from treatment with antidepressants, over 30% of them are (at least partly) treatment-resistant (2). It is likely that the lack of treatment efficacy arises from the gaps in our understanding of MDD etiology (3).

One of the risk factors for the development of MDD is exposure to psychosocial stress. Currently, it is estimated that 20-25% of individuals exposed to highly stressful events develop MDD (4; 5). Notably, recent evidence suggests that (neuro)inflammatory processes may be involved in the physiopathology of MDD (6). In this sense, it is possible that psychosocial stress and persistent immunological activation might contribute (either additively or in parallel) to treatment resistance to conventional antidepressants (4; 7–11).

Microglia and astrocytes are involved in the immunological response of the central nervous system. These cells are known to undergo a series of events, commonly known as "glial activation", in response to brain disturbances such as neuronal damage or infection. These events include cell proliferation, morphological changes, increased expression of specific cell surface markers, production of cytokines and other inflammatory mediators (12). Depending on the type of stimulus and its duration, microglia activation can be classified as acute or chronic (13). Stress could be such a stimulus for glia activation. Glucocorticoids released as a consequence of psychosocial stress can bind to corticoid receptors on microglia and induce a shift towards a pro-inflammatory phenotype (14; 15). Chronically activated microglia produce well-known pro-inflammatory cytokines (16) such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α), all of which can affect the hypothalamic-pituitary-adrenal (HPA) axis and alter central serotonin levels (17) and thus could ultimately trigger mood disorders (18).

Nowadays, a widely accepted biomarker for activated microglia and astrocytes is the translocator protein (TSPO) (19). Under normal conditions, TSPO expression is low, but the receptor is highly overexpressed upon activation by an inflammatory stimulus. Therefore, TSPO overexpression has been used as a glial activation biomarker (20), measured noninvasively by means of positron emission tomography (PET) using ^{11}C-PK11195 as the tracer (21). At the same time, PET offers the possibility to image other (patho)physiological processes that are seemingly altered in MDD patients, such as brain glucose metabolism (22). Glucose metabolism can be measured with the tracer ^{18}F-FDG.

A growing body of preclinical evidence has implicated microglia activation in the neuroinflammatory response to psychosocial stress (23–26). Repeated social defeat (RSD) constitutes a model of psychosocial stress in rats with a high ethological validity (27). RSD is able to induce adverse physiological, behavioural and neuronal deficits, which resemble certain core symptoms of depression (9; 28). However, the effects of RSD on glial activation and brain metabolism have not been assessed *in vivo* and the long-term effects of RSD are largely unknown.

Therefore, the aim of this study was to longitudinally investigate the short- and long-term effects of psychosocial stress on the development of glial activation and brain metabolism in the RSD rat model of stress-induced depressive behaviour, using non-invasive PET imaging. To confirm the validity of the animal model, behavioural changes and corticosterone levels were assessed.

Materials and Methods

Experimental Animals

Male outbred Wistar Unilever rats (n=40, 8 weeks, 261±15g; Harlan, Horst, The Netherlands) were randomly divided in two groups: control (n=20) and social defeat (SoD, n=20). Sample size was calculated based on previous studies using ^{11}C-PK11195 PET for glial activation detection in rats (20; 29). The rats were individually housed during the experiment and kept in humidity-controlled, thermo-regulated (21±2°C) rooms under a 12:12 hour light:dark cycle with lights on at 7 a.m. After experimental day 25, rats were housed in pairs for the 6-month follow-up in order to prevent social isolation stress effects(30–32). Rats had *ad libitum* access to food and water, and were weighed every day.

Animal experiments were performed in accordance with the Dutch Experimental Animals Act (Wet op Dierenproeven; WoD) of 1977 and its later amendments. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Dier Experimenten Comissie – DEC), protocol DEC 6828A and 6828B, and are reported according to the ARRIVE guidelines (33).

Study design

The overall design of the study is depicted in detail on Fig. 1. Wistar rats (SoD group) were subjected to RSD on day 0–4. Controls were handled similarly, but not exposed to

aggressive residents. In each group, 10 rats were randomly selected for PET imaging and followed-up longitudinally with ^{11}C-PK11195 and ^{18}F-FDG on days -1, 6, 11 and 25 (same rats scanned with both tracers at all time points). Behavioural assessments were performed on days -2, 5 and 24 (short-term follow-up). The previously scanned rats were re-evaluated after 3 and 6 months (long-term follow-up) in order to determine the persistence of behavioural alterations, glial activation and differences in brain metabolism. During the follow up, the rats were weighed once a week.

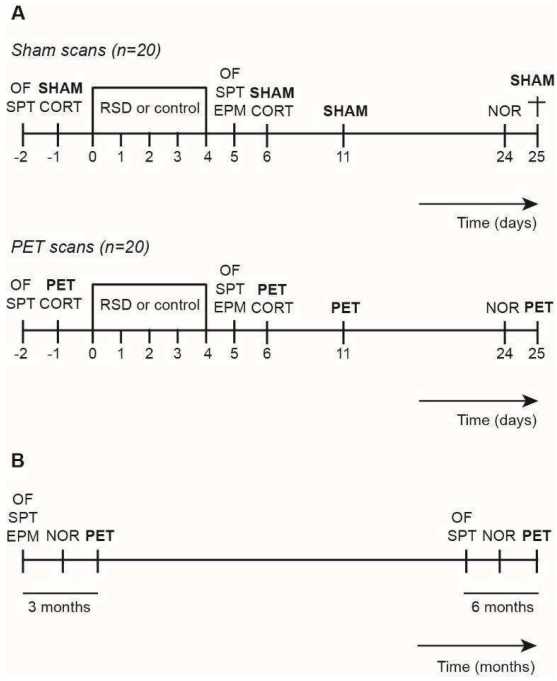


Figure 1: Study design. **(A)** Short-term follow-up: SoD (n=20) and control rats (n=20) were subjected to repeated social defeat from day 0–4. Open field (OF) and sucrose preference test (SPT) were carried out at day -2 and day 5. The elevated plus maze (EPM) was conducted on day 5. The novel object recognition test (NOR) was performed on day 24. PET scans with 11C-PK11195 and 18FFDG or sham scans were performed on day -1, 6, 11 and 25, with collection of serum samples for corticosterone (CORT) measurements on day -1 and 6. On day 25, 10 SoD and 10 control rats that underwent sham scans were terminated for brain collection and pro-inflammatory cytokines quantification. **(B)** Long-term follow-up: SoD (n=10) and control (n=10) rats were followed during six months after the cessation of RSD, with OF, SPT, EPM, NOR and PET scans being repeated after three and six months.

The remaining 10 rats per group were handled exactly the same, but were subjected to sham scans (anesthetized for the same period). On day 25, the rats that underwent sham scans were terminated and the brains were collected for the quantification of pro-inflammatory cytokines.

Repeated Social Defeat

SoD rats were introduced into the cage of a dominant (resident) male outbred Long Evans rat (502±36g; Harlan, Indianapolis, USA). The male Long Evans rats were housed in a separate experimental room in large cages (80x50x40 cm) with a Long Evans female rat, with ligated oviducts, to stimulate territorial aggression (27). The residents were trained and screened for aggressive behaviour at least three times prior to the experiment (8). Only residents that attacked an intruder within 1 min were used for the actual social defeat experiment.

The RSD experiment always took place between 16:00 and 18:00 p.m. Prior to RSD, females were removed from the cage of the resident. The experimental rat (intruder) was placed in the cage of the resident and they were allowed to interact for a period of 10 min or shorter if the intruder assumed a supine (submissive) position for at least 3 seconds. After submission (or 10 min exposure), the intruder was placed inside a wire mesh cage to avoid further physical contact, but still allowing intense visual, auditory and olfactory interactions for a total exposure period of 60 min. The social defeat protocol was repeated on 5 consecutive days using different residents. Control rats were placed in a new clean cage without resident for 60 min on 5 consecutive days.

Body weight gain (g)

Body weight gain (g) was calculated for each rat as the difference between the body weight at a given time point minus the weight on experimental day 0 (first day of RSD).

Behavioural Tests

Open field, elevated plus maze and novel object recognition tests were recorded on video for further analysis using Ethovision XT8.5 software (Noldus Information Technology, Wageningen, The Netherlands).

Sucrose Preference (SPT)

The sucrose preference test was used to assess anhedonia, a sign of depressive behaviour (8). Prior to the experiment, rats were habituated 4 times by exposure to a 1% sucrose solution for 1h. At baseline and after 5 days of RSD, a bottle with water and one with 1% sucrose solution were randomly placed in the cage of the rat. The preference for sucrose

was calculated as the total intake of sucrose solution divided by the total liquid intake and multiplied by 100% (34).

Open field (OF)

To investigate the effects of social defeat on explorative and anxiety-related behaviour, the rats were placed inside a square box (100x100x40 cm) for 10 min on day -2 and 5. The time spent in the centre of the arena relative to the time spent at the borders (a proxy measurement for anxiety), and the total distance moved (locomotor and explorative activity) were documented.

Elevated plus-maze (EPM)

A standard EPM with 52 cm arms extending from a 9x9 cm central area, 62 cm above the floor, was used to assess anxiety-like behaviour on day 5 and month 3 (35). Each session of 5 min was started by placing the rat in the central area facing the closed arms of the maze (36). The percentage of time spent in the open and closed arms, and in the centre was measured. The EPM could not be applied in the 6-month follow-up since the size of the animals impeded their mobility in the apparatus.

Novel object recognition (NOR)

A novel object recognition test was performed to evaluate visual memory (37) and the long-lasting memory impairment induced by RSD on day 24, month 3 and 6 (38). Rats were placed in a square box (50x50x40 cm) with two identical objects (plastic bottles or Lego cubes) (39). They were allowed to explore the objects for 3 min. The objects were removed and after 2 h one familiar and one new object were presented to the rat for 3 min. The preference index (PI) was calculated as the ratio between time spent on exploring the new object and the total time spent on object exploration (40).

Corticosterone Levels

For corticosterone quantification, rats were anesthetized with isoflurane mixed with medical air and 0.5 mL of whole blood was quickly collected from the tail vein on day -1 and 6. Samples were always collected at 10 a.m. The whole blood was allowed to cloth for 15 min and centrifuged at 6.000 rpm (3.5g) for 8 min at room temperature to obtain serum samples. Samples were stored at -20°C until further analysis by radioimmunoassay. Corticosterone (Sigma Chemical Co., Missouri, USA.) was used as

standard and ^{3}H-corticosterone as tracer (Perkin & Elmer, Massachusetts, USA). The sensitivity of the assay was 3 nM. The intra- and inter-assay variations were 6% and 9.6%, respectively.

PET Imaging

PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Both ^{11}C-PK11195 and ^{18}F-FDG PET scans were performed on the same day for each investigated time point. ^{11}C-PK11195 PET scans were always carried out in the morning (between 10:00-11:00 a.m.). For the procedure, rats were anesthetized with isoflurane mixed with medical air (5% for induction, 2% for maintenance) and ^{11}C-PK11195 was injected via the penile vein (66±29 MBq, 1.4±2.3 nmol). Immediately after injection, rats were allowed to wake up and recover in their home cage. ^{18}F-FDG PET scans were carried out in the afternoon (between 15:00-16:00 p.m.), respecting an interval of at least 10 half-lives ($t^1/_2$) of ^{11}C isotope decay. Rats were deprived from food for 4-6 h, injected intraperitoneally (21; 41) with ^{18}F-FDG (31±8 MBq), and returned to their home cage afterwards. For both ^{11}C-PK11195 and ^{18}F-FDG PET, rats were anesthetized 45 min after tracer injection and placed in prone position into the camera with the head in the field of view. A 30-min static scan was acquired, the body temperature was maintained at 37°C with heating pads, heart rate and blood oxygen saturation were monitored, and eye salve was applied to prevent conjunctival dehydration. A transmission scan was obtained using a ^{57}Co point source for attenuation and scatter correction.

PET scans were iteratively reconstructed (OSEM2D, 4 iterations and 16 subsets) into a single frame after being normalized and corrected for attenuation and decay of radioactivity. Images with a 128x128x95 matrix, a pixel width of 0.632 mm, and a slice thickness of 0.762 mm were obtained. PET images were automatically co-registered to a functional ^{11}C-PK11195 or ^{18}F-FDG rat brain template (42), which was spatially aligned with a stereotaxic T2-weighted MRI template in Paxinos space (43) using VINCI 4.26 software (Max Planck Institute for Metabolism Research, Germany). Aligned images were resliced into cubic voxels (0.2 mm) and converted into standardized uptake value (SUV) images: SUV= [tissue activity concentration (MBq/g) x body weight (g)] / [injected dose (MBq)], assuming a tissue density of 1 g/ml. ^{18}F-FDG uptake was not corrected for blood glucose levels (21; 44).

Tracer uptake was calculated in several predefined volumes-of-interest (VOI). VOIs were selected based on previous findings (22; 23; 45–51), taking the size of the brain regions into consideration. Due to the limited resolution of the small animal PET scanner (1.4 mm) (52), small brain regions were excluded to minimize partial volume effects (53). Therefore, the investigated regions were the amygdala/piriform complex, brainstem, cerebellum, cingulate cortex, entorhinal cortex, frontal association cortex, hippocampus, hypothalamus, insular cortex, medial prefrontal cortex, motor/somatosensory cortex, orbitofrontal cortex and striatum.

Enzyme linked immunoassay (ELISA) for pro-inflammatory cytokines in the brain

On day 25, rats were terminated under deep anaesthesia by transcardial perfusion with phosphate-buffered saline pH 7.4. Brains were collected and rapidly frozen and stored at -80°C. Frontal cortex, hippocampus, cerebellum and parietal/temporal/occipital cortex were dissected and prepared as published (39). Pro-inflammatory cytokines IL-6, TNF-α (Biolegend, San Diego, USA) and IL-1β (Thermo Scientific, Rockford, USA) concentrations were determined by ELISA according to the manufacturer's instructions. Total protein concentration in the brain areas was quantified through Bradford Assay and the cytokine levels corrected for the amount of protein after measurement (54).

Statistical Analysis

Statistical analyses were performed with the SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY). Continuous data are expressed as mean ± standard error of the mean (SEM). Data expressed as percentage were square root arcsine transformed prior to statistical analysis (55; 56). Differences in variables from behavioural and biochemical tests were tested through a two-sided paired or independent samples *t-test* and the effect size of the differences between groups was calculated through Cohen's *d* (57; 58). The Generalized Estimating Equations (GEE) model (59) was used to account for repeated measurements in the longitudinal design and missing data in body weight and PET measurements. For the statistical model of the body weight gain, "group", "day of measurement" and the interaction "group × day of measurement" were included as variables. The GEE model for ^{11}C-PK11195 and ^{18}F-FDG uptake (SUV) was applied individually for each brain region, including the variables "group", "day of scan" and the interaction "group × day of scan" in the model. The data was further explored through pairwise comparison of "group × day of scan" in each brain

region for all scan time points combined. The AR(1) working correlation matrix was selected according to the quasi-likelihood under the independence model information criterion value. Wald's statistics and associated *p*-values were considered statistically significant at $p<0.05$. A Bonferroni-Holm correction was used to adjust significance levels for multiple comparisons (60).

Results

As a consequence of methodological issues, 2 control rats did not survive a PET scan and a humane endpoint was applied to one rat due to a lethal wound during the RSD protocol.

RSD reduced bodyweight gain, normalizing only after 5 weeks

The bodyweight of the rats was measured daily until day 25 and weekly thereafter. No significant differences in bodyweight between groups was found before the start of RSD (control: 266±19g, and SoD: 261±18g, $p=0.44$). For the first 25 days, a significant main effect was found for the factors group ($p<0.001$) and day of measurement ($p<0.001$), and for the interaction group × day of measurement ($p<0.001$), showing that RSD significantly reduced bodyweight gain. More specifically, the reduction in bodyweight gain was already apparent on experimental day 2 (control: 8.8±1.4g vs. SoD: 4.2±1.3g, $p=0.01$), with no recovery to control levels until day 25 (control: 100.9±3.9g vs. SoD: 80.8±2.8g, $p<0.001$). When comparing the bodyweight gain from 5 weeks after the RSD until week 28 (6 months), there was a statistically significant main effect on day of measurement ($p<0.001$), and in the interaction day of measurement × group ($p<0.001$), but no difference was found between groups (Supplementary Fig. 1).

RSD provoked acute anxiety-like and depressive-like behaviour without cognition impairment

Behavioural tests were performed at baseline and at several time-points after RSD. Social defeat caused a reduced preference for sucrose (Fig. 2-A). At baseline, rats had a 96±1% preference for sucrose, whereas after the RSD (day 5) the preference decreased to 74±4% ($p<0.001$, $d=1.1$). At the 3- and 6-month follow up the sucrose preference of the SoD group was restored to the baseline value (Fig. 2-B). The sucrose preference of the control group remained constant over time.

The anxiety-like behaviour and explorative activity were investigated at baseline and immediately after the RSD protocol (day 5), using the OF. No significant differences

between groups were found at baseline for distance moved (control: 3.8±0.2m vs. SoD: 3.8±0.2m, p=0.83, d=0.01) and time spent in the centre of the arena (control: 89±7s vs. SoD: 80±7s, p=0.49, d=0.2). On day 5, rats exposed to RSD demonstrated anxiety-like behaviour through decreased exploration (control: 3.4±0.3m vs. SoD: 2.6±0.2m, p<0.001, d=0.7) and a diminished time spent in the centre of the arena (control: 63±13s vs. SoD: 42±10s, p<0.001, d=0.5) when compared to controls (Fig. 2-C and 2-D). At 3 and 6 months after RSD, the anxiety-like behaviour of SoD rats had normalized, as the OF test did not reveal any significant difference between groups (Fig. 2-E and 2-F).

Anxiety-like behaviour was additionally assessed with the EPM. On day 5, rats in the SoD group spent a significantly lower percentage of time in the open arms (control: 14±2% vs. SD: 9±2%, p<0.05, d=0.6) and a higher percentage of time in the closed arms (control: 61±3% vs. SD: 71±4%, p<0.05, d=0.7) as compared to controls, showing that rats exposed to RSD were more anxious. No significant difference between groups in time spent in the centre was found (control: 25±2% vs. SoD: 20±2%, p=0.114, d=0.6; Fig. 1-G). No significant difference between groups was observed anymore at month 3 (Fig. 2-H).

To assess whether RSD had long-lasting effects on memory, the NOR test was performed on day 24, month 3 and 6. No significant differences were found between groups (Fig. 2-I and 2-J).

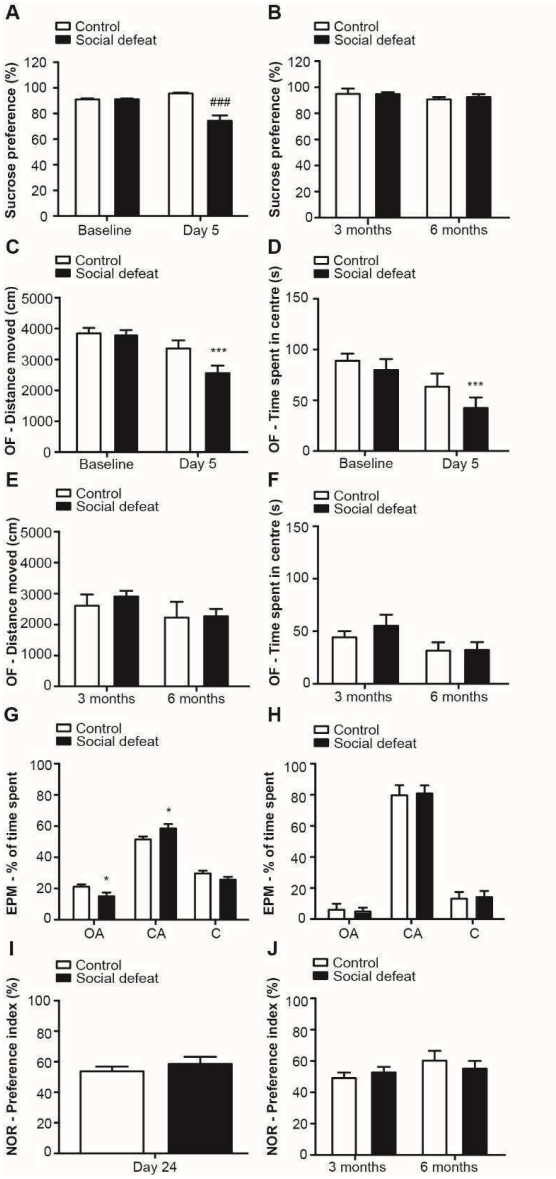


Figure 2: RSD-induced behavioural alterations in SoD rats in the short-term follow-up. **(A)** Anhedonic-like behaviour was demonstrated in SoD rats through a within-group comparison of the sucrose preference test (SPT) on baseline and day 5, $^{###}p<0.001$. **(B)** No differences in SPT of control and SoD rats on the 3- and 6-month follow-up. Anxiety-like behaviour was demonstrated in SoD rats in the open field test (OF) through **(C)** decreased distance moved on day 5 as compared to control rats, $***p<0.001$ and **(D)** decreased total time spent in the centre of the arena, $***p<0.001$. In the 3- and 6-month follow-up, **(E)** no differences were found in distance moved or **(F)** time spent in the centre of the OF arena between groups. The elevated plus maze (EPM) confirmed the anxiety behaviour of SoD rats on day 5, with **(G)** decreased the percentage of time spent in the open arms (OA), $*p<0.05$, and increased the percentage of time spent in the closed arms (CA), $*p<0.05$. No differences were found regarding the percentage of time spent in the centre (C). **(H)** In the 3-month evaluation, no differences were found in the percentage of time spent in the OA, CA or C between groups. RSD did not affect cognition, measured through the novel object recognition (NOR) test

and expressed as percentage of preference index (PI). **(I)** No between group difference on PI was found on day 24. **(J)** PI was not significantly different between groups on the 3- and 6-month follow-up.

Serum corticosterone increased significantly in response to social defeat

Serum concentrations of corticosterone were measured before (day -1) and after RSD (day 6). No significant differences were found between groups on day -1 (ctrl: 235±45 nmol/L vs. SoD: 186±43 nmol/L, p=0.44). Socially defeated rats had significantly increased corticosterone levels after the RSD (day -1: 186±43 nmol/L vs. day 6: 560±114 nmol/L, $p<0.01$, $d=1.00$), whereas corticosterone levels in control rats were not affected (day -1: 235±45 nmol/L vs. day 6: 207±40 nmol/L, $p=0.675$, $d=0.1$).

Stress-induced glial activation was detected at short-term follow-up after RSD but not at long-term

Between-group comparison of the ^{11}C-PK11195 PET data revealed no significant differences in tracer uptake between groups at baseline or immediately after RDS (day 6). However, an increased tracer uptake (suggestive of the presence of activated glial cells) was observed in defeated rats on experimental day 11 and 25 (Table 1). The regions that presented significantly increased uptake on day 11 were the orbitofrontal cortex (+38%, $p<0.001$), entorhinal cortex (+30%, $p=0.001$), insular cortex (+30%, $p=0.002$), medial prefrontal cortex (+26%, $p<0.001$) and cerebellum (+23%, $p=0.001$), as compared to the control group (Fig 3-A). On day 25, only the frontal association cortex had increased uptake (+23%, $p=0.008$). Furthermore, no between-group differences in ^{11}C-PK11195 uptake were found in any brain region at months 3 and 6. Analysis of the effect of time on tracer uptake in brain regions of control and defeated rats revealed a global increase in the uptake of ^{11}C-PK11195 at month 3 and 6 when compared to baseline levels (Table 2).

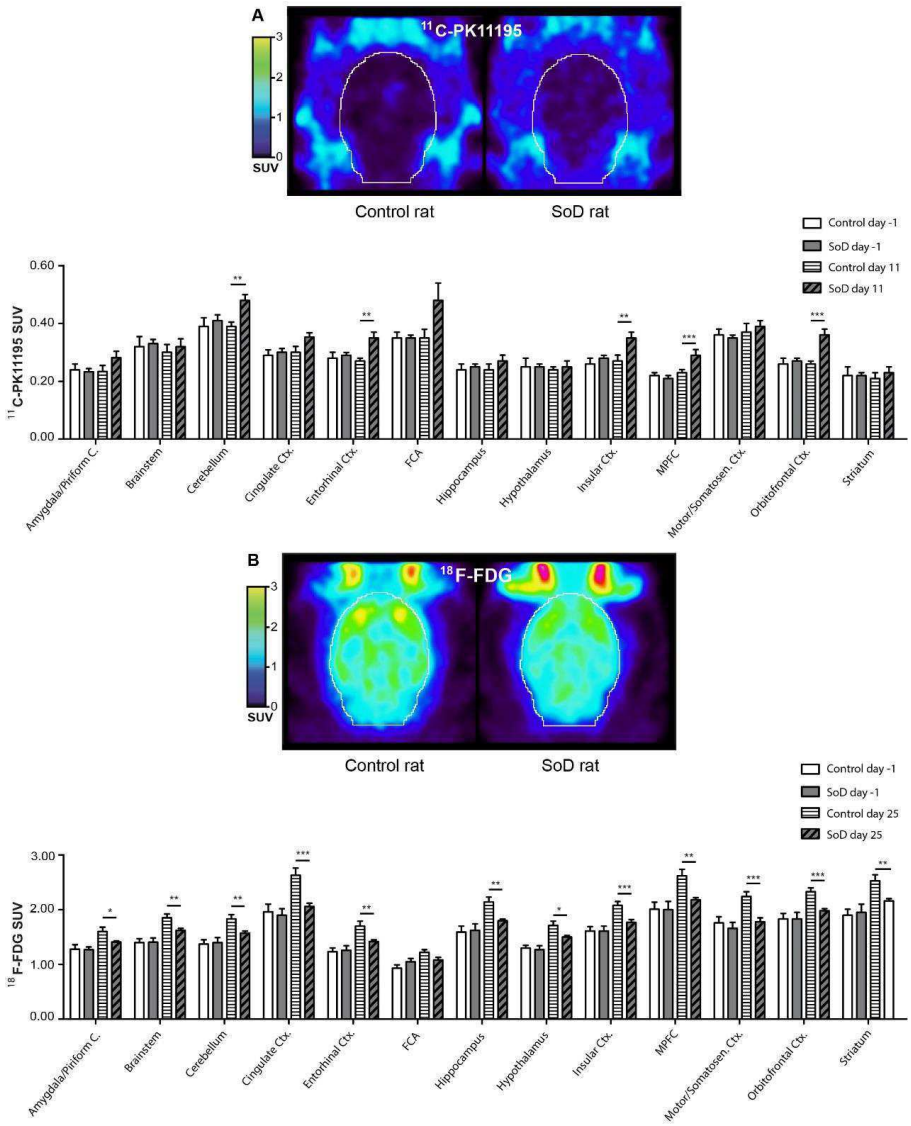


Figure 3: (A) ^{11}C-PK11195 PET scan of a representative control and defeated rat on experimental day 11, followed by a graphical representation of ^{11}C-PK11195 SUV on baseline and day 11. *p<0.05, **p<0.01 and ***p<0.001. **(B)** ^{18}F-FDG PET scan of a representative control and defeated rat on experimental day 25, followed by a graphical representation of ^{18}F-FDG SUV on baseline and day 25. *p<0.05, **p<0.01 and ***p<0.001.

Brain glucose metabolism alterations in defeated rats found at short-term follow-up normalized within 3 months

Several brain regions demonstrated alterations in brain glucose metabolism in SoD rats (Table 3). On day 6, SoD rats had lower ^{18}F-FDG uptake in the motor/somatosensory (-19%, p=0.006), cingulate (-17%, p=0.03) and entorhinal cortex (-17%, p=0.04) than controls. On day 25 (Fig. 3-B), a global decrease in tracer uptake was found in the cingulate cortex (-22%, p<0.001), motor/somatosensory cortex (-21%, p<0.001), medial prefrontal cortex (-17%, p=0.001), entorhinal cortex (-16%, p=0.006), hippocampus (-16%, p=0.001), insular cortex (-15%, p<0.001), orbitofrontal cortex (-15%, p<0.001), striatum (-15%, p=0.002), cerebellum (-14%, p=0.009), hypothalamus (-13%, p=0.013), brainstem (-12%, p=0.003) and amygdala/piriform complex (-11%, p=0.024). In contrast, no differences in ^{18}F-FDG uptake between groups were observed at baseline, 3 months or 6 months after RDS.

Table 1 – ^{11}C-PK11195 SUV values of the short-term follow-up for individual brain regions of control (n=8) and SoD (n=9) rats at baseline, day 6, 11 and 25.

	Baseline			Day 6			Day 11			Day 25		
	Control	SoD		Control	SoD		Control	SoD		Control	SoD	
Brain Regions	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***p***
Amygdala/Piriform complex	0.24 ± 0.02	0.23 ± 0.02	n.s	0.23 ± 0.02	0.23 ± 0.01	n.s	0.23 ± 0.01	0.28 ± 0.02	n.s	0.25 ± 0.02	0.24 ± 0.02	n.s
Brainstem	0.32 ± 0.04	0.33 ± 0.01	n.s	0.31 ± 0.03	0.32 ± 0.02	n.s	0.30 ± 0.02	0.32 ± 0.03	n.s	0.32 ± 0.04	0.31 ± 0.03	n.s
Cerebellum	0.39 ± 0.03	0.41 ± 0.02	n.s	0.41 ± 0.03	0.47 ± 0.03	n.s	0.39 ± 0.02	0.48 ± 0.02	0.001	0.45 ± 0.03	0.51 ± 0.04	n.s
Cingulate cortex	0.29 ± 0.02	0.30 ± 0.01	n.s	0.30 ± 0.01	0.33 ± 0.02	n.s	0.30 ± 0.02	0.35 ± 0.02	n.s	0.34 ± 0.02	0.41 ± 0.02	n.s
Entorhinal cortex	0.28 ± 0.02	0.29 ± 0.01	n.s	0.29 ± 0.03	0.30 ± 0.02	n.s	0.27 ± 0.01	0.35 ± 0.02	0.001	0.31 ± 0.03	0.31 ± 0.02	n.s
Frontal association cortex	0.35 ± 0.02	0.35 ± 0.01	n.s	0.33 ± 0.02	0.35 ± 0.01	n.s	0.35 ± 0.03	0.48 ± 0.06	n.s	0.34 ± 0.02	0.42 ± 0.02	0.008
Hippocampus	0.24 ± 0.02	0.25 ± 0.01	n.s	0.25 ± 0.02	0.28 ± 0.02	n.s	0.24 ± 0.02	0.27 ± 0.02	n.s	0.28 ± 0.03	0.29 ± 0.02	n.s
Hypothalamus	0.25 ± 0.03	0.25 ± 0.01	n.s	0.26 ± 0.02	0.26 ± 0.01	n.s	0.24 ± 0.01	0.25 ± 0.02	n.s	0.29 ± 0.04	0.29 ± 0.02	n.s
Insular cortex	0.26 ± 0.02	0.28 ± 0.01	n.s	0.26 ± 0.02	0.28 ± 0.01	n.s	0.27 ± 0.02	0.35 ± 0.02	0.002	0.27 ± 0.03	0.27 ± 0.02	n.s
Medial Prefrontal cortex	0.22 ± 0.01	0.21 ± 0.01	n.s	0.23 ± 0.02	0.26 ± 0.01	n.s	0.23 ± 0.01	0.29 ± 0.02	<0.001	0.25 ± 0.03	0.32 ± 0.02	n.s
Motor/Somatosensory cortex	0.36 ± 0.02	0.35 ± 0.01	n.s	0.36 ± 0.02	0.37 ± 0.02	n.s	0.37 ± 0.03	0.39 ± 0.02	n.s	0.43 ± 0.02	0.48 ± 0.03	n.s
Orbitofrontal cortex	0.26 ± 0.02	0.27 ± 0.01	n.s	0.26 ± 0.01	0.29 ± 0.02	n.s	0.26 ± 0.01	0.36 ± 0.02	<0.001	0.27 ± 0.02	0.31 ± 0.02	n.s
Striatum	0.22 ± 0.03	0.22 ± 0.01	n.s	0.23 ± 0.02	0.23 ± 0.02	n.s	0.21 ± 0.02	0.23 ± 0.02	n.s	0.26 ± 0.03	0.25 ± 0.02	n.s

Table 2 – Effect of time in ^{11}C-PK11195 uptake values in control (n=8) and SoD (n=9) rats during the long-term follow-up (3 and 6 months) for individual brain regions as compared to baseline uptake levels.

	Control					SoD				
	Day -1	3 months		6 months		Day -1	3 months		6 months	
Brain Regions	**Mean ± SE**	**Mean ± SE**	***P***	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	***p***
Amygdala/Piriform complex	0.24 ± 0.02	0.31 ± 0.01	0.005	0.35 ± 0.01	<0.001	0.23 ± 0.02	0.29 ± 0.01	0.001	0.33 ± 0.01	<0.001
Brainstem	0.32 ± 0.04	0.42 ± 0.03	0.011	0.49 ± 0.03	<0.001	0.33 ± 0.01	0.40 ± 0.01	0.001	0.47 ± 0.01	<0.001
Cerebellum	0.39 ± 0.03	0.55 ± 0.03	<0.001	0.61 ± 0.02	<0.001	0.41 ± 0.02	0.61 ± 0.02	<0.001	0.65 ± 0.04	<0.001
Cingulate cortex	0.29 ± 0.02	0.48 ± 0.02	<0.001	0.55 ± 0.04	<0.001	0.30 ± 0.01	0.48 ± 0.02	<0.001	0.56 ± 0.03	<0.001
Entorhinal cortex	0.28 ± 0.02	0.33 ± 0.02	0.008	0.37 ± 0.01	<0.001	0.29 ± 0.01	0.34 ± 0.01	0.001	0.35 ± 0.01	<0.001
Frontal association cortex	0.35 ± 0.02	0.43 ± 0.04	n.s.	0.45 ± 0.04	0.012	0.35 ± 0.01	0.45 ± 0.02	<0.001	0.49 ± 0.03	<0.001
Hippocampus	0.24 ± 0.02	0.36 ± 0.02	<0.001	0.42 ± 0.01	<0.001	0.25 ± 0.01	0.38 ± 0.01	<0.001	0.42 ± 0.01	<0.001
Hypothalamus	0.25 ± 0.03	0.37 ± 0.02	0.001	0.44 ± 0.02	<0.001	0.25 ± 0.01	0.32 ± 0.02	0.020	0.39 ± 0.01	<0.001
Insular cortex	0.26 ± 0.02	0.32 ± 0.02	0.006	0.37 ± 0.02	<0.001	0.28 ± 0.01	0.32 ± 0.01	n.s.	0.34 ± 0.01	<0.001
Medial Prefrontal cortex	0.22 ± 0.01	0.40 ± 0.03	<0.001	0.46 ± 0.03	<0.001	0.21 ± 0.01	0.40 ± 0.01	<0.001	0.48 ± 0.02	<0.001
Motor/Somatosensory cortex	0.36 ± 0.02	0.53 ± 0.03	<0.001	0.56 ± 0.04	<0.001	0.35 ± 0.01	0.53 ± 0.03	<0.001	0.56 ± 0.03	<0.001
Orbitofrontal cortex	0.26 ± 0.02	0.36 ± 0.02	<0.001	0.41 ± 0.02	<0.001	0.27 ± 0.01	0.39 ± 0.01	<0.001	0.44 ± 0.02	<0.001
Striatum	0.22 ± 0.03	0.34 ± 0.01	<0.001	0.40 ± 0.01	<0.001	0.22 ± 0.01	0.34 ± 0.01	<0.001	0.40 ± 0.01	<0.001

A within-group comparison in control rats showed a significant increase in the ^{18}F-FDG uptake in all investigated brain regions on day 6 and 25, as compared to baseline levels (Table 4). In SoD rats, increased ^{18}F-FDG uptake was only found on day 25 in the brainstem (+13%, p=0.009), entorhinal cortex (+13%, p=0.03) and hypothalamus (+10%, p=0.016). Further analysis of the effect of time in the long-term follow-up showed a significant increase in ^{18}F-FDG in all brain regions for both groups at month 3 and 6, as compared to baseline.

Elevated IL-1β levels were found in the frontal cortex 3 weeks after RSD

A significant increase in the levels of IL-1β was found in the frontal cortex of defeated rats (Supplementary Fig. 2-A), when compared to control rats (controls: 74±6 pg/mg vs. SoD: 122±14 pg/mg, p=0.012, d=1.55) at day 25. No differences between groups were found in hippocampus, cerebellum and parietal/temporal/occipital cortex. No significant differences in IL-6 and TNF-α levels (p≥0.05) were found in any of the brain regions (Supplementary Fig. 2-B and C). However, the Cohen's effect size values for IL-6 and TNF-α levels in frontal cortex (d=0.63 and 0.55, respectively) suggest a trend towards increased expression of these cytokines.

Table 3 - ^{18}F-FDG SUV values of the short-term follow-up for individual brain regions of control (n=8) and SoD (n=9) rats at baseline, day 6, 11 and 25.

	Baseline			Day 6			Day 11			Day 25		
	Control	SoD		Control	SoD		Control	SoD		Control	SoD	
Brain Regions	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***P***	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***p***
Amygdala/Piriform complex	1.28 ± 0.08	1.27 ± 0.05	n.s.	1.45 ± 0.07	1.28 ± 0.07	n.s.	1.28 ± 0.06	1.43 ± 0.12	n.s.	1.60 ± 0.08	1.41 ± 0.02	0.024
Brainstem	1.40 ± 0.07	1.41 ± 0.08	n.s.	1.64 ± 0.09	1.47 ± 0.07	n.s.	1.47 ± 0.08	1.68 ± 0.12	n.s.	1.85 ± 0.07	1.62 ± 0.04	0.003
Cerebellum	1.37 ± 0.08	1.40 ± 0.09	n.s.	1.65 ± 0.08	1.48 ± 0.09	n.s.	1.47 ± 0.09	1.67 ± 0.13	n.s.	1.83 ± 0.08	1.57 ± 0.04	0.009
Cingulate cortex	1.96 ± 0.14	1.90 ± 0.12	n.s.	2.30 ± 0.13	1.90 ± 0.12	0.030	1.97 ± 0.15	2.11 ± 0.16	n.s.	2.63 ± 0.13	2.06 ± 0.06	<0.001
Entorhinal cortex	1.23 ± 0.07	1.26 ± 0.08	n.s.	1.49 ± 0.09	1.24 ± 0.07	0.040	1.27 ± 0.07	1.42 ± 0.11	n.s.	1.70 ± 0.09	1.42 ± 0.03	0.006
Frontal association cortex	0.98 ± 0.06	1.05 ± 0.06	n.s.	1.16 ± 0.07	1.11 ± 0.06	n.s.	0.98 ± 0.06	1.18 ± 0.07	n.s.	1.22 ± 0.05	1.08 ± 0.05	n.s.
Hippocampus	1.59 ± 0.11	1.62 ± 0.12	n.s.	1.90 ± 0.11	1.67 ± 0.11	n.s.	1.66 ± 0.10	1.85 ± 0.15	0.030	2.14 ± 0.09	1.80 ± 0.03	0.001
Hypothalamus	1.30 ± 0.05	1.27 ± 0.07	n.s.	1.49 ± 0.09	1.32 ± 0.08	n.s.	1.32 ± 0.08	1.50 ± 0.11	n.s.	1.71 ± 0.08	1.50 ± 0.03	0.013
Insular cortex	1.61 ± 0.08	1.61 ± 0.09	n.s.	1.90 ± 0.10	1.66 ± 0.09	n.s.	1.66 ± 0.09	1.79 ± 0.15	0.030	2.08 ± 0.07	1.77 ± 0.05	<0.001
Medial Prefrontal cortex	2.01 ± 0.13	2.00 ± 0.15	n.s.	2.31 ± 0.13	2.03 ± 0.14	n.s.	2.03 ± 0.14	2.26 ± 0.17	n.s.	2.62 ± 0.12	2.18 ± 0.04	0.001
Motor/Somatosensory cortex	1.76 ± 0.11	1.66 ± 0.11	n.s.	2.06 ± 0.11	1.67 ± 0.09	0.006	1.79 ± 0.10	1.86 ± 0.13	n.s.	2.24 ± 0.09	1.78 ± 0.07	<0.001
Orbitofrontal cortex	1.83 ± 0.10	1.83 ± 0.12	n.s.	2.09 ± 0.11	1.89 ± 0.12	n.s.	1.81 ± 0.11	2.06 ± 0.15	0.003	2.33 ± 0.07	1.98 ± 0.04	<0.001
Striatum	1.90 ± 0.11	1.95 ± 0.15	n.s.	2.22 ± 0.12	1.94 ± 0.13	n.s.	1.94 ± 0.12	2.18 ± 0.18	n.s.	2.53 ± 0.11	2.16 ± 0.04	0.002

Table 4 - Effect of time in ^{18}F-FDG SUV values in control (n=8) and SoD (n=9) rats during the long-term follow-up (3 and 6 months).

	Control					SoD				
	Day -1	3 months		6 months		Day -1	3 months		6 months	
Brain Regions	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	***p***	**Mean ± SE**	**Mean ± SE**	***P***	**Mean ± SE**	***p***
Amygdala/Piriform complex	1.27 ± 0.05	1.59 ± 0.04	<0.001	1.81 ± 0.08	<0.001	1.28 ± 0.08	1.78 ± 0.10	0.001	1.91 ± 0.10	<0.001
Brainstem	1.40 ± 0.07	1.87 ± 0.04	<0.001	2.15 ± 0.09	<0.001	1.41 ± 0.08	2.06 ± 0.10	<0.001	2.17 ± 0.11	<0.001
Cerebellum	1.37 ± 0.08	1.93 ± 0.05	<0.001	2.21 ± 0.10	<0.001	1.40 ± 0.08	2.16 ± 0.12	<0.001	2.27 ± 0.14	<0.001
Cingulate cortex	1.96 ± 0.14	2.63 ± 1.10	0.001	2.92 ± 0.17	<0.001	1.90 ± 0.14	2.82 ± 0.17	<0.001	2.99 ± 0.16	<0.001
Entorhinal cortex	1.23 ± 0.07	1.76 ± 0.05	<0.001	2.01 ± 0.11	<0.001	1.26 ± 0.07	1.95 ± 0.12	<0.001	2.12 ± 0.12	<0.001
Frontal association cortex	0.98 ± 0.06	1.19 ± 0.04	0.004	1.39 ± 0.04	<0.001	1.05 ± 0.06	1.28 ± 0.05	0.005	1.40 ± 0.06	<0.001
Hippocampus	1.59 ± 0.11	2.14 ± 0.07	<0.001	2.42 ± 0.12	<0.001	1.62 ± 0.12	2.38 ± 0.13	<0.001	2.54 ± 0.15	<0.001
Hypothalamus	1.30 ± 0.05	1.75 ± 0.06	<0.001	2.01 ± 0.09	<0.001	1.27 ± 0.07	2.00 ± 0.11	<0.001	2.13 ± 0.11	<0.001
Insular cortex	1.61 ± 0.08	2.03 ± 0.04	<0.001	2.30 ± 0.09	<0.001	1.61 ± 0.09	2.08 ± 0.10	0.003	2.21 ± 0.10	<0.001
Medial Prefrontal cortex	2.01 ± 0.13	2.61 ± 0.10	0.001	2.97 ± 0.14	<0.001	2.00 ± 0.15	2.89 ± 0.16	<0.001	3.07 ± 0.17	<0.001
Motor/Somatosensory cortex	1.76 ± 0.11	2.24 ± 0.06	0.001	2.50 ± 0.11	<0.001	1.66 ± 0.11	2.26 ± 0.11	<0.001	2.37 ± 0.10	<0.001
Orbitofrontal cortex	1.83 ± 0.10	2.20 ± 0.06	0.005	2.50 ± 0.08	<0.001	1.83 ± 0.12	2.39 ± 0.11	0.004	2.54 ± 0.13	<0.001
Striatum	1.90 ± 0.11	2.55 ± 0.07	<0.001	2.82 ± 0.15	<0.001	1.95 ± 0.15	2.86 ± 0.16	<0.001	3.00 ± 0.17	<0.001

Discussion

For the first time, we have demonstrated *in vivo* that psychosocial stress in rats transiently induces depressive- and anxiety-like behaviour associated with glial activation and altered brain glucose metabolism, as measured by PET. Yet, these effects had normalized during the 3 and 6 months follow-up.

The RSD protocol effectively exposed rats to recurrent stress as was confirmed by increased corticosterone levels, decreased bodyweight gain, and depressive- and anxiety-like behaviour. These findings are in line with previous studies, confirming the validity of the model (8; 61–63). However, we now observed for the first time that these effects of RSD on bodyweight and behaviour did not persist at 3 and 6 months after RSD. Furthermore, we observed that defeated rats did not reveal long-lasting memory deficits measurable in the NOR test. Previous studies focused on the cognitive alterations shortly after the termination of the stressful condition and reported that high levels of corticosterone impaired object recognition memory (64–66). Although we found that corticosterone levels were elevated 2 days after the 5-day RSD protocol, we did not measure corticosterone levels at the time of the NOR test (day 24). It is plausible that corticosterone levels had already normalized 3 weeks after RSD. A recent study by McKim et al. (67) subjected mice to RSD for six consecutive days and tested the effects on memory with the Barnes maze. They reported an increased number of errors to find the escape hole of the maze for defeated rats at day 2, but not at day 28. Put together, these results suggest that RSD causes only early, transient deficits in short-term memory recall (67).

PET imaging with the TSPO tracer ^{11}C-PK11195 demonstrated the presence of glial activation 7 days after RSD in the cerebellum, entorhinal cortex, insular cortex, medial prefrontal cortex and the orbitofrontal cortex. The medial prefrontal cortex and orbitofrontal cortex are associated with depressive behaviour and reward (68; 69), whereas the cerebellum and insular cortex have been related to anxiety (48; 70; 71), and the entorhinal cortex is linked with conscious memory and spatial navigation (71; 72). Three weeks after RSD, glial activation was only evident in the frontal association cortex, a brain area associated with depression (73). Interestingly, increased levels of IL-1β were temporally and spatially consistent with this glial activation. IL-1β seems to be the key mediator between increased corticosterone levels as a consequence of psychosocial stress and neuroinflammatory processes (15; 74–76). Overall, the observed glial activation was in accordance with previous preclinical studies that have evaluated brain cytokine

expression in conjunction with microglia activation after RSD (9; 77; 78). However, in the 3- and 6-month follow-up of our study, no differences in tracer uptake between groups were detected anymore, indicating that RSD-induced glial activation is transient.

We observed that ^{18}F-FDG brain uptake was decreased in the motor cortex of SoD rats on day 6, which is in agreement with the decreased locomotor activity observed in the OF test. The decreased ^{18}F-FDG uptake in the cingulate cortex combined with the reduced sucrose preference suggests that RSD reduced motivation and induced anhedonia (79). Overall, the global decrease in ^{18}F-FDG uptake in the brain of SoD rats on day 25 is in line with the results reported in unipolar depressive patients (22; 80–82).

An interesting insight arises from the finding that longitudinal within-group comparisons revealed an increase in ^{11}C-PK11195 uptake over a 6-month time period both in control and SoD rats. This is in agreement with previous studies that demonstrated age-related microglia activation in healthy rodents (83) and humans (84). There was also a time-related increase in ^{18}F-FDG uptake, which differed between groups. In contrast to controls, SoD rats had a stable uptake during the short-term follow-up, which only reached the levels of the control group during the long-term follow-up. This suggests that RSD delays rather than hampers brain metabolism maturation (85).

Our most interesting finding was the seemingly “transient” nature of the effects of RSD. However, this does not mean that there are no long-term effects of RDS at all, and further efforts should be encouraged into elucidating whether the observed transient changes are leading to other deleterious effects. To our knowledge, this is the first study that reports a 6-month follow-up after RSD along with non-invasive (PET) imaging evaluation. Buwalda et al. evaluated the long-term effects (3 months) of adolescent exposure to RSD (86) and showed no differences between controls and defeated animals in physiological (body temperature and corticosterone levels) and behavioural (EPM and social interaction) parameters. These findings are in agreement with our study that also did not reveal any long-lasting negative behavioural effects of social defeat (or at least, not measurable with the reported methods). Still, the question remains whether exposure to psychosocial stress conveys hitherto uninvestigated long-term effects mediated by (neuro)inflammation, which may be relevant in the pathogenesis and treatment of (treatment-resistant) MDD.

Due to its longitudinal design, this study has some limitations. First, we did not confirm PET findings by immunohistochemical analysis of alterations in the morphology of microglia and/or astrocytes. However, indirect measurement of the pro-inflammatory

glial phenotype was confirmed by the quantification of pro-inflammatory cytokines in the brain on day 25. Second, corticosterone levels were only measured in serum during two timepoints (before and immediately after RSD) in order to confirm the biochemical effect of the RSD protocol in the model. Therefore, we do not have any information about the longitudinal changes in corticosterone levels during the whole period of the study. Third, recent studies have shown that the TSPO tracer ^{11}C-PK11195 is not the most sensitive candidate for detecting mild glial activation. Tracers like ^{11}C-CB184 (87), ^{11}C-PBR28 (20) and ^{18}F-DPA-714 (88) are second generation TSPO tracers with superior characteristics in comparison with ^{11}C-PK11195 in terms of affinity and/or nonspecific binding. Therefore, for future preclinical studies, a second generation TSPO tracer should be considered. The second generation, however, are sensitive to polymorphism in the TSPO receptor in humans. Forth, the SUV is a semi-quantitative measurement of tracer uptake, with the advantage of enabling individual monitoring over time and simplicity of the analysis (89). However, the SUV is sensitive for changes such as blood flow and tracer delivery. Moreover, it is positively correlated to the subject's body weight. To overcome differences in body weight that might influence SUV values, it has been clinically proposed to use the body surface area or lean body mass instead of body weight in the SUV equation (90). However, this was not validated in the preclinical setting. Additionally, in order to perform a fully quantitative determination of tracer binding to its receptor (e.g. TSPO), performing the kinetic modelling of ^{11}C-PK11195 requires a terminal procedure with arterial blood sampling for radioactivity measurement of blood and plasma, since no reference region devoid of TSPO is available within the brain. Due to the longitudinal nature of the study, such methodology was not feasible.

In conclusion, psychosocial stress in rats, in the form of RSD, transiently induces depressive- and anxiety-like behaviour, provokes immune activation in the central nervous system, and significantly diminishes brain glucose metabolism. PET imaging proved a useful tool to noninvasively monitoring the effects of stress in a longitudinal study design. This study supports the hypothesis of a mechanistic role of (neuro)inflammation in the development of depressive behaviour. Nevertheless, further research is warranted to elucidate how the transient effects of psychosocial stress can lead to persistent depressive behaviour, as observed in (treatment-resistant) patients with MDD.

Acknowledgements

The authors would like to thank David Vállez García for the statistical support and Natalia M. Peñaranda Fajardo, for technical assistance in the biochemical analysis.

Disclosure / Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The Authors declare that there is no conflict of interest.

References

1. Marcus M, Yasami T, van Ommeren M, et al. Depression: A Global Public Health Concern Developed. In: *65th World Health Assembly*. Geneva, Switzerland, 2012, pp. 6–8. Palais des Nations, Geneva, Switzerland, 21–26 May 2012.
2. Bschor T, Ising M, Erbe S, et al. Impact of citalopram on the HPA system. A study of the combined DEX/CRH test in 30 unipolar depressed patients. *J Psychiatr Res* 2012; 46: 111–117.
3. Friedman A. Jump-starting natural resilience reverses stress susceptibility. *Science (80)* 2014; 346: 555–555.
4. Cohen S, Janicki-Deverts D, Miller GE. Psychological Stress and Disease. *JAMA* 2007; 298: 1685–1687.
5. de Boer SF, Buwalda B, Koolhaas JM. Untangling the neurobiology of coping styles in rodents: Towards neural mechanisms underlying individual differences in disease susceptibility. *Neurosci Biobehav Rev* 2017; 74: 401–422.
6. Carvalho LA, Juruena MF, Papadopoulos AS, et al. Clomipramine In Vitro Reduces Glucocorticoid Receptor Function in Healthy Subjects but not in Patients with Major Depression. *Neuropsychopharmacology* 2012; 33: 3182–3189.
7. Rossetti AC, Papp M, Gruca P, et al. Stress-induced anhedonia is associated with the activation of the inflammatory system in the rat brain: Restorative effect of pharmacological intervention. *Pharmacol Res* 2016; 103: 1–12.
8. Patki G, Solanki N, Atrooz F, et al. Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. *Brain Res* 2013; 1539: 73–86.
9. Wohleb ES, McKim DB, Sheridan JF, et al. Monocyte trafficking to the brain with stress and inflammation: a novel axis of immune-to-brain communication that influences mood and behavior. *Front Neurosci* 2015; 8: 1–17.
10. Ramirez K, Niraula A, Sheridan JF. GABAergic modulation with classical benzodiazepines prevent stress-induced neuro-immune dysregulation and behavioral alterations. *Brain Behav Immun* 2015; 51: 1–15.
11. Weber MD, Godbout JP, Sheridan JF. Repeated Social Defeat, Neuroinflammation, and Behavior: Monocytes Carry the Signal. *Neuropsychopharmacology* 2016; 42: 1–51.
12. Loggia ML, Chonde DB, Akeju O, et al. Evidence for brain glial activation in chronic pain patients. *Brain* 2015; 138: 604–615.
13. Czeh M, Gressens P, Kaindl AM. The yin and yang of microglia. *Dev Neurosci* 2011; 33: 199–209.
14. Chijiwa T, Oka T, Lkhagvasuren B, et al. Prior chronic stress induces persistent polyI:C-induced allodynia and depressive-like behavior in rats: Possible involvement of glucocorticoids and microglia. *Physiol Behav* 2015; 147: 264–273.

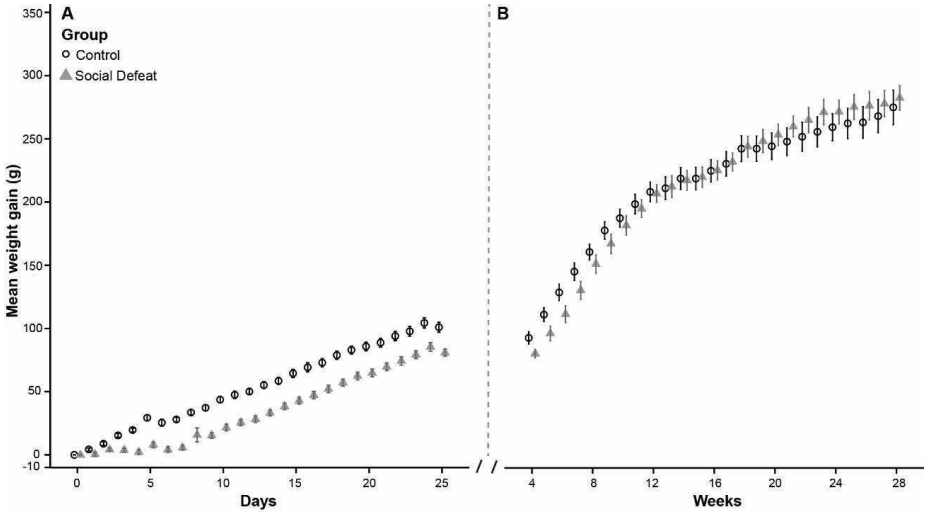
15. Walker F, Nilsson M, Jones K. Acute and Chronic Stress-Induced Disturbances of Microglial Plasticity, Phenotype and Function. *Curr Drug Targets* 2013; 14: 1262–1276.
16. Venneti S, Lopresti B, Wiley C. Molecular imaging of microglia / macrophages in the brain. *Glia* 2013; 61: 10–23.
17. Beishuizen A, Thijs LG. Endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J Endotoxin Res* 2003; 9: 3–24.
18. Rosenblat JD, Cha DS, Mansur RB, et al. Inflamed moods: A review of the interactions between inflammation and mood disorders. *Prog Neuro-Psychopharmacology Biol Psychiatry* 2014; 53: 23–34.
19. Papadopoulos V, Baraldi M, Guilarte TR, et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol Sci* 2006; 27: 402–409.
20. Parente A, Feltes PK, Vallez Garcia D, et al. Pharmacokinetic Analysis of 11C-PBR28 in the Rat Model of Herpes Encephalitis: Comparison with (R)-11C-PK11195. *J Nucl Med* 2016; 57: 785–791.
21. Vállez García D, Dierckx RAJO, Doorduin J. Three months follow-up of rat mild traumatic brain injury: a combined [18F]FDG and [11C]PK11195 PET study. *J Neurotrauma* 2016; 33: 1–39.
22. Su L, Cai Y, Xu Y, et al. Cerebral metabolism in major depressive disorder: a voxel-based meta-analysis of positron emission tomography studies. *BMC Psychiatry* 2014; 14: 321.
23. McKim DB, Weber MD, Niraula A, et al. Microglial recruitment of IL-1β-producing monocytes to brain endothelium causes stress-induced anxiety. *Mol Psychiatry*; 1–11. Epub ahead of print, 2017. DOI: 10.1038/mp.2017.64.
24. Ramirez K, Fornaguera-Trías J, Sheridan JF. Stress-Induced Microglia Activation and Monocyte Trafficking to the Brain Underlie the Development of Anxiety and Depression. In: *Brain Imaging in Behavioral Neuroscience*, pp. 155–172.
25. Wohleb ES, Hanke ML, Corona AW, et al. β-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat. *J Neurosci* 2011; 31: 6277–6288.
26. Wohleb ES, Powell ND, Godbout JP, et al. Stress-Induced Recruitment of Bone Marrow-Derived Monocytes to the Brain Promotes Anxiety-Like Behavior. *J Neurosci* 2013; 33: 13820–13833.
27. Koolhaas JM, Coppens CM, de Boer SF, et al. The Resident-intruder Paradigm: A Standardized Test for Aggression, Violence and Social Stress. *J Vis Exp* 2013; 77 : e4367.
28. Patki G, Solanki N, Atrooz F, et al. Novel mechanistic insights into treadmill exercise based rescue of social defeat-induced anxiety-like behavior and memory impairment in rats. *Physiol Behav* 2014; 130: 135–144.
29. Doorduin J, Klein HC, Dierckx RA, et al. [11C]-DPA-713 and [18F]-DPA-714 as new PET tracers for TSPO: a comparison with [11C]-(R)-PK11195 in a rat model of herpes encephalitis. *Mol Imaging Biol* 2009; 11: 386–398.
30. Serra M, Pisu MG, Floris I, et al. Social isolation-induced changes in the hypothalamic–pituitary–adrenal axis in the rat. *Stress* 2005; 8: 259–264.
31. Buwalda B, Geerdink M, Vidal J, et al. Social behavior and social stress in adolescence: A focus on animal models. *Neurosci Biobehav Rev* 2011; 35: 1713–1721.
32. Ma X, Jiang D, Jiang W, et al. Social Isolation-Induced Aggression Potentiates Anxiety and Depressive-Like Behavior in Male Mice Subjected to Unpredictable Chronic Mild Stress. *PLoS One* 2011; 6: e20955.
33. Kilkenny C, Browne WJ, Cuthill IC, et al. Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLoS Biol* 2010; 8: e1000412.
34. Hurley LL, Akinfiresoye L, Kalejaiye O, et al. Antidepressant effects of resveratrol in an animal model of depression. *Behav Brain Res* 2014; 268: 1–7.
35. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* 2007; 2: 322–328.
36. Gonzalez LE, File SE. A five minute experience in the elevated plus-maze alters the state of the benzodiazepine receptor in the dorsal raphe nucleus. *J Neurosci* 1997; 17: 1505–1511.

37. Dere E, Huston JP, De Souza Silva MA. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neurosci Biobehav Rev* 2007; 31: 673–704.
38. Elizalde N, Gil-Bea FJ, Ramírez MJ, et al. Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. *Psychopharmacology (Berl)* 2008; 199: 1–14.
39. Hovens IB, van Leeuwen BL, Nyakas C, et al. Prior infection exacerbates postoperative cognitive dysfunction in aged rats. *Am J Physiol - Regul Integr Comp Physiol* 2015; 309: R148–R159.
40. Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn Process* 2012; 13: 93–110.
41. Wong K-P, Sha W, Zhang X, et al. Effects of Administration Route, Dietary Condition, and Blood Glucose Level on Kinetics and Uptake of 18F-FDG in Mice. *J Nucl Med* 2011; 52: 800–807.
42. Vállez García D, Casteels C, Schwarz AJ, et al. A Standardized Method for the Construction of Tracer Specific PET and SPECT Rat Brain Templates : Validation and Implementation of a Toolbox. *PLoS One* 2015; 10: 1–21.
43. Schwarz AJ, Danckaert A, Reese T, et al. A stereotaxic MRI template set for the rat brain with tissue class distribution maps and co-registered anatomical atlas: Application to pharmacological MRI. *Neuroimage* 2006; 32: 538–550.
44. Boellaard R, Delgado-Bolton R, Oyen WJ, et al. FDG PET/CT: EANM procedure guidelines for tumour imaging: version 2.0. *Eur J Nucl Med Mol Imaging* 2015; 42: 328–354.
45. Setiawan E, Wilson AA, Mizrahi R, et al. Role of Translocator Protein Density, a Marker of Neuroinflammation, in the Brain During Major Depressive Episodes. *JAMA Psychiatry* 2015; 72: 268.
46. Weber MD, Godbout JP, Sheridan JF. Repeated Social Defeat, Neuroinflammation, and Behavior: Monocytes Carry the Signal. *Neuropsychopharmacology* 2017; 42: 46–61.
47. Kim Y-K, Won E. The influence of stress on neuroinflammation and alterations in brain structure and function in major depressive disorder. *Behav Brain Res* 2017; 329: 6–11.
48. Phillips JR, Hewedi DH, Eissa AM, et al. The cerebellum and psychiatric disorders. *Front public Heal* 2015; 3: 66.
49. Wei K, Xue H, Guan Y, et al. Analysis of glucose metabolism of 18F-FDG in major depression patients using PET imaging: Correlation of salivary cortisol and α-amylase. *Neurosci Lett* 2016; 629: 52–57.
50. Hinwood M, Tynan RJ, Day TA, et al. Repeated Social Defeat Selectively Increases FosB Expression and Histone H3 Acetylation in the Infralimbic Medial Prefrontal Cortex. *Cereb Cortex* 2011; 21: 262–271.
51. Yu T, Guo M, Garza J, et al. Cognitive and neural correlates of depression-like behaviour in socially defeated mice: an animal model of depression with cognitive dysfunction. *Int J Neuropsychopharmacol* 2011; 14: 303–317.
52. Marx C, Lex B, Calaminus C, et al. Conflict Processing in the Rat Brain: Behavioral Analysis and Functional μPET Imaging Using [F]Fluorodeoxyglucose. *Front Behav Neurosci* 2012; 6: 4.
53. Lehnert W, Gregoire M-C, Reilhac A, et al. Characterisation of partial volume effect and region-based correction in small animal positron emission tomography (PET) of the rat brain. *Neuroimage* 2012; 60: 2144–2157.
54. Ledeboer A, Sloane EM, Milligan ED, et al. Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain* 2005; 115: 71–83.
55. Studebaker GA. A 'Rationalized' Arcsine Transform. *J Speech Lang Hear Res* 1985; 28: 455–462.
56. Mikaelsson MA, Constância M, Dent CL, et al. Placental programming of anxiety in adulthood revealed by Igf2-null models. *Nat Commun* 2013; 4: 2311.
57. Francis G. Equivalent statistics and data interpretation. *Behav Res Methods*. Epub ahead of print, 2016. DOI: 10.3758/s13428-016-0812-3.
58. Johnson KE, McMorris BJ, Raynor LA, et al. What big size you have! Using effect sizes to

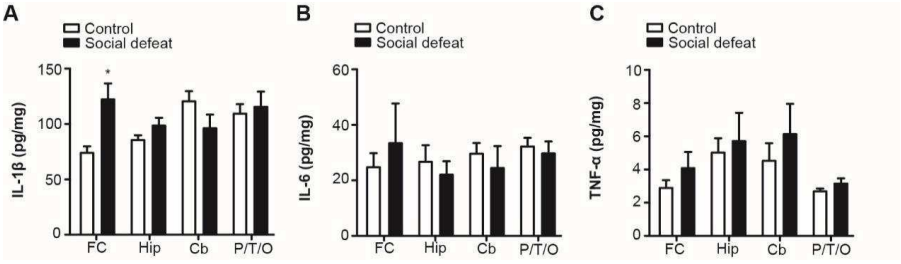
determine the impact of public health nursing interventions. *Appl Clin Inform* 2013; 4: 434–444.
59. Hardin JW, Hilbe JM. *Generalized Estimating Equations*. 2nd ed. Boca Raton, FL: Chapman & Hall/CRC, 2012.
60. Streiner DL. Best (but oft-forgotten) practices: The multiple problems of multiplicity-whether and how to correct for many statistical tests. *Am J Clin Nutr* 2015; 102: 721–728.
61. Pulliam J, Dawaghreh A, Alema-Mensah E, et al. Social Defeat Stress Produces Prolonged Alterations in Acoustic Startle and Body Weight Gain in Male Long Evans Rats. *J Psychiatr Res* 2010; 44: 1–14.
62. Koolhaas JM, Meerlo P, De Boer SF, et al. The temporal dynamics of the stress response. *Neurosci Biobehav Rev* 1997; 21: 775–782.
63. Harris R. Chronic and acute effects of stress on energy balance: are there appropriate animal models? *Am J Physiol Comp Physiol* 2015; 308: R250–R265.
64. Atsak P, Hauer D, Campolongo P, et al. Glucocorticoids interact with the hippocampal endocannabinoid system in impairing retrieval of contextual fear memory. *Proc Natl Acad Sci* 2012; 109: 3504–3509.
65. Vargas-López V, Torres-Berrio A, González-Martínez L, et al. Acute restraint stress and corticosterone transiently disrupts novelty preference in an object recognition task. *Behav Brain Res* 2015; 291: 60–66.
66. Luine V. Recognition memory tasks in neuroendocrine research. *Behav Brain Res* 2015; 285: 158–164.
67. McKim DB, Niraula A, Tarr AJ, et al. Neuroinflammatory Dynamics Underlie Memory Impairments after Repeated Social Defeat. *J Neurosci* 2016; 36: 2590–2604.
68. McEwen B, Morrison J. The Brain on Stress: Vulnerability and Plasticity of the Prefrontal Cortex over the Life Course. *Neuron* 2013; 79: 16–29.
69. Cheng W, Rolls ET, Qiu J, et al. Medial reward and lateral non-reward orbitofrontal cortex circuits change in opposite directions in depression. *Brain* 2016; 139: 3296–3309.
70. Belin-Rauscent A, Daniel M-L, Puaud M, et al. From impulses to maladaptive actions: the insula is a neurobiological gate for the development of compulsive behavior. *Mol Psychiatry* 2015; 21: 1–9.
71. Strange BA, Witter MP, Lein ES, et al. Functional organization of the hippocampal longitudinal axis. *Nat Rev Neurosci* 2014; 15: 655–669.
72. Eichenbaum H, Yonelinas AR, Ranganath C. The Medial Temporal Lobe and Recognition Memory. *Annu Rev Neurosci* 2007; 30: 123–152.
73. Shelton RC, Claiborne J, Sidoryk-Wegrzynowicz M, et al. Altered expression of genes involved in inflammation and apoptosis in frontal cortex in major depression. *Mol Psychiatry* 2011; 16: 751–762.
74. Frank MG, Watkins LR, Maier SF. Stress- and glucocorticoid-induced priming of neuroinflammatory responses: Potential mechanisms of stress-induced vulnerability to drugs of abuse. *Brain Behav Immun* 2011; 25: S21–S28.
75. Frank MG, Baratta M V., Sprunger DB, et al. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. *Brain Behav Immun* 2007; 21: 47–59.
76. Sorrells SF, Caso JR, Munhoz CD, et al. The Stressed CNS: When Glucocorticoids Aggravate Inflammation. *Neuron* 2009; 64: 33–39.
77. Wohleb ES, McKim DB, Shea DT, et al. Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain. *Biol Psychiatry* 2014; 75: 970–981.
78. Wohleb ES, Fenn AM, Pacenta AM, et al. Peripheral innate immune challenge exaggerated microglia activation, increased the number of inflammatory CNS macrophages, and prolonged social withdrawal in socially defeated mice. *Psychoneuroendocrinology* 2012; 37: 1491–1505.
79. Devinsky O, Morrell MJ, Vogt BA. Contributions of anterior cingulate cortex to behaviour. *Brain* 1995; 118: 279–306.
80. Saxena S, Brody AL, Ho ML, et al. Cerebral metabolism in major depression and obsessive-

compulsive disorder occurring separately and concurrently. *Biol Psychiatry* 2001; 50: 159–170.
81. Martinot J, Hardy P, Feline A. Left prefrontal glucose hypometabolism in the depressed state: a confirmation. *Am J Psychiatry* 1990; 147: 1313–1317.
82. Biver F, Goldman S, Delvenne V, et al. Frontal and Parietal Metabolic Disturbances in Unipolar Depression. *Biol Psychiatry* 1994; 36: 381–388.
83. Liu B, Le KX, Park M, et al. In Vivo Detection of Age- and Disease-Related Increases in Neuroinflammation by 18 F-GE180 TSPO MicroPET Imaging in Wild-Type and Alzheimer's Transgenic Mice. *J Neurosci* 2015; 35: 15716–15730.
84. Schuitemaker A, van der Doef TF, Boellaard R, et al. Microglial activation in healthy aging. *Neurobiol Aging* 2012; 33: 1067–1072.
85. Choi H, Choi Y, Kim KW, et al. Maturation of metabolic connectivity of the adolescent rat brain. *Elife* 2015; 4: 1–12.
86. Buwalda B, Stubbendorff C, Zickert N, et al. Adolescent social stress does not necessarily lead to a compromised adaptive capacity during adulthood: A study on the consequences of social stress in rats. *Neuroscience* 2013; 249: 258–270.
87. Vállez Garcia D, F J De Vries E, Toyohara J, et al. Evaluation of [11C]CB184 for imaging and quantification of TSPO overexpression in a rat model of herpes encephalitis. *Eur J Nucl Med Mol Imaging* 2015; 42: 1106–1118.
88. Boutin H, Prenant C, Maroy R, et al. [18F]DPA-714: Direct Comparison with [11C]PK11195 in a Model of Cerebral Ischemia in Rats. *PLoS One 2013*; 8: e56441.
89. Lodge MA. Repeatability of SUV in Oncologic 18 F-FDG PET. *J Nucl Med* 2017; 58: 523–532.
90. Adams MC, Turkington TG, Wilson JM, et al. A Systematic Review of the Factors Affecting Accuracy of SUV Measurements. *Am J Roentgenol* 2010; 195: 310–320.

Supplementary material



Supplementary figure 1: (A) Bodyweight gain (g) of control (n=18) and SoD rats (n=19) from day 0 to 25 of the short-term protocol, with significant decreased bodyweight gain in SoD rats evident already on day 2 (p<0.01) and with no recovery up to day 25 (p<0.001); **(B)** No significant difference in bodyweight gain (g) of control (n=8) and SoD rats (n=9) was found from week 5 to 28 of the follow-up protocol.



Supplementary figure 2: Assessment of the pro-inflammatory cytokines in the frontal cortex (FC), hippocampus (Hip), cerebellum (Cb) and parietal/temporal/occipital cortex (P/T/O) in the brains of control and defeated rats on day 25. **(A)** IL-1β levels were increased in the frontal cortex of SoD rats, *p<0.05. No significant differences were found in the other investigated brain areas. **(B)** Quantification of IL-6 revealed no significant differences between groups in any brain regions. A moderate effect size was found in the frontal cortex (d=0.63) of SoD rats. **(C)** TNF-α levels did not differ between groups in any brain regions; a moderate effect size was found in the frontal cortex of SoD rats (d=0.55).

Pharmacokinetic analysis of ^{11}C-PBR28 in the rat model of herpes encephalitis (HSE): comparison with (R)-^{11}C-PK11195

Author(s): Andrea Parente[§], Paula Kopschina Feltes[§], David Vállez García, Jurgen WA Sijbesma, Cristina M Moriguchi Jeckel, Rudi AJO Dierckx, Erik FJ de Vries, Janine Doorduin.

[§]Contributed equally to this work.

CHAPTER 4

as published in the Journal of Nuclear Medicine. Parente A et al. (2016) *J Nucl Med.* 57: 785-791.

Abstract

^{11}C-PBR28 is a second generation TSPO tracer with supposedly superior characteristics than the most commonly used tracer for neuroinflammation, *(R)*-^{11}C-PK11195. Despite its use in clinical research, no studies on the imaging properties and pharmacokinetic analysis of ^{11}C-PBR28 in rodent models of neuroinflammation have been published yet. Therefore, this study aims to evaluate ^{11}C-PBR28 as a tool for detection and quantification of neuroinflammation in pre-clinical research and to compare its imaging properties with *(R)*-^{11}C-PK11195. The herpes simplex encephalitis (HSE) model was used for induction of neuroinflammation in male Wistar rats. Six or seven days after virus inoculation, a dynamic ^{11}C-PBR28 or *(R)*-^{11}C-PK11195 PET scan with arterial blood sampling was performed. Pharmacokinetic modeling was performed on the PET data and analyzed using volumes of interest (VOIs) and voxel-based approach. VOI- and voxel-based analysis of ^{11}C-PBR28 images showed overexpression of TSPO in brain regions known to be affected in the HSE rat model. ^{11}C-PBR28 was metabolized faster than *(R)*-^{11}C-PK11195, with a metabolic half-life in plasma of 5 and 21 min, respectively. Overall, ^{11}C-PBR28 was more sensitive than *(R)*-^{11}C-PK11195 in detecting neuroinflammation. The binding potential (BP_{ND}) of ^{11}C-PBR28 was significantly higher ($P < 0.05$) in the medulla (176%), pons (146%), midbrain (101%), hippocampus (85%), thalamus (73%), cerebellum (54%) and hypothalamus (49%) in HSE rats than in control rats, while *(R)*-^{11}C-PK11195 only showed a higher BP_{ND} in the medulla (32%). The BP_{ND} in control animals was not significantly different between tracers, suggesting that non-specific binding of both tracers is similar. ^{11}C-PBR28 was more sensitive than *(R)*-^{11}C-PK11195 in the detection of TSPO overexpression in the HSE rat model, as more brain regions with significantly increased tracer uptake could be found, irrespective of the data analysis method used. These results suggest that ^{11}C-PBR28 should be able to detect more subtle changes in microglia activation in pre-clinical models of neuroinflammation.

Keywords: Neuroinflammation, herpes simplex encephalitis, rat model, Positron Emission Tomography, pharmacokinetic analysis

Introduction

Microglia are the resident macrophages of the central nervous system (1). These immune cells are activated by inflammatory stimuli, such as pathogens or neuronal damage, and initiate a cascade of inflammatory responses. When microglia are activated, the expression of the 18 kDa translocator protein (TSPO) (2) on the outer mitochondrial membrane is increased. This increase in TSPO expression is also observed in infiltrating macrophages and activated astrocytes, cell types that both participate in the neuroinflammatory response. Under normal conditions TSPO expression in the brain is low. Therefore, TSPO overexpression can be used as a neuroinflammatory biomarker, which can be measured with noninvasive imaging techniques like Positron Emission Tomography (PET) (3).

The oldest and most commonly used PET tracer for the detection of neuroinflammation is the TSPO ligand *(R)*-^{11}C-PK11195, which has been used in clinical and preclinical studies of various diseases and to evaluate new treatment strategies. However, this PET tracer has some limitations, including a low signal-to-noise ratio, poor bioavailability in brain tissue, high nonspecific binding, high variability in the pharmacokinetics and metabolism between subjects, high binding to plasma proteins, and low sensitivity to visualize mild inflammation (4-6).

To overcome some of the drawbacks associated with *(R)*-^{11}C-PK11195, second generation TSPO PET tracers like ^{11}C-PBR28 have now been applied in clinical studies. ^{11}C-PBR28 has better intrinsic characteristics for a PET tracer than *(R)*-^{11}C-PK11195, such as a higher affinity (K_i=0.2 nM vs 0.8 nM) and lower lipophilicity (LogD=3.01±0.11 vs 3.95±0.18) (7). Consequently, ^{11}C-PBR28 shows a higher TSPO specific signal, which is beneficial for the follow-up of treatment strategies and the detection of mild neuroinflammation. Despite its superior imaging characteristics, ^{11}C-PBR28 is still not the ideal TSPO tracer due to its sensitivity to the genotype of a single nucleotide polymorphism in the human TSPO gene (rs6971), with allele frequency of about 30% in Caucasians (8). Other second-generation high-affinity TSPO ligands, such as ^{18}F-FEPPA (9), ^{18}F-PBR06, ^{18}F-PBR111, ^{18}F-DPA-714, ^{11}C-DPA-113 and ^{11}C-DAA1106 (10-12), are also to some extent sensitive to this polymorphism, which is a major limitation for their use in clinical studies.

To our knowledge, there are no studies that have demonstrated the presence of TSPO polymorphism in rodents. Therefore, ^{11}C-PBR28 could be an attractive PET tracer for preclinical imaging studies in animal models of neuroinflammation. However, only

two studies have evaluated ^{11}C-PBR28 for PET imaging of neuroinflammation in rodent models (13, 14). None of those studies compared the ^{11}C-PBR28 imaging results with those of *(R)*-^{11}C-PK11195.

The aim of the present study was to evaluate ^{11}C-PBR28 as a TSPO PET tracer for preclinical imaging in the herpes simplex encephalitis model (HSE) (15). In this rat model, neuroinflammation is caused by intranasal inoculation of the herpes simplex virus type-1 (HSV-1) (15, 16) and does not require a surgical procedure that could damage the integrity of the blood-brain barrier. The *in vivo* pharmacokinetics and metabolism of ^{11}C-PBR28 were investigated and compared with *(R)*-^{11}C-PK11195.

Materials and Methods

Rats

Male outbred Wistar-Unilever rats (age 6-8 weeks, weight 299±25 g) were obtained from Harlan (Horst, The Netherlands). The rats were allowed to acclimatize for at least seven days before the start of the experiment. Rats were housed individually in Makrolon cages, containing a layer of wood shavings. The room was kept on constant temperature (21±2 °C) with a 12-12h light-dark regimen. Water and commercial chow were available *ad libitum*.

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 (DEC 6264A and 6264C).

Animal Model

A HSV-1 strain was obtained from a clinical isolate, cultured in Vero cells and assayed for plaque-forming units (PFU) per mL. Rats were slightly anaesthetized with 5% isoflurane mixed with medical air and 50 µL of phosphate-buffered saline (PBS) containing 1x10^7 PFU of HSV-1 was pipetted into each nostril (15). The same procedure was applied to control rats using PBS without the virus. Clinical symptoms were scored daily by the same observer.

Study Design

Rats were randomly divided in the control group (n=6) and HSE group (n=6). ^{11}C-PBR28 PET scans with arterial blood sampling were performed on day 6 or 7 after inoculation.

The *(R)*-^{11}C-PK11195 PET data was acquired in a previous study using identical methods (16), but completely reanalyzed for this study.

Tracer synthesis

^{11}C-PBR28 was synthesized following the previously described procedure (17), with slight modifications. The precursor was dissolved in 300 µL of dimethyl-sulfoxide instead of acetonitrile, and 10 mg of potassium hydroxide was used as base instead of sodium hydride. The use of potassium hydroxide required the addition of 200 µL of 0.1M hydrochloric acid after the reaction for neutralization. A filtration step was added before high performance liquid chromatography (HPLC) purification. The final product (pH 6.5-7) was obtained in 39±6% radiochemical yield (corrected for decay), with a radiochemical purity of 100% and a specific activity of 196±35 GBq/µmol.

PET imaging with arterial blood sampling

Rats were anesthetized with isoflurane in medical air (5% for induction, 2-3% for maintenance). A cannula was placed in the femoral artery for blood sampling, while another was inserted in the femoral vein for tracer injection. The rats were placed into the PET camera (Focus 220, Siemens Medical Solutions Inc., United States) with their head in the field of view. Body temperature was maintained with heating pads, and heart rate and oxygen saturation were monitored during the scan. A transmission scan was acquired using a ^{57}Co point source for attenuation and scatter correction. ^{11}C-PBR28 (68±21 MBq; 0.67±0.11 nmol) was injected over 1 min, using an automatic pump at a speed of 1 mL/min, and a 91-min dynamic PET scan was acquired.

During the first 60-min of the scan, 16 blood samples of 0.1 mL were taken at 10, 20, 30, 40, 50, 60, 90, 120, 180, 300, 450, 600, 900, 1800, 2700, 3600 s after tracer injection. After collection of the blood samples, the same volume of heparinized saline was injected to prevent large changes in blood pressure. A 25 µL aliquot of whole blood was taken from each sample for radioactivity measurement (whole blood curve). The remainder of the sample was centrifuged at 13,000 rpm (16,000×g) for 8 min and 25 µL of plasma was taken for radioactivity measurement. The radioactivity in blood and plasma was measured with a gamma counter (LKB-Wallac, Finland) and corrected for decay.

Tracer displacement

Displacement of ^{11}C-PBR28 was evaluated by administration of an excess of PK11195 during the PET scan. Thus, 5 mg/kg unlabeled PK11195 in 200 µL of dimethyl-sulfoxide was intravenously injected over a period of 1 min via the venous cannula at 61 min post tracer injection. PET acquisition was continued for another 30 min without blood sampling.

Metabolite analysis

Measurement of the percentage of intact tracer in plasma was performed on blood samples (0.6 mL) collected at 3, 5, 7.5, 10, 15, 30, 45 or 60 min post tracer injection. Two or three samples were collected from each animal. Immediately after collection, the blood samples were placed on ice to inhibit tracer metabolism (18). Centrifugation and collection of the plasma sample was performed as described above. Plasma was diluted and mixed with an equal volume of acetonitrile. The samples were centrifuged for 3 min at 5,300 rpm (3,000×g). The supernatant was filtered through a Millipore Millex-HV filter (4 mm, pore size 0.45 µm) and analyzed by HPLC using an Alltima RP-C18 column (5 µm, 10 mm x 250 mm) and a mobile phase consisting of acetonitrile/water (50/50) at a flow of 5 mL/min. Fractions of 30 s were collected and measured in the gamma counter.

The metabolite data of all animals was grouped to generate a single population curve, since no statistical difference in tracer metabolism and in parent fraction of each tracer between the groups was found. The data points of the percentage of intact tracer vs. time were fitted with a one-phase exponential function. The individual plasma radioactivity values were corrected for the percentage of intact tracer and used together with the whole blood for pharmacokinetic analysis.

PET image reconstruction and preparation

The list-mode data from the first 60 min of the emission scan was reconstructed into 21 frames (6x10, 4x30, 2x60, 1x120, 1x180, 4x300, 3x600 seconds). For the displacement study, the last 31 min of the PET scan were reconstructed into 18 frames (1x60, 6x10, 4x30, 2x60, 1x120, 1x180, 4x300 seconds). Emission sinograms were iteratively reconstructed (OSEM2D, 4 iterations and 16 subsets) after being normalized and corrected for attenuation and decay of radioactivity.

PET images were analyzed using PMOD 3.5 software (PMOD Technologies Ltd, Switzerland). The scans were automatically registered to tracer-specific PET templates

(19). Volumes of interest (VOI) of several brain regions were constructed based of previously defined structures (19). The brain radioactivity concentration was calculated from the VOIs to generate time-activity curves (TACs) and expressed as standardized uptake values (SUVs): [tissue activity concentration (MBq/g) x body weight (g)] / [injected dose (MBq)]. The 50-60 min time frame was used for VOI- and voxel-based statistical analysis (20).

Pharmacokinetic analysis

Pharmacokinetic modeling analysis was performed in PMOD, using the whole blood and metabolite corrected plasma curves as input functions. Visual inspection showed a better fit for Logan graphical analysis, confirming the reversible behavior of the tracer (21), using a t* of 15 min, and used to calculate the distribution volume (V_T). In addition, the reversible two-tissue compartment model (2TCM) was calculated with the equation

$$\frac{dC_1(t)}{dt} = K_1 C_p(t) - (k_2 + k_3)\, C_1(t) + k_4 C_2(t)$$

$$\frac{dC_2(t)}{dt} = k_3 C_1(t) - k_4 C_2(t)$$

Where C_p, C_1 and C_2 represent the tracer concentration in plasma, tissue compartment 1 and 2, respectively. A fixed blood volume of 3.6% (22) was used for the calculation, and V_T and non-displaceable binding potential (BP_{ND} calculated as k_3/k_4) were obtained (23).

Statistical analysis

Results are presented as mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics 20. Differences between groups were analyzed by independent samples t-tests and considered to be significant with $P < 0.05$, without correction for multiple comparisons.

Voxel-based analysis was performed using SPM12 (Wellcome Trust Centre for Neuroimaging, United Kingdom) and SAMIT toolbox (19). Images were smoothed with a 1.2 mm isotropic Gaussian kernel. Statistical analysis was performed using a two-sample t-test design (control *vs.* HSE) without global normalization. For evaluation of group differences, T-map data was interrogated at $P < 0.005$ (uncorrected) and extent

threshold of 200 voxels. Only those clusters with $P < 0.05$ corrected for family-wise error were considered significant.

The magnitude of difference between groups was assessed using the Cohen's *d* effect size index, calculated for VOI-analysis as d= (mean HSE-mean control)$/\sqrt{(\text{SD HSE}^2+\text{SD control}^2)/2}$, and for voxel-based analysis as $d = (2\,T - value)/\sqrt{df}$.

Results

VOI-based analysis

The uptake of ^{11}C-PBR28 in several brain regions of HSE rats corresponded with the distribution pattern of the viral infection (Fig. 1) (15). VOI-based analysis showed significantly higher whole brain ^{11}C-PBR28 uptake in HSE rats than in control rats (+44%, $P = 0.032$, Table 1). Analysis of individual brain regions revealed an increased uptake of ^{11}C-PBR28 in the pons (+150%, $P = 0.016$), medulla (+144%, $P = 0.015$) and hypothalamus (+44%, $P = 0.034$).

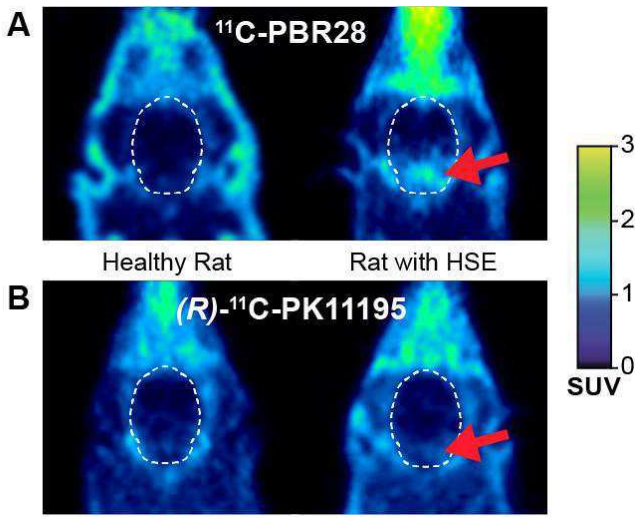


Figure 1: Transaxial ^{11}C-PBR28 **(A)** and *(R)*-^{11}C-PK11195 **(B)** PET images (30-60 min) of the head of a control rat and an HSE rat. The arrow shows increased uptake in the region of the pons and medulla.

Table 1: ^{11}C-PBR28 uptake (50-60 min), expressed as SUV (mean±SD), obtained by PET imaging of control and HSE groups.

	Control	HSE	*d*
Amygdala	0.45±0.08	0.56±0.10	
Cerebellum	0.64±0.11	0.98±0.37	
Frontal Cortex	0.53±0.05	0.73±0.24	
Hippocampus	0.42±0.06	0.56±0.14	
Hypothalamus	0.42±0.10	0.61±0.14*	1.56
Medulla	0.64±0.11	1.56±0.52*	2.45
Midbrain	0.44±0.06	0.76±0.30	
Pons	0.50±0.11	1.25±0.44*	2.34
Septum	0.47±0.05	0.53±0.08	
Striatum	0.38±0.06	0.43±0.08	
Thalamus	0.41±0.04	0.54±0.13	
Whole brain	0.52±0.07	0.75±0.17*	1.77

* $P < 0.05$, *d*: Cohen's effect size

Voxel-based analysis

Voxel-based analysis showed a large cluster with a significantly higher ^{11}C-PBR28 uptake in the HSE group than in the control group (Fig. 2 and Table 2). This cluster included bilaterally the pons, medulla, midbrain, hippocampus, cerebellum, and hypothalamus.

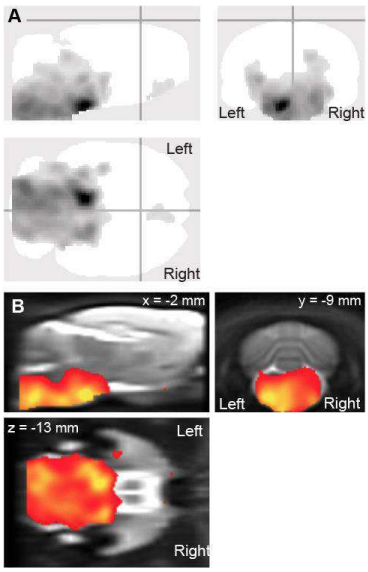


Figure 2: ^{11}C-PBR28 voxel-based analysis results displayed as "glass brain", showing areas with significantly higher uptake in the HSE group than in the control group ($P < 0.05$ family-wise error corrected at cluster level).

Table 2: Brain regions showing increased ^{11}C-PBR28 uptake in the voxel-based analysis.

	Number of Voxels	T-value (mean±SD)	*D*
Medulla	6886	4.58±0.81	3.05
Pons	5241	4.46±0.80	2.97
Midbrain	2485	3.76±0.42	2.51
Hippocampus	1041	3.83±0.49	2.55
Cerebellum	886	3.55±0.26	2.37
Hypothalamus	623	3.65±0.33	2.43

d: Cohen's effect size

Displacement

The TACs of medulla and frontal cortex are shown in Fig. 3, representing an infected and a non-infected brain region respectively. Injection of 5 mg/kg of PK11195 at 60 min caused an initial increase in tracer uptake in all brain regions due to the release of ^{11}C-PBR28 from peripheral organs with TSPO expression, such as lungs, heart, glands and blood vessels (15). ^{11}C-PBR28 uptake in the medulla of HSE rats was significantly lower 10 min after PK11195 injection than just before displacement (51% and 68% at 10 and 30 minutes after displacement, respectively, $P < 0.05$). No significant reduction in ^{11}C-PBR28 uptake in the medulla of control rats was observed. Moreover, injection of PK11195 did not significantly reduce ^{11}C-PBR28 uptake in the frontal cortex of HSE or control rats.

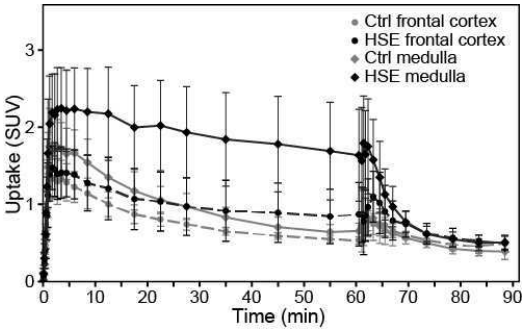


Figure 3: ^{11}C-PBR28 TACs of the medulla and frontal cortex from HSE and control groups. Rats were injected with 5 mg/kg PK11195 60 min after tracer injection to displace bound tracer from translocator protein (TSPO).

Tracer metabolism

Metabolite analysis revealed that ^{11}C-PBR28 was metabolized faster than *(R)*-^{11}C-PK11195 (Fig. 4), with 50% of plasma radioactivity consisting of metabolites at 5 and 21 min after injection of ^{11}C-PBR28 and *(R)*-^{11}C-PK11195, respectively. The whole blood

and metabolite-corrected plasma curves showed that ^{11}C-PBR28 presented higher whole blood, but substantially lower plasma activity, after correction for metabolites, than *(R)*-^{11}C-PK11195.

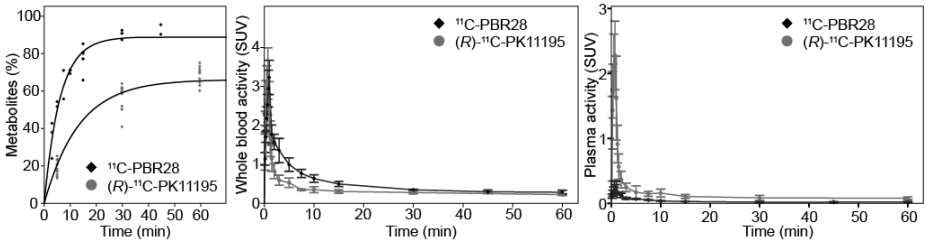


Figure 4: Percentage of metabolites present in **(A)** plasma and **(B)** whole blood, and **(C)** metabolite-corrected plasma curves of ^{11}C-PBR28 and *(R)*-^{11}C-PK11195.

Kinetic Modeling

For both ^{11}C-PBR28 and *(R)*-^{11}C-PK11195 the V_T obtained from the 2TCM showed an excellent correlation ($r^2 = 0.95$ and $r^2 = 0.98$, respectively; $P < 0.001$) with the V_T obtained from Logan graphical analysis (Fig. 5). V_T values of ^{11}C-PBR28 were approximately 5-fold higher than those of *(R)*-^{11}C-PK11195, irrespective of the group or brain area.

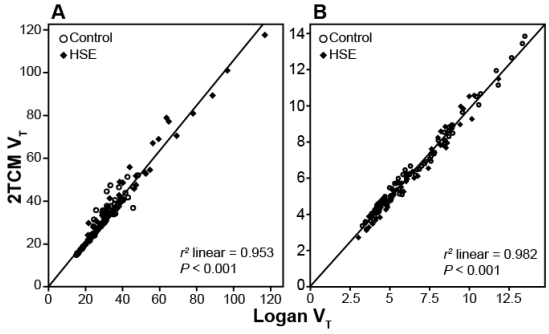


Figure 5: Correlation of the distribution volume (V_T) of individual brain regions determined by 2TCM and Logan graphical analysis for **(A)** ^{11}C-PBR28 and **(B)** *(R)*-^{11}C-PK11195.

Since ^{11}C-PBR28 and *(R)*-^{11}C-PK11195 are receptor tracers, BP_{ND} was chosen as the main outcome parameter. No statistical differences were found between the BP_{ND} of ^{11}C-PBR28 and *(R)*-^{11}C-PK11195 in any brain regions of control rats. For both tracers, whole brain BP_{ND} was significantly higher in HSE rats than in controls (Table 3). The BP_{ND} of ^{11}C-PBR28 was significantly higher in several brain regions of HSE rats than in control rats, in particular in the medulla (+176%, $P < 0.001$), pons (+146%, $P < 0.001$),

midbrain (+101, $P = 0.001$), hippocampus (85%, $P < 0.05$), thalamus (+73%, $P < 0.05$), cerebellum (+54%, $P < 0.05$), and hypothalamus (+49%, $P < 0.05$). In contrast, *(R)*-^{11}C-PK11195 only showed a significantly higher BP_{ND} in the medulla (+32%, $P < 0.01$) of HSE rats as compared to controls.

Table 3: ^{11}C-PBR28 and *(R)*-^{11}C-PK11195 binding potential (mean±SD) of control and HSE rats.

	^{11}C-PBR28			(R)-^{11}C-PK11195		
	Control	HSE	*D*	Control	HSE	*d*
Amygdala	1.24±0.12	2.03±0.48*	2.3	1.67±0.35	1.84±0.54	
Cerebellum	1.94±0.34	3.00±0.70*	1.9	2.10±0.53	2.09±0.57	
Cortex Frontal	1.55±0.45	2.61±0.93		1.90±0.61	2.01±0.59	
Hippocampus	1.08±0.26	2.00±0.63**	1.9	1.42±0.30	1.83±0.63	
Hypothalamus	1.12±0.27	1.69±0.34*	1.9	1.63±0.35	1.42±0.50	
Medulla	1.43±0.26	3.95±0.55***	5.9	1.74±0.30	2.30±0.25**	2.0
Midbrain	1.12±0.27	2.26±0.52***	2.8	1.62±0.60	2.20±0.81	
Pons	1.30±0.42	3.19±0.42***	5.0	1.88±0.45	2.05±0.66	
Septum	1.19±0.42	1.87±0.55		1.67±0.35	1.49±0.57	
Striatum	1.04±0.24	1.87±0.55		1.22±0.29	1.30±0.53	
Thalamus	1.05±0.24	1.81±0.60*	1.7	1.29±0.30	1.68±0.43	
Whole brain	1.53±0.36	2.63±0.47**	2.6	1.48±0.33	1.73±0.60	

*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, *d*: Cohen's effect size

Correlation between tracer uptake parameters

To assess whether a simplified procedure without blood sampling could be applied to quantify tracer uptake, the SUV values of ^{11}C-PBR28 and *(R)*-^{11}C-PK11195 in different brain regions were correlated with the V_T and BP_{ND} obtained from Logan and 2TCM kinetic analysis, respectively (Fig. 6). The SUV values of ^{11}C-PBR28 showed a moderate correlation ($r^2 = 0.463$, $P < 0.001$) with BP_{ND} values. In contrast, a strong correlation was found between the SUV and V_T of ^{11}C-PBR28 ($r^2 = 0.87$, $P < 0.001$). For *(R)*-^{11}C-PK11195 only modest correlations were found between the SUV and the BP_{ND} ($r^2 = 0.133$, $P < 0.001$) and between the SUV and the V_T ($r^2 = 0.143$, $P < 0.001$).

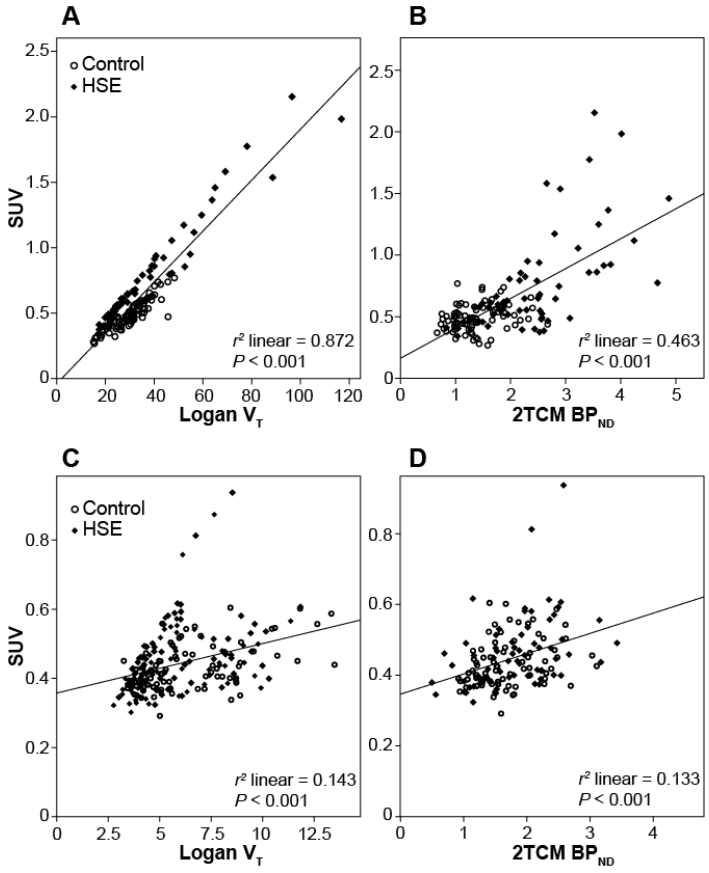


Figure 6: Correlations between **(A)** SUV and V_T values and **(B)** SUV and BP_{ND} values of ^{11}C-PBR28, and between **(C)** SUV and V_T values and **(D)** SUV and BP_{ND} values of *(R)*-^{11}C-PK11195, in HSE and control rats.

Discussion

^{11}C-PBR28 is a second-generation PET tracer for TSPO imaging that has already been applied in clinical studies, but surprisingly has not been fully evaluated in a rodent model of neuroinflammation yet. In this study, the performance of ^{11}C-PBR28 for the pre-clinical imaging of neuroinflammation was evaluated with the HSE model, with *(R)*-^{11}C-PK11195 tracer used for comparison purposes. In the HSE model, nasal infection with HSV-1 induces strong activation of microglia 6-7 days after infection, in particular in the pons and medulla (15,16,24). ^{11}C-PBR28 was able to detect the activation of microglia in more brain regions and proved to be more sensitive than *(R)*-^{11}C-PK11195. This difference between tracers might be due to the higher affinity of ^{11}C-PBR28 for TSPO compared to *(R)*-^{11}C-PK11195. VOI-based analysis of predefined brain regions

demonstrated an increased ^{11}C-PBR28 uptake in the medulla, pons and hippocampus in HSE rats when compared to controls. The enhanced ^{11}C-PBR28 uptake in these brain regions could be displaced by administration of 5 mg/kg PK11195, resulting in tracer concentrations that were comparable to controls. This demonstrates that the increased uptake of ^{11}C-PBR28 in the infected brain areas represents increased specific binding to TSPO and is not solely due to other inflammatory phenomena, such as increased cerebral blood flow (13).

Voxel-based analysis, compared with VOI-based, has the capacity to identify affected brain region not limited to pre-defined regions. In this study, voxel-based analysis showed more brain regions with increased ^{11}C-PBR28 uptake than VOI-based analysis. Besides the medulla, pons and hypothalamus, significantly increased ^{11}C-PBR28 uptake was found in the midbrain, hippocampus and cerebellum. These results indicate that voxel-based analysis is a more sensitive method to detect focal neuroinflammation.

For the pharmacokinetic modeling, blood sampling and metabolite analysis was performed for both tracers. ^{11}C-PBR28 proved to be metabolized substantially faster than *(R)*-^{11}C-PK11195. However, only polar metabolites of ^{11}C-PBR28 were formed and these radioactive metabolites practically do not enter the brain, as demonstrated by Briard *et al.* (7). At 30 min after injection, 97.6% of the radioactivity in the brain consisted of intact tracer, with the small percentage of metabolites in the brain likely originating from the blood compartment. Interestingly, the activity of ^{11}C-PBR28 in plasma is much lower than in whole blood. This might be explained by the presence of TSPO receptors in red blood cells, which can bind the tracer. This binding seems to be more important for the second generation TSPO tracers with higher affinity for TSPO (*e.g.* ^{11}C-PBR28) than for *(R)*-^{11}C-PK11195 (25).

The 2TCM is considered the most suitable model for pharmacokinetic analysis of the receptor ligands ^{11}C-PBR28 (26) and *(R)*-^{11}C-PK11195 (27). BP_{ND} was used as the main outcome since it represents the specific binding of the tracer to the TSPO receptor. ^{11}C-PBR28 was able to detect a statistically significant increase in BP_{ND} in affected brain regions, such as the medulla, pons, cerebellum, midbrain, thalamus, hippocampus and hypothalamus. In contrast, the BP_{ND} of *(R)*-^{11}C-PK11195 was significantly increased only in the medulla of HSE rats. Comparison of the BP_{ND} of both tracers in control animals showed no significant difference, suggesting that binding of both tracers under normal physiological conditions is similar (16). V_T values of ^{11}C-PBR28 calculated by Logan

analysis and 2TCM were highly correlated but seem less suitable as outcome parameter because their high inter-subject variability (14,28) (Suppl. Table 1 and Suppl. Figure 1), which may be attributed to variations in the *K1/k2* (perfusion may be altered in neuroinflammatory processes) or to variations in plasma availability of ^{11}C-PBR28 (20). Consequently, V_T comparison between groups was not performed in the current study. A possible limitation of the current study is the lack of measurement of the plasma free fraction (fP). However, previously high variability in fP was found (25-35%) (29), increasing the inter-subject variability in V_T. Consequently, the added error by including fP was greater than the correction it represented.

To simplify the imaging procedure while retaining reliable quantitative information, the V_T and BP_{ND} values were correlated with SUV values, which can easily be obtained without blood sampling. SUV values of ^{11}C-PBR28 showed a moderate correlation with BP_{ND}, but are strongly correlated with V_T. This can be explained by the fact that SUV and V_T can both be influenced by different factors (*e.g.* the delivery of the tracer or the cerebral blood flow), whereas BP_{ND} is only dependent on specific receptor binding and its release. Therefore, SUV values might better reflect the total distribution volume than the binding potential for ^{11}C-PBR28. For *(R)*-^{11}C-PK11195, the SUV showed a poor correlation with both BP_{ND} and V_T.

Conclusion

The present study demonstrated that ^{11}C-PBR28 was able to detect TSPO overexpression in the encephalitic rat brain model. The most sensitive analysis methods to detect infected brain areas were either voxel-based analysis of static scans or the assessment of BP_{ND} by full pharmacokinetic analysis of dynamic PET data. ^{11}C-PBR28 has a better sensitivity towards areas with overexpression of TSPO than *(R)*-^{11}C-PK11195. ^{11}C-PBR28 not only detected more brain regions with neuroinflammation, but also showed a larger increase in BP_{ND} in infected areas than *(R)*-^{11}C-PK11195. A higher sensitivity for detection of TSPO overexpression implies that milder neuroinflammation and smaller changes might be better detected; therefore, disease processes and novel treatment strategies could be better monitored in pre-clinical models.

Acknowledgments

The authors thank to Bram Maas, Rolf Zijlma, Luís Juarez Orozco and Inês Farinha Antunes for their support.

Disclosure

The scholarship of Andrea Parente was financed by Siemens. The other authors declare no conflict of interest.

References

1. Frick LR, Williams K, Pittenger C. Microglial dysregulation in psychiatric disease. *Clin Dev Immunol*. 2013; 2013 :1-10.
2. Papadopoulos V, Baraldi M, Guilarte TR, et al. Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. *Trends Pharmacol Sci*. 2006; 27:402-409.
3. Venneti S, Lopresti BJ, Wiley C a. The peripheral benzodiazepine receptor (Translocator protein 18kDa) in microglia: from pathology to imaging. *Prog Neurobiol*. 2006;80:308-322.
4. Schweitzer PJ, Fallon BA, Mann JJ, Kumar JSD. PET tracers for the peripheral benzodiazepine receptor and uses thereof. *Drug Discov Today*. 2010; 15:933-942.
5. Trapani A, Palazzo C, De Candia M, Lasorsa FM, Trapani G. Targeting of the translocator protein 18 kDa (TSPO): A valuable approach for nuclear and optical imaging of activated microglia. *Bioconjug Chem*. 2013; 24:1415-1428.
6. Chauveau F, Boutin H, Van Camp N, Dollé F, Tavitian B. Nuclear imaging of neuroinflammation: A comprehensive review of [11C]PK11195 challengers. *Eur J Nucl Med Mol Imaging*. 2008; 35:2304-2319.
7. Briard E, Zoghbi SS, Imaizumi M, et al. Synthesis and evaluation in monkey of two sensitive 11C-labeled aryloxyanilide ligands for imaging brain peripheral benzodiazepine receptors in vivo. *J Med Chem*. 2008; 51:17-30.
8. Kreisl WC, Jenko KJ, Hines CS, et al. A genetic polymorphism for translocator protein 18 kDa affects both in vitro and in vivo radioligand binding in human brain to this putative biomarker of neuroinflammation. *J Cereb Blood Flow Metab*. 2012; 33:53-58.
9. Setiawan E, Wilson AA, Mizrahi R, et al. Role of Translocator Protein Density, a Marker of Neuroinflammation, in the Brain During Major Depressive Episodes. *JAMA Psychiatry*. 2015:1-8.
10. Owen DRJ, Gunn RN, Rabiner EA, et al. Mixed-affinity binding in humans with 18-kDa translocator protein ligands. *J Nucl Med*. 2011; 52:24-32.
11. Owen DR, Yeo AJ, Gunn RN, et al. An 18-kDa Translocator Protein (TSPO) polymorphism explains differences in binding affinity of the PET radioligand PBR28. *J Cereb Blood Flow Metab*. 2012; 32:1-5.
12. Owen DR, Howell OW, Tang S-P, et al. Two binding sites for [3H]PBR28 in human brain: implications for TSPO PET imaging of neuroinflammation. *J Cereb Blood Flow Metab*. 2010; 30:1608-1618.
13. Imaizumi M, Kim HJ, Zoghbi SS, et al. PET imaging with [11C]PBR28 can localize and quantify upregulated peripheral benzodiazepine receptors associated with cerebral ischemia in rat. *Neurosci Lett*. 2007; 411:200-205.
14. Walker MD, Dinelle K, Kornelsen R, et al. [11C]PBR28 PET imaging is sensitive to neuroinflammation in the aged rat. *J Cereb Blood Flow Metab*. 2015; 35:1331-1338.
15. Doorduin J, Klein HC, Dierckx RA, James M, Kassiou M, de Vries EFJ. [11C]-DPA-713 and [18F]-DPA-714 as new PET tracers for TSPO: A comparison with [11C]-(R)-PK11195 in a rat model of herpes encephalitis. *Mol Imaging Biol*. 2009; 11:386-398.
16. Vállez Garcia D, de Vries EFJ, Toyohara J, et al. Evaluation of [11C]CB184 for imaging and quantification of TSPO overexpression in a rat model of herpes encephalitis. *Eur J Nucl Med Mol Imaging*. 2015;42:1106-1118.

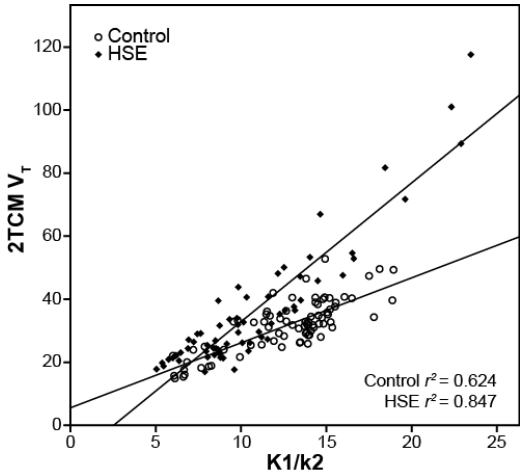
17. Wang M, Yoder KK, Gao M, et al. Fully automated synthesis and initial PET evaluation of [11C]PBR28. *Bioorganic Med Chem Lett*. 2009; 19:5636-5639.
18. Yin P, Peter A, Franken H, et al. Preanalytical aspects and sample quality assessment in metabolomics studies of human blood. *Clin Chem*. 2013; 59:833-845.
19. Vállez Garcia D, Casteels C, Schwarz AJ, Dierckx RAJO, Koole M, Doorduin J. A standardized method for the construction of tracer specific PET and SPECT rat brain templates: validation and implementation of a toolbox. *PLoS One*. 2015;10: e0122363.
20. Tóth M, Doorduin J, Häggkvist J, et al. Positron Emission Tomography studies with [11C]PBR28 in the Healthy Rodent Brain: Validating SUV as an Outcome Measure of Neuroinflammation. *PLoS One*. 2015;10: e0125917.
21. Logan J, Fowler JS, Volkow ND, et al. Graphical analysis of reversible radioligand binding from time-activity measurements applied to [N-11C-methyl]-(-)-cocaine PET studies in human subjects. *J Cereb Blood Flow Metab*. 1990; 10:740-747.
22. Julien-Dolbec C, Tropres I, Montigon O, et al. Regional response of cerebral blood volume to graded hypoxic hypoxia in rat brain. *Br J Anaesth*. 2002; 89:287-293.
23. Innis RB, Cunningham VJ, Delforge J, et al. Consensus nomenclature for in vivo imaging of reversibly binding radioligands. *J Cereb Blood Flow Metab*. 2007; 27:1533-1539.
24. Esiri MM, Drummond CWE, Morris CS. Macrophages and microglia in HSV-1 infected mouse brain. *J Neuroimmunol*. 1995; 62:201-205.
25. Rizzo G, Veronese M, Tonietto M, Zanotti-Fregonara P, Turkheimer FE, Bertoldo A. Kinetic modeling without accounting for the vascular component impairs the quantification of [(11)C]PBR28 brain PET data. *J Cereb Blood Flow Metab*. 2014; 34:1060-1069.
26. Owen DR, Guo Q, Kalk NJ, et al. Determination of [11C]PBR28 binding potential in vivo: a first human TSPO blocking study. *J Cereb Blood Flow Metab*. 2014; 34:989-994.
27. Kropholler MA, Boellaard R, Schuitemaker A, et al. Development of a tracer kinetic plasma input model for (R)-[11C]PK11195 brain studies. *J Cereb Blood Flow Metab*. 2005; 25:842-851.
28. Suridjan I, Rusjan PM, Voineskos AN, et al. Neuroinflammation in healthy aging: A PET study using a novel Translocator Protein 18kDa (TSPO) radioligand, [18F]-FEPPA. *Neuroimage*. 2014; 84:868-875.
29. Bloomfield PS, Selvaraj S, Veronese M, et al. Microglial Activity in People at Ultra High Risk of Psychosis and in Schizophrenia: An [11C]PBR28 PET Brain Imaging Study. *Am J Psychiatry*. 2015: appi.ajp.2015.1.

Supplemental data

Supplemental table 1. Volume of distribution of ^{11}C-PBR28 and *(R)*-^{11}C-PK11195 calculated by 2TCM in control and herpes encephalitis (HSE) rats (mean±SD).

	^{11}C-PBR28		*(R)*-^{11}C-PK11195	
	Control	HSE	Control	HSE
Amygdala	28.96±7.17	29.32±7.24	6.02±2.06	6.27±2.40
Cerebellum	41.81±9.45	50.83±16.84	8.57±3.06	7.63±2.84
Cortex Frontal	34.44±4.94	39.01±16.34	7.57±3.27	6.75±2.60
Hippocampus	26.68±6.16	29.34±9.30	5.33±1.78	5.06±1.91
Hypothalamus	28.36±7.04	31.17±10.01	5.66±1.79	5.12±1.63
Medulla	40.92±9.44	82.86±29.42*	7.74±3.01	7.64±1.46
Midbrain	30.35±6.44	40.24±13.87	5.80±1.96	5.50±1.74
Pons	33.73±8.44	64.06±24.08*	6.64±2.31	6.75±1.52
Septum	31.18±7.13	27.09±6.74	5.95±2.16	5.07±2.07
Striatum	25.38±6.24	21.79±5.63	5.03±2.18	4.03±1.37
Thalamus	27.87±6.24	29.01±8.54	5.25±1.92	4.24±1.23
Whole brain	32.51±6.82	38.43±10.26	6.47±2.74	6.27±2.40

*$P < 0.05$



Supplemental figure 1. Correlation between V_T obtained with the 2TCM and K_1/k_2 for ^{11}C-PBR28.

Supplemental table 2: Kinetic parameters obtained through the 2TCM, for ^{11}C-PBR28 and ^{11}C-PK11195, in control and HSE rats.

	^{11}C-PBR28																			
Control	K_1			%SE	k_2			%SE	k_3			%SE	k_4			%SE	V_T 2TCM			%SE	BP_{ND}			%SE	R^2
Amygdala	6.59	±	2.46	8.41	0.53	±	0.21	14.32	0.08	±	0.04	21.92	0.06	±	0.03	15.28	28.96	±	7.17	3.28	1.25	±	0.12	12.18	0.94
Cerebellum	5.74	±	2.52	6.67	0.42	±	0.18	11.67	0.07	±	0.03	15.34	0.04	±	0.02	15.59	41.81	±	9.45	5.46	1.94	±	0.34	10.12	0.95
Cortex Frontal	6.19	±	1.72	6.43	0.47	±	0.16	10.67	0.07	±	0.03	16.97	0.04	±	0.01	15.29	34.44	±	4.94	3.93	1.55	±	0.45	8.98	0.95
Hippocampus	6.80	±	2.29	6.90	0.56	±	0.22	11.72	0.08	±	0.05	21.41	0.07	±	0.03	17.50	26.68	±	6.16	3.58	1.08	±	0.26	12.52	0.95
Hypothalamus	8.35	±	2.97	11.99	0.65	±	0.24	21.73	0.11	±	0.05	32.21	0.10	±	0.04	16.57	28.36	±	7.04	2.63	1.13	±	0.27	20.85	0.96
Medulla	9.12	±	4.45	7.75	0.57	±	0.31	14.31	0.11	±	0.05	19.61	0.07	±	0.02	11.16	40.92	±	9.44	2.17	1.43	±	0.26	12.25	0.95
Midbrain	9.03	±	3.38	8.17	0.64	±	0.22	15.14	0.11	±	0.03	22.69	0.10	±	0.02	11.04	30.35	±	6.44	1.67	1.12	±	0.24	14.98	0.96
Pons	9.30	±	3.82	11.44	0.69	±	0.34	22.22	0.15	±	0.08	29.87	0.11	±	0.05	14.22	33.73	±	8.44	2.28	1.30	±	0.42	20.61	0.96
Septum	6.39	±	1.77	8.41	0.45	±	0.11	14.38	0.06	±	0.02	23.48	0.05	±	0.02	17.64	31.18	±	7.13	3.96	1.19	±	0.15	13.70	0.94
Striatum	6.35	±	1.67	8.20	0.55	±	0.22	14.55	0.08	±	0.05	25.34	0.08	±	0.04	20.17	25.38	±	6.24	4.53	1.04	±	0.24	17.55	0.95
Thalamus	7.92	±	2.57	7.32	0.59	±	0.19	12.60	0.09	±	0.03	22.57	0.08	±	0.02	13.24	27.87	±	5.21	2.05	1.05	±	0.24	13.09	0.96
Average	7.15	±	2.56	8.14	0.54	±	0.21	14.47	0.09	±	0.04	22.18	0.07	±	0.03	15.49	32.03	±	7.14	3.55	1.35	±	0.28	13.99	0.95

Units: K_1: ml/cm^3/min; k_2, k_3 and k_4: min^{-1}; V_T: ml/cm^3; BP_{ND}: unitless.

	^{11}C-PBR28																				
HSE	K_1			%SE	k_2			%SE	k_3			%SE	k_4			%SE	V_T 2TCM			%SE	BP_{ND}			%SE	R^2
Amygdala	6.01	±	2.60	8.20	0.63	±	0.25	15.23	0.13	±	0.08	17.70	0.07	±	0.04	11.50	29.32	±	7.24	3.27	2.04	±	0.48	12.31	0.96
Cerebellum	6.75	±	3.92	8.09	0.60	±	0.30	15.59	0.17	±	0.11	13.97	0.05	±	0.03	9.31	50.83	±	16.84	3.62	3.00	±	0.70	11.27	0.97
Cortex Frontal	5.71	±	2.65	7.94	0.53	±	0.18	14.79	0.12	±	0.05	15.90	0.05	±	0.03	10.83	39.01	±	16.34	3.70	2.61	±	0.94	11.05	0.96
Hippocampus	6.61	±	3.71	8.08	0.72	±	0.44	14.07	0.14	±	0.10	15.28	0.07	±	0.04	10.38	29.34	±	9.30	3.18	2.00	±	0.63	10.61	0.98
Hypothalamus	7.43	±	2.95	7.74	0.66	±	0.24	14.11	0.15	±	0.09	17.03	0.08	±	0.04	9.58	31.17	±	10.01	2.21	1.69	±	0.34	11.58	0.98
Medulla	9.08	±	4.82	8.84	0.61	±	0.38	18.38	0.18	±	0.09	15.35	0.05	±	0.03	8.73	82.86	±	29.42	3.36	3.95	±	0.55	12.92	0.97
Midbrain	9.51	±	5.03	9.25	0.87	±	0.61	15.37	0.19	±	0.13	16.49	0.08	±	0.05	10.44	40.24	±	13.87	2.90	2.26	±	0.52	11.11	0.97
Pons	8.67	±	3.25	9.14	0.67	±	0.35	18.13	0.20	±	0.13	15.05	0.06	±	0.03	7.75	64.06	±	24.08	2.59	3.19	±	0.33	12.59	0.97
Septum	6.76	±	3.91	7.82	0.73	±	0.45	13.37	0.12	±	0.07	18.72	0.07	±	0.04	14.04	27.09	±	6.74	3.75	1.87	±	0.55	11.73	0.96
Striatum	6.06	±	3.53	10.01	0.73	±	0.46	17.89	0.14	±	0.11	21.88	0.09	±	0.05	13.14	21.79	±	5.63	3.06	1.54	±	0.67	14.61	0.97
Thalamus	7.85	±	4.42	8.86	0.79	±	0.44	15.56	0.15	±	0.10	17.61	0.08	±	0.05	9.94	29.01	±	8.54	2.32	1.81	±	0.60	12.00	0.97
Average	6.95	±	3.54	8.34	0.66	±	0.35	15.35	0.15	±	0.09	16.42	0.06	±	0.04	10.45	39.51	±	13.03	3.18	2.41	±	0.61	11.70	0.97

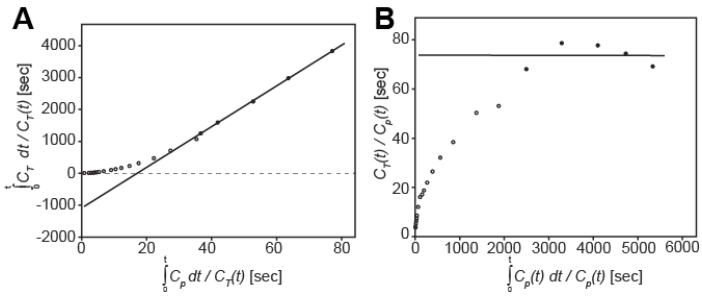
Units: K_1: ml/cm^3/min; k_2, k_3 and k_4: min^{-1}; V_T: ml/cm^3; BP_{ND}: unitless.

	(R)-^{11}C-PK11195																				
Control	K_1			%SE	k_2			%SE	k_3			%SE	k_4			%SE	V_T 2TCM			%SE	BP_{ND}			%SE	R^2
Amygdala	1.37	±	0.52	8.76	0.67	±	0.34	16.47	0.14	±	0.10	22.97	0.08	±	0.05	14.61	6.02	±	2.06	3.47	1.67	±	0.32	14.27	0.95
Cerebellum	1.44	±	0.39	6.42	0.56	±	0.23	12.32	0.11	±	0.03	16.55	0.06	±	0.02	11.43	8.57	±	3.06	3.22	2.11	±	0.48	10.24	0.96
Cortex Frontal	1.28	±	0.35	6.33	0.52	±	0.19	12.37	0.11	±	0.04	18.57	0.06	±	0.03	13.10	7.57	±	3.27	3.52	1.90	±	0.56	11.15	0.96
Hippocampus	1.45	±	0.46	7.28	0.68	±	0.22	14.52	0.15	±	0.09	21.78	0.10	±	0.07	12.83	5.33	±	1.78	2.71	1.42	±	0.27	13.99	0.97
Hypothalamus	1.75	±	0.64	8.29	0.89	±	0.47	16.32	0.22	±	0.18	20.93	0.13	±	0.09	11.16	5.66	±	1.79	2.43	1.64	±	0.32	14.55	0.97
Medulla	1.81	±	0.57	7.78	0.69	±	0.27	15.47	0.17	±	0.07	19.90	0.10	±	0.05	10.86	7.74	±	3.01	2.50	1.74	±	0.28	13.55	0.97
Midbrain	2.11	±	0.93	8.31	1.06	±	0.76	16.10	0.28	±	0.31	20.06	0.15	±	0.11	10.67	5.80	±	1.92	2.22	1.62	±	0.53	14.13	0.97
Pons	1.88	±	0.75	9.05	0.88	±	0.50	18.59	0.26	±	0.20	22.06	0.13	±	0.07	10.78	6.74	±	2.31	2.39	1.88	±	0.41	16.30	0.96
Septum	1.48	±	0.61	9.24	0.74	±	0.44	17.45	0.18	±	0.17	23.09	0.10	±	0.08	14.14	5.95	±	2.16	3.36	1.67	±	0.32	15.15	0.94
Striatum	1.33	±	0.44	6.31	0.59	±	0.23	12.21	0.12	±	0.07	20.82	0.11	±	0.07	13.20	5.25	±	1.92	2.63	1.22	±	0.27	12.68	0.97
Thalamus	1.61	±	0.46	6.79	0.68	±	0.19	13.36	0.15	±	0.09	21.45	0.12	±	0.07	12.36	5.56	±	1.90	2.35	1.29	±	0.27	13.51	0.97
Average	1.59	±	0.55	7.69	0.72	±	0.35	15.02	0.17	±	0.12	20.74	0.10	±	0.07	12.29	6.38	±	2.29	2.80	1.65	±	0.37	13.59	0.96

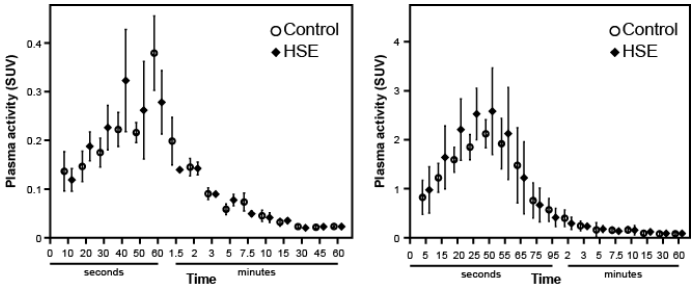
Units: K_1: ml/cm^3/min; k_2, k_3 and k_4: min^{-1}; V_T: ml/cm^3; BP_{ND}: unitless.

	(R)-^{11}C-PK11195																				
HSE	K_1			%SE	k_2			%SE	k_3			%SE	k_4			%SE	V_T 2TCM			%SE	BP_{ND}			%SE	R^2
Amygdala	1.08	±	0.40	7.13	0.66	±	0.38	16.86	0.24	±	0.20	26.27	0.12	±	0.08	16.75	5.18	±	1.79	4.23	1.84	±	0.54	17.22	0.93
Cerebellum	1.26	±	0.42	6.45	0.57	±	0.30	15.36	0.20	±	0.14	22.36	0.10	±	0.06	15.29	7.63	±	2.84	4.58	2.09	±	0.57	15.05	0.93
CortexFrontal	1.03	±	0.36	7.08	0.51	±	0.26	17.53	0.19	±	0.15	26.65	0.10	±	0.07	19.81	6.75	±	2.60	6.40	2.01	±	0.59	18.97	0.92
Hippocampus	1.21	±	0.52	8.27	0.79	±	0.57	19.77	0.31	±	0.32	29.58	0.15	±	0.12	18.55	5.06	±	1.91	4.64	1.83	±	0.63	20.78	0.94
Hypothalamus	1.34	±	0.56	8.01	0.71	±	0.46	18.68	0.25	±	0.24	32.57	0.16	±	0.11	20.85	5.12	±	1.63	4.42	1.42	±	0.50	21.22	0.95
Medulla	1.46	±	0.38	7.97	0.68	±	0.29	20.03	0.29	±	0.18	24.46	0.12	±	0.07	12.83	7.64	±	1.46	3.34	2.30	±	0.25	18.74	0.95
Midbrain	1.77	±	0.70	9.57	1.27	±	0.89	19.35	0.46	±	0.38	25.10	0.18	±	0.12	15.78	5.50	±	1.74	4.01	2.20	±	0.81	17.69	0.96
Pons	1.47	±	0.41	8.95	0.75	±	0.42	22.26	0.31	±	0.24	29.37	0.14	±	0.08	16.04	6.75	±	1.52	3.86	2.05	±	0.66	21.67	0.95
Septum	1.10	±	0.50	8.93	0.60	±	0.37	21.88	0.20	±	0.17	39.71	0.13	±	0.09	26.32	5.07	±	2.07	6.09	1.49	±	0.57	26.71	0.93
Striatum	1.07	±	0.41	7.57	0.62	±	0.33	19.03	0.24	±	0.22	32.48	0.17	±	0.12	19.51	4.24	±	1.23	3.91	1.30	±	0.53	22.45	0.95
Thalamus	1.49	±	0.61	9.01	0.93	±	0.47	22.29	0.38	±	0.27	30.23	0.21	±	0.14	14.84	4.70	±	1.25	3.14	1.68	±	0.43	22.64	0.97
Average	1.30	±	0.48	8.09	0.73	±	0.43	19.37	0.28	±	0.23	28.98	0.14	±	0.10	17.87	5.79	±	1.82	4.42	1.84	±	0.55	20.29	0.94

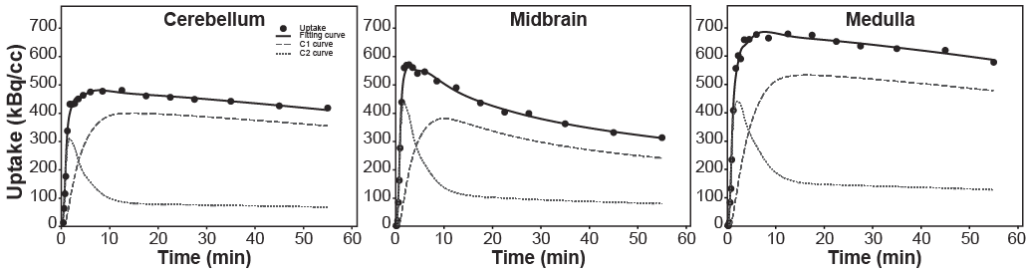
Units: K_1: ml/cm^3/min; k_2, k_3 and k_4: min^{-1}; V_T: ml/cm^3; BP_{ND}: unitless.



Supplemental figure 2: Comparison between Logan (A) and Patlak (B) graphical analysis in the medulla region for ^{11}C-PBR28, with t*=15 min.



Supplemental figure 3: Metabolite-corrected plasma input curves for ^{11}C-PBR28 (left) and *(R)*-^{11}C-PK11195 (right), in control and HSE rats.



Supplemental figure 4: Fit of the 2TCM of ^{11}C-PBR28 in the cerebellum, midbrain and medulla in the HSE rat.

Glial, metabolic and behavioral response to recurrent psychosocial stress: PET imaging in stress-sensitized and stress-naïve aged rats

Author(s): Paula Kopschina Feltes, Janine Doorduin, Luis Eduardo Juárez-Orozco LE, David Vállez García, Rudi AJO Dierckx, Cristina M. Moriguchi-Jeckel, Erik FJ de Vries.

CHAPTER 5

In preparation for submission.

Abstract

Background: Early life adversities increases the vulnerability to psychiatric conditions later in life. However, it is still unknown how stress exposure influences the neurobiological response to a secondary stressful event at an older age. The present study aimed to evaluate glial, brain-metabolic and behavioral response to repeated social defeat (RSD) in stress-sensitized (SS, previously exposed to 5-day RSD protocol during adolescence) and stress-naïve (SN) aged rats through positron emission tomography (PET).

Methods: Fourteen-month old SN (n=8) and SS (n=10) Wistar rats underwent a 5-day RSD protocol, repeated PET imaging with ^{11}C-PBR28 (glial activation) and ^{18}F-FDG (brain metabolism), and behavioral and biochemical assessments.

Results: RSD at old age induced anhedonic-like behavior in SS rats only, while anxiety was present in both groups. RSD in aged SN rats increased corticosterone levels, whereas recurrence of RSD blunted the corticosterone response in SS rats. RSD increased ^{11}C-PBR28 uptake levels in SN rats, whereas re-exposure to RSD diminished tracer uptake in the brain of SS rats. Higher brain levels of the cytokines IL-1β and IL-10 were found in SN rats after RSD, as compared to SS rats. RSD caused hypometabolism in the brains of both groups.

Conclusion: Recurrence of RSD in aged SS rats induced depressive- and anxiety-like behavior, despite diminished corticosterone and brain inflammatory responses, as compared to SN rats. In contrast to SN rats, the immune response in SS rats was not correlated with corticosterone levels, pointing towards an alternative pathway for coping with detrimental stressful stimuli or exhaustion of the brain immune cells in sensitized animals.

Keywords: chronic stress, neuroinflammation, brain metabolism, PET imaging, repeated social defeat.

Introduction

Psychosocial stress is a predominant environmental risk factor for several psychiatric disorders, including major depressive disorder (MDD) (1). It is estimated that 20-25% of individuals exposed to highly stressful events develop MDD (2). Interestingly, trauma exposure at a young age increases the likelihood of fulfilling the criteria for MDD at any point in life (3). It has been hypothesized that such exposures may modify the individual's immune, endocrine, neural and behavioral responsiveness to recurrent stressful conditions at later ages (4).

Stimuli such as chronic stress can activate microglia and astrocytes, the brain's immune cells, which can subsequently release pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (5). Pro-inflammatory cytokines produced during glial activation might influence central levels of neurotransmitters (6). In addition, stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis can cause an increase in glucocorticoid levels. These glucocorticoids can cross the blood-brain barrier (6) and act on receptors located in vulnerable brain regions. The brain is highly sensitive to stress and altered glucocorticoid levels during crucial periods of development (such as adolescence), mainly in brain areas as the medial prefrontal cortex (MPFC), cingulate cortex and orbitofrontal cortex (OBFC) (7). Interestingly, the prefrontal cortex (PFC) shows higher levels of glucocorticoid receptor mRNA in adolescence than during any other period of development (8), suggesting that the PFC may be especially sensitive to glucocorticoid regulation during this period. The aforementioned stress-sensitive brain regions are amply associated with reward, emotional regulation, and fear extinction and therefore appear to be of relevance for stress recovery (7). Negative neurobiological changes during developmental periods might have long-lasting detrimental effects and increase vulnerability to depression (9). As a potential reflection of these detrimental effects, stress has been found to decrease brain metabolism in several brain regions in animal models of stress (10). Notably, a similar decrease in brain metabolism is also found in patients with unipolar depressive disorder (11–14). Hence, immune activation and cytokine release in the central nervous system and abnormalities in the HPA axis have been suggested as key factors in the development and recurrence of depression (15). Although these mechanisms may not apply to all patients, they may be of particular interest for the subgroup of treatment-resistant MDD patients (6).

The repeated social defeat (RSD) rat model has been widely used to mimic psychosocial stress in rodents, due to its high ethological validity (16; 17). RSD is able to provoke immune dysregulation, coupled with depressive- and anxiety-like behavior that resembles the MDD phenotype. Previous studies in adolescent rats demonstrated glial (mainly microglia) activation in response to RSD, in conjunction with an elevation of pro-inflammatory cytokine levels (18–20). These alterations were transient and resolved within a month (21; 22). However, such a stressful event may have primed pro-inflammatory microglial responses to a subsequent stress exposure (23). Consequently, transient effects of psychosocial stress early in life may translate into long-term (persistent) immunological, behavioral and brain metabolic disturbances, as the ones observed in (treatment-resistant) MDD patients. However, there is still no evidence available that supports such conjecture. Furthermore, it is also unknown whether an early exposure to psychosocial stress would influence the response to a secondary stressful event later in life.

Therefore, the aim of the present study was to evaluate the glial, brain-metabolic and behavioral response to repeated social defeat (RSD) in 1) stress-sensitized (SS) (i.e. with a history of previous exposure to psychosocial stress during adolescence) and 2) stress-naive (SN) aged rats through repeated neuroimaging. Positron emission tomography (PET) offers the opportunity to longitudinally image the (patho)physiological processes that are seemingly altered in MDD patients and animal models. PET has been successfully used to evaluate glial activation (24) with the translocator protein receptor (TSPO) tracer ^{11}C-PBR28 (25), and to assess brain glucose metabolism (11) with the glucose analogue 2'-[^{18}F]fluoro-2'-deoxyglucose (^{18}F-FDG).

Materials and Methods

Experimental Animals

Male outbred Wistar Unilever rats of fourteen months were used for the present study (n=18, 577±11g). They were purchased at the age of seven weeks from Harlan Laboratories (Horst, The Netherlands) and were allowed to age under monitored conditions during twelve months. Rats were kept in humidity-controlled, thermo-regulated (21±2°C) rooms under a 12:12 hour light:dark cycle with lights on at 7 a.m. Rats had *ad libitum* access to food and water. During the RDS protocol, rats were housed individually and divided into two groups: stress-naïve (SN, n=8) and stress-sensitized (SS, n=10) rats.

Animal experiments were performed in accordance with Dutch Regulations for Animal Welfare. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen (protocol DEC 6828A and 6828B).

Study design

The overall design of the study is depicted in Fig. 1. SS rats were subjected to five consecutive days of RSD when they were eight weeks old (22). During the RSD protocol, both groups were handled similarly. However, SN rats were only placed in another cage, but not exposed to an aggressive resident rat. After the RSD protocol at adolescence, rats were allowed to age for 12 months and housed in pairs to prevent isolation stress (26; 27). At the age of 14 months, rats from both groups (SN and SS) underwent a 5-day RSD protocol (day 0–4), PET scans with ^{11}C-PBR28 and ^{18}F-FDG (days -1, 6, 11 and 25), behavioral tests and biochemical assessments. Body weight was measured daily between day 0 and 25. On day 25, the rats were terminated and the brains were collected for the quantification of pro- and anti-inflammatory cytokines.

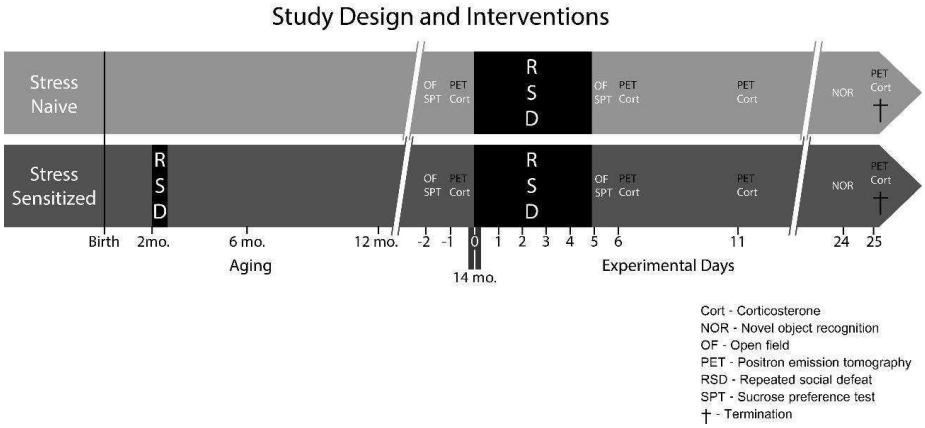


Figure 1: Stress-naïve (SN) and stress-sensitized (SS) rats were subjected to a 5-day RSD protocol (day 0–4) at the age of 14 months. PET scans with ^{11}C-PBR28 and ^{18}F-FDG were conducted on experimental days -1, 6, 11 and 25. In order to evaluate post-RSD behavioral alterations, the sucrose preference test (SPT) and open field (OF) were conducted on days -2 and 5. Corticosterone levels were assessed on days -1, 6, 11 and 25. On day 25, after the PET scans, rats were terminated and the brains were collected for the quantification of pro- and anti-inflammatory cytokines.

Repeated Social Defeat

RSD was carried out through the introduction of SN and SS rats (intruder) into the cage of a dominant (resident) male outbred Long Evans rat (537±28g; Harlan, Indianopolis,

USA). All the intruders were exposed to the same residents (using a different resident for each social defeat conflict to avoid habituation). The male Long Evans rats were housed in a separate experimental room in large cages (80x50x40 cm) with a tubal ligated Long Evans female rat to stimulate territorial aggression (16). The Long Evans residents were screened for aggressive behavior at least three times prior to the experiment (28; 29). Only residents with an attack latency shorter than 30 s were used for the actual social defeat experiment (30).

The social defeat experiment was conducted as previously described (16), and it always took place between 16:00–18:00 p.m. Briefly, the females were removed from the cage of the resident before the introduction of the experimental rat (intruder). The total duration of the interaction between the resident and the intruder was of 60 min, but the physical interaction was limited to a maximum period of 10 min, or shorter, if the intruder assumed a supine (submissive) position for at least 3 seconds. Hereafter, the intruder was placed in a wire mesh cage inside the cage of the resident to avoid further physical contact, but still allowing intense visual, auditory and olfactory interactions for the remainder of the 60-min stress period. The social defeat protocol was repeated on 5 consecutive days using different residents.

Body weight gain

Body weight gain was calculated individually for each rat as the difference between the body weight at a certain time point minus the body weight on experimental day 0 (first day of RSD).

Behavioral Tests

To assess RDS-induced behavioral changes, the open field (anxiety, locomotion) (31), novel object recognition (visual memory) (32) and sucrose preference test (anhedonia) (29) were performed. The open field and novel object recognition tests were recorded on video for further analysis using Ethovision XT8.5 software (Noldus Information Technology, Wageningen, The Netherlands).

Open field (OF)

Rats were placed inside a square box (100x100x40 cm) for 10 min. The time spent in the center of the arena relative to the time spent at the borders (anxiety), and the total distance moved (locomotion) were documented.

Novel object recognition (NOR)

On day 24, rats were placed in a square box (50x50x40 cm) with two identical objects (plastic bottles or Lego cubes) (33; 34). They were allowed to explore the objects for 3 min. The objects were removed and after 2 h one familiar and one new object were presented to the rat for 3 min. The preference index (PI) was calculated as the ratio between time spent on exploring the new object and the total time spent on object exploration.

Sucrose Preference (SPT)

Rats were habituated to a 1% sucrose solution for 1h during 4 days prior to the experiment. At baseline and after 5 days of RSD, rats were exposed to two bottles placed randomly in the cage, one containing water and one with 1% sucrose. Preference for sucrose was calculated as the total intake of sucrose solution divided by the total liquid intake and multiplied by 100% (29; 35).

Corticosterone Levels

For determination of corticosterone levels, rats were anesthetized with isoflurane mixed with oxygen and 0.5 mL of whole blood was quickly collected from the tail vein on day -1, 6, 11 and 25. Samples were always collected at 10 a.m. to avoid circadian fluctuations. The blood was allowed to clot for 15 min and centrifuged at 6.000 rpm (3.5g) for 8 min at room temperature to obtain serum samples. Samples were stored at -20°C until further analysis by radioimmunoassay. Corticosterone (Sigma Chemical Co., Missouri, USA.) was used as standard and ^{3}H-corticosterone as tracer (Perkin & Elmer, Massachusetts, USA).

PET

PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). ^{11}C-PBR28 PET scans were always carried out in the morning. The rats were anesthetized with isoflurane mixed with oxygen (5% for induction, 2% for maintenance) and ^{11}C-PBR28 was injected via the penile vein (73±34 MBq, 1.25±1.91 nmol). Immediately after injection, rats were allowed to wake up and recover in their home cage. Rats were anesthetized 45 min after tracer injection and placed in prone position into the camera with the head in the field of view. A 30-min static scan was

acquired. The body temperature was maintained at 37°C with heating pads, heart rate and blood oxygen saturation were monitored, and eye salve was applied to prevent conjunctival dehydration. After completion of the emission scan, a transmission scan was obtained using a ^{57}Co point source enabling attenuation and scatter correction of PET images.

After at least 10 half-lives of the radioisotope ^{11}C, a ^{18}F-FDG PET scan was acquired. Between scans, rats were deprived of food for 4-6 h. Rats were injected intraperitoneally (36; 37) with ^{18}F-FDG (27±5 MBq) and returned to their home cage. After 45 min, ^{18}F-FDG PET acquisition was performed as described above for ^{11}C-PBR28 PET.

The reconstruction of the scans was performed iteratively (OSEM2D, 4 iterations and 16 subsets) into a single frame after being normalized and corrected for attenuation and decay of radioactivity. Images with a 128x128x95 matrix, a pixel width of 0.632 mm, and a slice thickness of 0.762 mm were obtained. PET images were automatically co-registered to a functional ^{11}C-PBR28 or ^{18}F-FDG rat brain template (25; 38), using PMOD 3.6 software (PMOD technologies Ltd., Switzerland). Aligned images were resliced into cubic voxels (0.2 mm) and converted into standardized uptake value (SUV) images: SUV = [tissue activity concentration (MBq/g) x body weight (g)] / [injected dose (MBq)], assuming a tissue density of 1g/mL. ^{18}F-FDG uptake was not corrected for blood glucose levels (39).

Tracer uptake was calculated in several predefined volumes-of-interest (VOI). VOIs were selected based on previous findings (11; 18; 24; 40–45), taking the size of the brain regions into consideration. Small brain regions were excluded to minimize partial volume effects (47). The investigated regions were the amygdala/piriform complex, brainstem, cerebellum, cingulate cortex, entorhinal cortex, frontal association cortex (FCA), hippocampus, hypothalamus, insular cortex, MPFC, motor/somatosensory cortex, OBFC, and striatum.

Enzyme linked immunoassay (ELISA) for pro-inflammatory cytokines in the brain

On day 25, rats were terminated under deep anesthesia by transcardial perfusion with phosphate-buffered saline pH 7.4. Brains were collected and rapidly frozen and stored at -80°C. Frontal cortex, hippocampus, cerebellum and parietal/temporal/occipital cortex were dissected and prepared as published (34). IL-6, IL-1β, and IL-10 (Thermo Scientific, Rockford, USA) concentrations were determined in these brain regions by ELISA

according to the manufacturer's instructions and the cytokine levels were corrected for the amount of proteins, as determined through a Bradford Assay.

Statistical Analysis

Statistical analyses were performed with IBM SPSS 23 software (IBM Corp, New York, USA). Continuous data are expressed as mean ± standard error of the mean (SEM). The Generalized Estimating Equations (GEE) model (48) was used for statistical analysis of body weight measurements, behavioral tests (OF and SPT), corticosterone levels, and PET data, in order to account for repeated measurements in the longitudinal design and missing data. The parameters "group", "day of measurement" and the interaction "group × day of measurement" were included as independent variables for the statistical analysis of body weight gain, corticosterone levels, and the behavioral tests. For the statistical analysis of ^{11}C-PBR28 and ^{18}F-FDG uptake (SUV), the GEE model was applied independently for each brain region, using the variables "group", "day of scan" and the interaction "group × day of scan" in the model. The data was further explored through pairwise comparison of "group × day of scan" in each brain region for all scan time points combined. The AR(1) working correlation matrix was selected according to the quasi-likelihood under the independence model information criterion value. Wald's statistics and associated *p*-values were considered statistically significant if $p<0.05$, after the sequentially rejective Bonferroni-Holm correction for multiple comparisons was applied to ensure that the Type I error resulting from multiple tests never exceeded the *p* set level of statistical significance at $\alpha= 0.05$ (49; 50). Spearman correlations were performed to investigate the relationship between corticosterone levels, cytokines, behavior and tracer uptake levels between groups at different timepoints. Between-group differences in the PI (NOR) and brain cytokines levels were assessed through the Mann-Whitney U test and the results were reported as the median and the 0.25-0.75 interquartile range (IQR).

Results

RSD significantly decreases body weight gain

No statistically significant difference in body weight between groups was found before the start of RSD (SN: 571±19 vs. SS: 581±12, $p=0.66$). The GEE analysis revealed a significant main effect for the factor "day of measurement" ($p<0.001$) and the interaction "day of measurement x group" ($p<0.001$), but not for "group". Pairwise analysis of the

data revealed between-group differences on experimental days 1 to 3 only, indicating that RSD affected body weight significantly more in SS rats than in SN rats (day 3, SN: -3.6±1.4g, vs. SS: -8.1±0.8g, p=0.007). From experimental day 4 onwards, no significant difference in body weight gain between groups was observed anymore (Fig. 2).

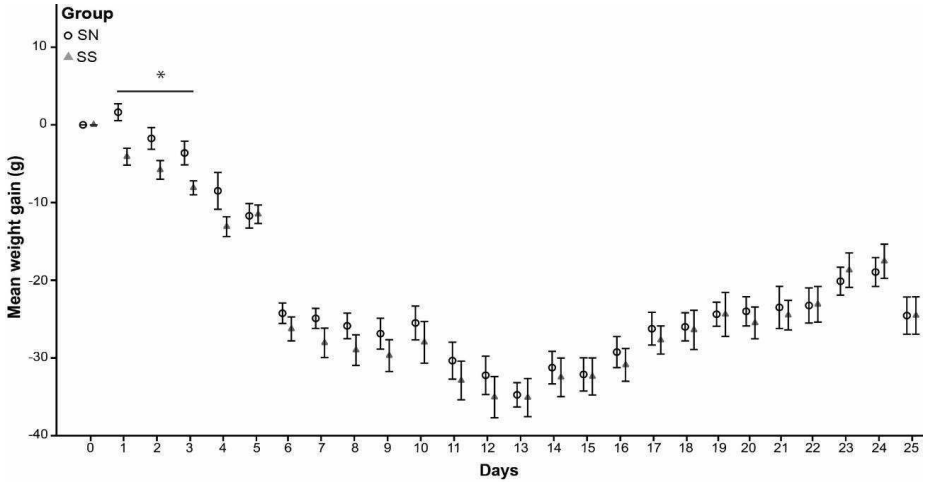


Figure 2: Body weight gain (g) of stress-naïve (SN) and stress-sensitized (SS) rats during the 25 days of the study protocol. Significant differences between the groups were apparent from day 1 to 3, with SS rats gaining less weight than SN rats (p<0.05). From day 4 onwards, both groups gained weight at the same rate, without returning to baseline levels until the end of the study. The dips in body weight gain on day 6, 11 and 25 are due to the PET procedures on these days. *p<0.05.

RSD induces anxiety-like behavior and decreases locomotor activity both in SN and SS rats, whereas RSD-induced anhedonia is only observed in SS rats

No significant differences in sucrose preference between groups were found at baseline. RSD provoked anhedonia in SS rats, as it reduced the sucrose preference from 93±2% on day -2 to 69±7% on day 5 (p=0.003). In contrast, no significant change in sucrose preference was observed after RSD in SN rats (90±4% to 84±5%, p=0.25). A trend towards significance was observed in the between-groups comparison on day 5 (p=0.060) (Fig. 3-A).

The anxiety-like behavior and locomotor activity were assessed with the OF test at baseline and after the RSD protocol (day 5). At baseline, SN rats moved a total distance of 2.2±0.5 m and spent 64±20 s in the center of the arena, whereas after RSD the total distance moved was reduced to 1.3±0.3 m (p<0.001) and the time spent in the center to 19±11s (p=0.016). A similar pattern was observed in SS rats, with a distance moved of

2.4±0.2 m and a time spent in the center of 43±9 s at baseline. On day 5, these measures were significantly reduced to 1.4±0.1 m and 9±2 s (p<0.001), respectively. No significant differences in locomotion or the time spent in the center were observed between both groups, neither at baseline, nor on day 5 (Fig. 3-B, C).

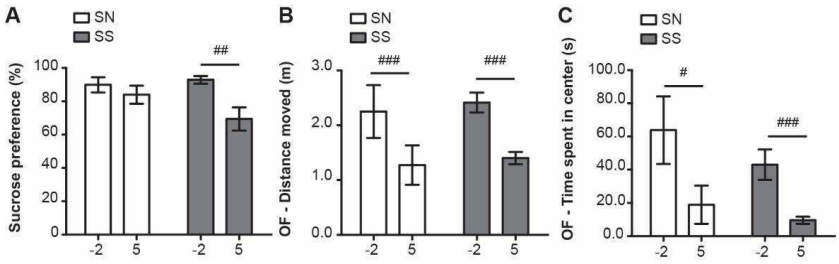


Figure 3: RSD-induced behavioral alterations in both SN and SS rats. **A:** Within-group comparison between baseline and day 5 showed anhedonic-like behavior through the sucrose preference test (SPT) in SS rats (p<0.01), but not in SN rats. $^{\#\#}p$<0.01. **B:** Anxiety-like behavior was demonstrated in both SN and SS rats in the open field test (OF), with a decreased distance moved on day 5 as compared to baseline (p<0.001) and **C:** decreased total time spent in the center of the arena. SS rats had a more pronounced decrease in total time spent in the center p<0.001 on day 5 when compared to baseline, than SN rats (p<0.05). $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001.

To evaluate effects on long-lasting memory impairment, the NOR test was performed on day 24. No significant differences in the PI were found between groups (SN: 48%, IQR 43-61 *vs.* SS: 56%, IQR 46-70, p=0.33).

RSD provokes a generalized decrease in glucose metabolism

No significant differences in brain glucose metabolism between SN and SS rats were found at baseline. On day 11, however, ^{18}F-FDG PET revealed several brain regions with lower glucose metabolism in SS rats than in SN rats. The affected brain regions were the amygdala (-58%, p<0.001), brainstem (-62%, p<0.001), cerebellum (-39%, p=0.002), entorhinal cortex (-69%, p<0.001), hippocampus (-35%, p=0.012), hypothalamus (-44%, p=0.001), insular cortex (-51%, p<0.001), OBFC (-36%, p=0.008) and striatum (-48%, p<0.001). On day 6 and 25, no significant differences in ^{18}F-FDG uptake between SS and SN rats were observed in any brain region anymore.

A within-group analysis was conducted to explore the effect of RSD on glucose metabolism over time, relative to baseline levels (Fig.4). SN rats did not show any significant effect of RSD on day 6 and 11, but had a general decrease in tracer uptake in the brain on day 25. The affected brain regions in SN rats were the amygdala (-13%, p=0.004), brainstem (-11% p=0.048), cerebellum (-11%, p=0.026), entorhinal cortex (-

16%, p=0.001), FCA (-12%, p=0.021), hippocampus (-12%, p=0.017), hypothalamus (-12%, p=0.02), insular cortex (-13%, p=0.014), motor / somatosensory cortex (-13%, p=0.009), and striatum (-10%, p=0.036). On the other hand, SS rats did not show any significant changes in ^{18}F-FDG uptake on day 6 and 25, but presented a large generalized decrease in glucose metabolism only on day 11. The brain regions of SS rats with decreased uptake on day 11 compared to baseline were the amygdala (-60%, p<0.001), brainstem (-64%, p<0.001), cerebellum (-38%, p<0.001), entorhinal cortex (-70%, p<0.001), hippocampus (-37%, p<0.001), hypothalamus (-48%, p<0.001), insular cortex (-53%, p<0.001), MPFC (-22%, p<0.001), OBFC (-36%, p<0.001) and striatum (-52%, p<0.001) (Table 1).

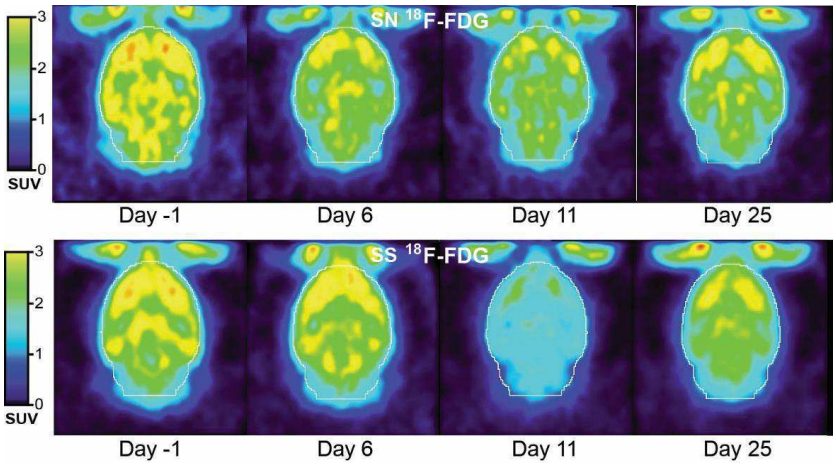


Figure 4: Representative ^{18}F-FDG PET images from SN and SS rats on day -1, 6, 11 and 25.

When investigating the correlation between regional ^{18}F-FDG uptake and behavioral alterations, no significant correlations were found at all.

Table 1 – ^{18}F-FDG SUV between-group comparison for individual brain regions of stress-naïve (SN, n=8) and stress-sensitized (SS, n=10) rats at baseline, day 6, 11 and 25.

	Baseline			Day 6			Day 11			Day 25		
	SN	SS		SN	SS		SN	SS		SN	SS	
Brain Regions	Mean ± SE	Mean ± SE	*p*	Mean ± SE	Mean ± SE	*p*	Mean ± SE	Mean ± SE	*p*	Mean ± SE	Mean ± SE	*P*
Amygdala/Piriform complex	2.35 ± 0.14	2.42 ± 0.13	n.s	2.08 ± 0.1	2.24 ± 0.12	n.s	2.28 ± 0.28	0.96 ± 0.10	<0.001	2.04 ± 0.08	2.29 ± 0.16	n.s
Brainstem	2.40 ± 0.14	2.45 ± 0.14	n.s	2.16 ± 0.12	2.37 ± 0.12	n.s	2.38 ± 0.30	0.87 ± 0.07	<0.001	2.13 ± 0.06	2.42 ± 0.17	n.s
Cerebellum	2.40 ± 0.15	2.37 ± 0.12	n.s	2.10 ± 0.09	2.25 ± 0.10	n.s	2.40 ± 0.27	1.47 ± 0.15	0.002	2.11 ± 0.07	2.28 ± 0.14	n.s
Cingulate cortex	3.09 ± 0.22	3.05 ± 0.22	n.s	2.68 ± 0.12	2.80 ± 0.18	n.s	3.07 ± 0.40	2.97 ± 0.34	n.s	2.77 ± 0.11	2.93 ± 0.23	n.s
Entorhinal cortex	2.41 ± 0.14	2.44 ± 0.14	n.s	2.08 ± 0.11	2.23 ± 0.14	n.s	2.33 ± 0.32	0.73 ± 0.07	<0.001	2.03 ± 0.08	2.28 ± 0.16	n.s
Frontal association cortex	3.27 ± 0.22	3.08 ± 0.18	n.s	2.88 ± 0.15	2.87 ± 0.15	n.s	3.20 ± 0.37	2.89 ± 0.33	n.s	2.87 ± 0.13	2.92 ± 0.19	n.s
Hippocampus	2.68 ± 0.16	2.68 ± 0.15	n.s	2.37 ± 0.11	2.53 ± 0.13	n.s	2.61 ± 0.32	1.69 ± 0.18	0.012	2.35 ± 0.09	2.62 ± 0.20	n.s
Hypothalamus	2.32 ± 0.14	2.36 ± 2.2	n.s	2.07 ± 0.12	2.20 ± 0.11	n.s	2.20 ± 0.27	1.23 ± 0.11	0.001	2.03 ± 0.07	2.25 ± 0.15	n.s
Insular cortex	3.07 ± 0.19	3.08 ± 0.17	n.s	2.64 ± 0.11	2.84 ± 0.16	n.s	2.98 ± 0.34	1.45 ± 0.16	<0.001	2.66 ± 0.11	2.95 ± 0.21	n.s
Medial Prefrontal cortex	3.18 ± 0.21	3.20 ± 0.21	n.s	2.84 ± 0.11	2.95 ± 0.17	n.s	3.11 ± 0.42	2.51 ± 0.29	n.s	2.88 ± 0.12	3.07 ± 0.25	n.s
Motor/Somatosensory cortex	2.94 ± 0.19	2.74 ± 0.17	n.s	2.51 ± 0.11	2.51 ± 0.14	n.s	2.90 ± 0.34	2.53 ± 0.30	n.s	2.56 ± 0.10	2.61 ± 0.16	n.s
Orbitofrontal cortex	3.51 ± 0.21	3.40 ± 0.21	n.s	3.11 ± 0.12	3.15 ± 0.17	n.s	3.41 ± 0.40	2.18 ± 0.25	0.008	3.19 ± 0.15	3.29 ± 0.24	n.s
Striatum	2.78 ± 0.17	2.96 ± 0.17	n.s	2.49 ± 0.1	2.76 ± 0.14	n.s	2.72 ± 0.34	1.41 ± 0.15	<0.001	2.49 ± 0.09	2.90 ± 0.23	n.s

RSD induces a different glial activation pattern in SS rats than in SN rats

A between-group comparison of the ^{11}C-PBR28 PET data revealed significant differences in tracer uptake between groups at baseline (day -1) in several brain regions. Increased ^{11}C-PBR28 uptake was found in the cerebellum (+30%, p<0.001), cingulate cortex (+30%, p=0.003), FCA (+30%, p=0.003), MPFC (+46%, p<0.001) and OBFC (+44%, p<0.001) of SS rats, as compared to SN rats. ^{11}C-PBR28 uptake at baseline was significantly lower in the entorhinal cortex (-14%, p=0.001) and hypothalamus (-26%, p<0.001) of SS rats than in SN rats. Smaller differences were found on day 6 and 25. On day 6, SS rats had a significantly increased uptake only in the cerebellum (+27%, p=0.023), whereas on day 25 SS rats even had significantly lower tracer uptake than SN rats in the cingulate cortex (-23%, p=0.024), hypothalamus (-25%, p=0.045) and motor / somatosensory cortex (-25%, p=0.012).

A within-group comparison (Table 2) demonstrated that SN rats presented an increase in ^{11}C-PBR28 uptake over time, which became significant 7 days after RSD (day 11) and persistent until the end of the experiment (day 25) in the cingulate cortex (+22%, p=0.022; and +45%, p<0.001, respectively), MPFC (+26, p<0.001; and +44%, p<0.001) and OBFC (+18%, p<0.001; and 40%, p=0.001), when compared to baseline. Three weeks after RSD (day 25), tracer uptake was also significantly increased in the FCA (25%, p=0.012). Conversely, a significant decrease in ^{11}C-PBR28 uptake was observed on day 6 in the brainstem (-25%, p=0.006), hippocampus (-29%, p<0.001), hypothalamus (-40%, p<0.001), insular cortex (-18%, p=0.008), motor/somatosensory cortex (-14%, p=0.039) and striatum (-27%, p<0.001), followed by normalization to baseline levels up to day 25. The entorhinal cortex also presented a decrease in uptake on day 6, but without recovery until the end of the experiment (-33%, p<0.001).

Taken together, these results suggest a migration of activated glia to RSD affected regions, followed by recovery in almost all glial depleted regions.

Table 2 – ^{11}C-PBR28 SUV within-group comparison for individual brain regions of stress-naïve (SN, n=8) and stress-sensitized (SS, n=10) rats at baseline, day 6, 11 and 25.

	SN						SS					
	Day -1	Day 6		Day 11		Day 25		Day -1	Day 6		Day 11		Day 25	
Brain Regions	**Mean ± SE**	**Mean ± SE**	***p***	**Mean ± SE**	***p***	**Mean ± SE**	***P***	**Mean ± SE**	**Mean ± SE**	***P***	**Mean ± SE**	***p***	**Mean ± SE**	***p***
Amygdala/Piriform complex	0.53 ± 0.06	0.40 ± 0.03	n.s.	0.46 ± 0.01	n.s.	0.52 ± 0.06	n.s.	0.50 ± 0.02	0.44 ± 0.03	n.s.	0.44 ± 0.02	n.s.	0.46 ± 0.02	n.s.
Brainstem	0.67 ± 0.03	0.50 ± 0.05	0.006	0.59 ± 0.02	0.004	0.63 ± 0.07	n.s.	0.67 ± 0.02	0.53 ± 0.03	0.001	0.59 ± 0.03	0.010	0.62 ± 0.04	n.s.
Cerebellum	0.69 ± 0.02	0.63 ± 0.05	n.s.	0.76 ± 0.03	n.s.	0.78 ± 0.06	n.s.	0.90 ± 0.03	0.80 ± 0.05	0.039	0.83 ± 0.02	0.021	0.83 ± 0.03	n.s.
Cingulate cortex	0.63 ± 0.01	0.66 ± 0.04	n.s.	0.77 ± 0.04	0.022	0.92 ± 0.06	<0.001	0.82 ± 0.06	0.75 ± 0.05	n.s.	0.73 ± 0.03	n.s.	0.73 ± 0.05	n.s.
Entorhinal cortex	0.58 ± 0.02	0.39 ± 0.03	<0.001	0.45 ± 0.01	<0.001	0.48 ± 0.04	0.009	0.50 ± 0.01	0.42 ± 0.03	0.02	0.46 ± 0.02	0.008	0.46 ± 0.02	n.s.
Frontal association cortex	0.91 ± 0.06	0.97 ± 0.08	n.s.	1.04 ± 0.04	n.s.	1.14 ± 0.07	0.012	1.18 ± 0.06	1.04 ± 0.08	0.01	1.04 ± 0.04	0.006	1.08 ± 0.05	n.s.
Hippocampus	0.56 ± 0.02	0.40 ± 0.03	<0.001	0.47 ± 0.01	0.002	0.53 ± 0.06	n.s.	0.56 ± 0.02	0.46 ± 0.02	<0.001	0.51 ± 0.02	0.033	0.52 ± 0.02	0.042
Hypothalamus	0.68 ± 0.03	0.41 ± 0.03	<0.001	0.48 ± 0.02	<0.001	0.58 ± 0.06	n.s.	0.50 ± 0.02	0.43 ± 0.03	0.018	0.46 ± 0.02	n.s.	0.44 ± 0.02	0.018
Insular cortex	0.50 ± 0.02	0.41 ± 0.03	0.008	0.42 ± 0.01	<0.001	0.51 ± 0.05	n.s.	0.54 ± 0.02	0.45 ± 0.02	0.003	0.47 ± 0.02	<0.001	0.48 ± 0.02	<0.001
Medial Prefrontal cortex	0.61 ± 0.03	0.66 ± 0.04	n.s.	0.77 ± 0.02	<0.001	0.88 ± 0.07	<0.001	0.89 ± 0.05	0.76 ± 0.04	0.003	0.78 ± 0.03	0.008	0.79 ± 0.03	<0.001
Motor/Somatosensory cortex	0.66 ± 0.03	0.57 ± 0.03	0.039	0.65 ± 0.03	n.s.	0.74 ± 0.04	n.s.	0.72 ± 0.05	0.65 ± 0.05	n.s.	0.64 ± 0.03	n.s.	0.60 ± 0.04	0.039
Orbitofrontal cortex	0.64 ± 0.02	0.69 ± 0.04	n.s.	0.76 ± 0.02	<0.001	0.90 ± 0.07	0.001	0.92 ± 0.03	0.76 ± 0.04	<0.001	0.80 ± 0.03	<0.001	0.82 ± 0.03	<0.001
Striatum	0.49 ± 0.02	0.36 ± 0.02	<0.001	0.42 ± 0.01	<0.001	0.47 ± 0.04	n.s.	0.50 ± 0.02	0.41 ± 0.02	<0.001	0.45 ± 0.02	0.006	0.44 ± 0.02	<0.001

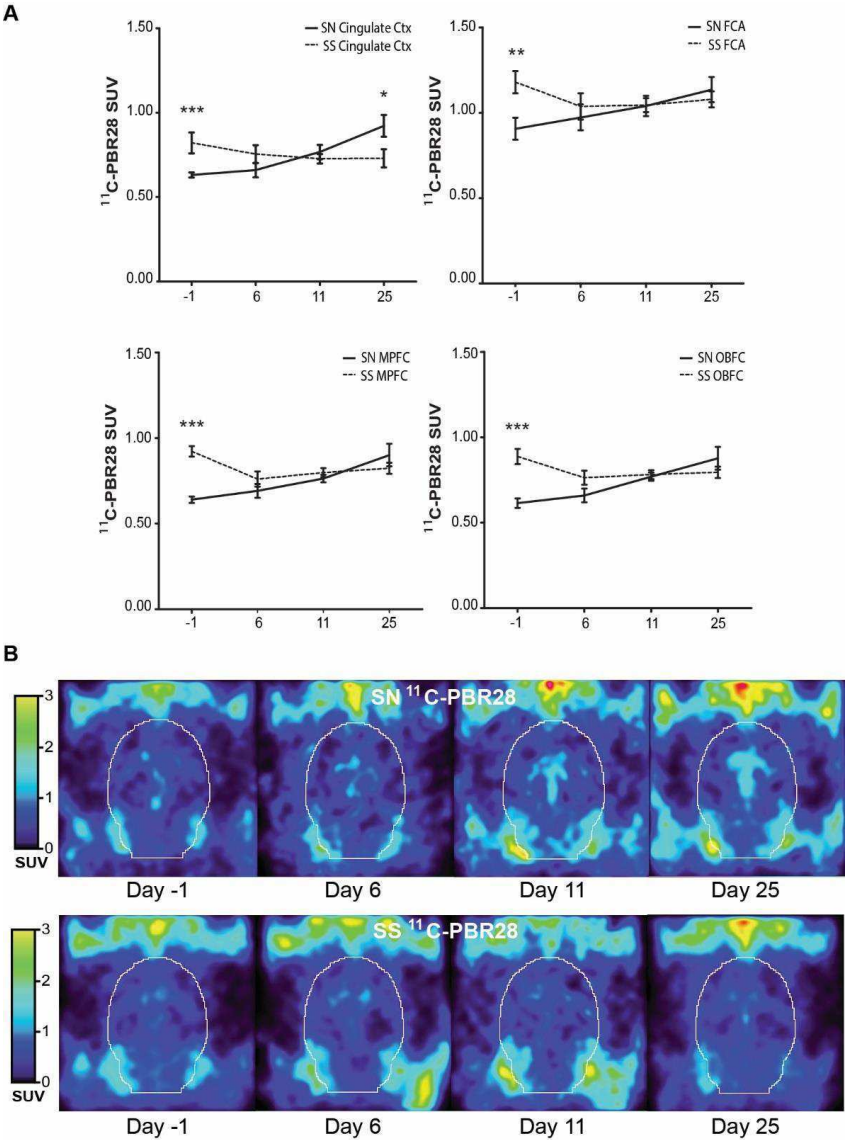


Figure 5: A - Graphical representation of the opposite behavior between SN and SS rats regarding ^{11}C-PBR28 uptake in the cingulate cortex, frontal cortex association (FCA), medial prefrontal cortex (MPFC) and orbitofrontal cortex (OBFC) on day -1, 6, 11 and 25. *p<0.05, **p<0.01 and ***p<0.001. **B:** Representative ^{11}C-PBR28 PET images from SN and SS rats on day -1, 6, 11 and 25.

Conversely, within-group comparisons in SS rats only showed diminished ^{11}C-PBR28 tracer uptake in multiple brain regions. Immediately after RSD (day 6), a significant decrease was found in the brainstem (-21%, p=0.001), cerebellum (-11%, p=0.039), entorhinal cortex (-16%, p=0.02), FCA (-12%, p=0.01), hippocampus (-18%, p<0.001), hypothalamus (-14%, p=0.018), insular cortex (-17%, p=0.003), MPFC (-15%,

p=0.003), OBFC (-17%, *p*<0.001) and striatum (-18%, *p*<0.001). On day 11, a similar decreased uptake pattern as on day 6 was observed. On day 25, lower ^{11}C-PBR28 uptake than at baseline was still observed in the hippocampus (-7%, *p*=0.042), hypothalamus (-12%, *p*=0.018), insular cortex (-11%, *p*<0.001), MPFC (-11%, *p*<0.001), motor / somatosensory cortex (-17%, *p*=0.039), OBFC (-11%, *p*<0.001) and striatum (-10%, *p*<0.001). RSD did not cause a significant increase in ^{11}C-PBR28 uptake in any brain region of SS rat. The opposite pattern of ^{11}C-PBR28 uptake over time in the cingulate cortex, FCA, MPFC and OBFC between SN and SS rats is depicted in Fig. 5. When investigating whether the ^{11}C-PBR28 uptake was related to the observed behavioral outcomes, no correlations were found at all.

RSD induces increased levels of IL-1β and IL-10 in the brain of SN rats

On day 25, significantly higher levels of IL-1β were observed in the cerebellum (SN: 618 pg/mg, IQR 352-959 *vs.* SS: 382 pg/mg, IQR 287-458, *p*=0.036), frontal cortex (SN: 601 pg/mg, IQR 316-796 *vs*. SS: 269 pg/mg, IQR 243–318, *p*=0.003), hippocampus (SN: 614 pg/mg, IQR 478-955 *vs*. SS: 295 pg/mg, IQR 259-387, *p*=0.004) (Fig.6-A) and in the parietal/temporal/occipital (P/T/O) cortex (SN: 576 pg/mg, IQR 467-947 *vs*. SS: 268 pg/mg, IQR 257-315, p=0.007) (Fig. 6-C) of SN rats than in SS rats. Also, IL-1β levels strongly correlated in a positive manner with ^{11}C-PBR28 uptake in the cingulate cortex of SN rats (r_s=0.94, *p*=0.005) (Fig. 6-D). No significant between-group differences in IL-6 levels were observed in any of the investigated brain regions. However, a trend towards significance was observed when comparing the levels of IL-6 in the cerebellum between SN and SS rats (SN: 2730 pg/mg, IQR 1878-4205 *vs*. SS: 1791 pg/mg, IQR 1134-2196, *p*=0.068). Moreover, IL-6 levels in the cerebellum of SN rats positively correlated with ^{11}C-PBR28 uptake (r_s=0.86, *p*=0.014) (Fig. 6-E). Due to technical issues, IL-10 levels were only measured in the P/T/O cortex. SN rats presented significantly higher levels of IL-10 in the P/T/O cortex (SN: 867 pg/mg, IQR 784-1547 *vs*. SS: 473 pg/mg, IQR 351-579, *p*=0.002) than SS rats.

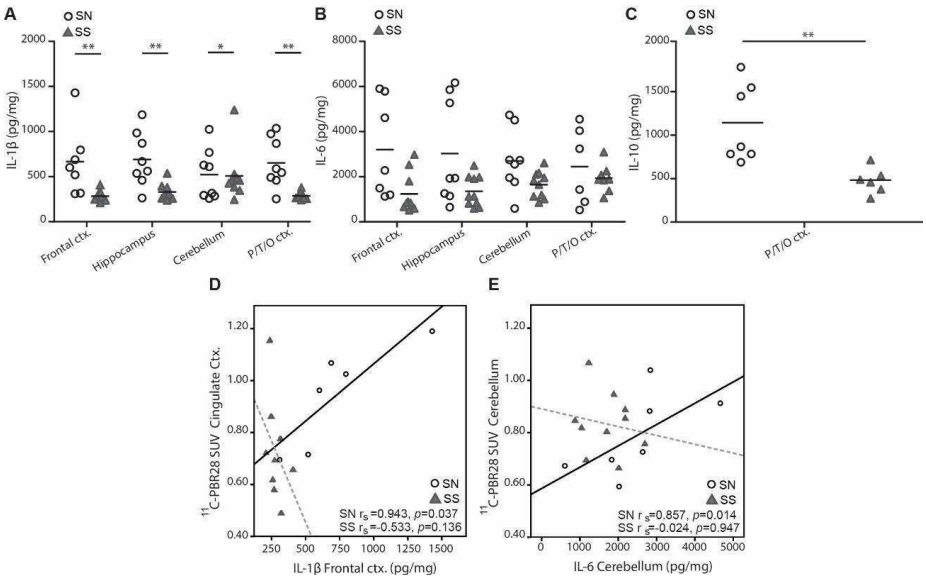


Figure 6 – A: Pro-inflammatory cytokines IL-1β and **B:** IL-6 levels in the frontal cortex, hippocampus, cerebellum and parietal/temporal/occipital cortex (P/T/O ctx.) of SN and SS rats. **C:** Anti-inflammatory cytokine IL-10 levels in the P/T/O ctx. between SN and SS rats. *p<0.05, **p<0.01. **D:** Spearman (r_s) correlation between ^{11}C-PBR28 uptake and IL-1β frontal cortex levels quantified through ELISA in the brain of SN and SS rats on day 25. **E:** Spearman (r_s) correlation between ^{11}C-PBR28 uptake and IL-6 cerebellar levels quantified through ELISA in brain of SN and SS rats on day 25.

RSD significantly increases corticosterone levels in SN rats, while a recurrence of RSD in SS rats blunts the corticosterone response

In order to investigate the effect of RSD on corticosterone release, blood samples were taken on day -1, 6, 11 and 25. A significant difference in corticosterone levels between groups was already found at baseline (SN: 240±38 nmol/L vs. SS: 383±56 nmol/L, p=0.036). The within-group analysis of corticosterone levels revealed a significant increase in the corticosterone levels in SN rats on day 11 (+61%, p=0.015) and 25 (+67%, p=0.040), whereas corticosterone levels were significantly decreased in SS rats on day 11 (-28%, p=0.036) and 25 (-51%, p<0.001), when compared to baseline levels. Consequently, significant between-group differences were found on day 11 (SN: 386±68 nmol/L vs. SS: 275±51 nmol/L, p=0.001) and day 25 (SN: 399±75 nmol/L vs. SS: 187±32 nmol/L, p<0.001) (Fig. 7-A).

Corticosterone levels are correlated with increased ^{11}C-PBR28 uptake in frontal cortical areas of SN rats

Positive correlations between corticosterone levels and ^{11}C-PBR28 uptake (SUV) on day 11 were found in the MPFC (r_s= 0.74, p=0.037) and in the OBFC (r_s= 0.88, p=0.004) of SN rats (Fig. 7-B and C, respectively). No significant correlations between corticosterone levels and tracer uptake in any other brain region were found at any time point. Also, no significant correlations between corticosterone levels and ^{11}C-PBR28 uptake were found in any brain region of SS rats at any time point. However, a very strong and positive correlation (r_s=1.0, p=0.01) between corticosterone levels and the anti-inflammatory cytokine IL-10 was found in the P/T/O cortex of SS rats. When investigating the relationship between ^{18}F-FDG and corticosterone levels, no significant correlations were found in any brain region of either SN or SS rats.

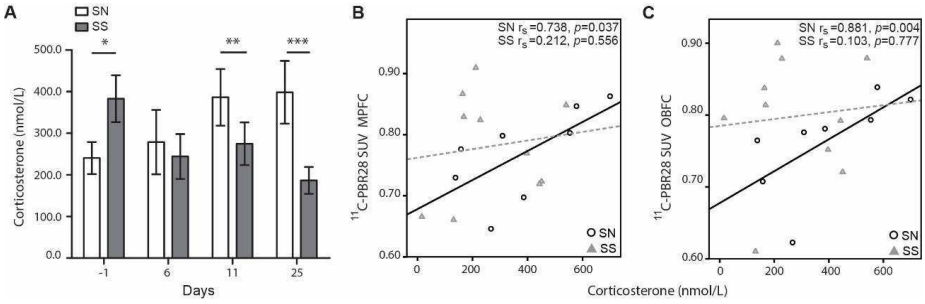


Figure 7 – A: Corticosterone levels were altered in a different manner in SN as compared to SS rats. A between-group difference was already apparent at baseline, with SS rats displaying higher corticosterone levels than SN rats (p<0.05). Corticosterone levels increased over time in SN rats, being significantly higher than SS rats on day 11 (p<0.01) and 25 (p<0.001). *p<0.05, **p<0.01 and ***p<0.001. **B**: Spearman correlation (r_s) between ^{11}C-PBR28 uptake (SUV) in the medial prefrontal cortex (MPFC) and corticosterone levels (nmol/L) on day 11 for SN and SS rats. **C:** Spearman correlation (r_s) between ^{11}C-PBR28 uptake (SUV) in the orbitofrontal cortex (OBFC) and corticosterone levels (nmol/L) on day 11 for SN and SS rats.

Discussion

Chronic stress may have long-lasting effects even after the stressor has been eliminated. So far, the cognitive and behavioral effects of RSD, as a model for psychosocial stress, have only been evaluated shortly after the stressful paradigm. In the present study, we demonstrated that a previous exposure to RSD during adolescence moderates glial activation, brain cytokine and corticosterone responses after a second exposure to the stressful paradigm in aged rats. Moreover, previous exposure to RSD provoked stress-induced depressive-like behavior in SS rats. In contrast, SN rats exposed to RSD had

increased levels of glial activation, production of pro-inflammatory cytokines and higher levels of corticosterone. RSD at old age induced a decrease in brain metabolism and anxiety-like behavior, irrespective of previous exposure to the psychosocial stressor.

The effects of a previous exposure to a stressful condition were already apparent in the body weight measurements during the exposure of the aged rats to RSD. SS rats lost significantly more weight during the 5-day RSD protocol than SN rats. However, from experimental day 4 onwards, both groups changed body weight at the same rate. Behaviourally, SS rats had a more exacerbated reaction to the recurrence of the stressful exposure. While both groups demonstrated anxiety-like behaviour and decreased locomotor activity in the OF test, only SS rats presented anhedonic-like behaviour. The absence of anhedonic behaviour in SN rats adds to the hypothesis that the adult brain is more resilient to stress-induced behavioural alterations than the adolescent brain (26). The previous exposure of SS rats to RSD during adolescence mainly affected areas linked with reward, such as the PFC and OBFC (7). Priming may have made these regions more vulnerable and therefore the secondary stressful stimuli might have provoked a more exacerbated depressive-like response. These results seem to be in agreement with the clinical observation that a history of stress exposures during adolescence can be a precursor to depression in adulthood (51). Previous exposure to RSD did not affect cognition in the NOR test, in accordance with other studies that evaluated long-term memory impairment in a rodent stress model (22; 52).

RSD significantly reduced brain glucose metabolism (^{18}F-FDG uptake) in both groups, although with different temporal patterns. SS rats showed a large global decrease in glucose metabolism on day 11, whereas SN rats presented a subtle decrease in glucose metabolism on day 25. Consequently, SS rats displayed lower global ^{18}F-FDG uptake on day 11 than SN rats. The reduction in brain glucose metabolism can be considered as a surrogate marker of brain activity and thus seems to reflect the reduction in general activity (depressive-like behaviour) observed after RSD. In general, these findings are in accordance with the reduced brain glucose metabolism observed in patients with MDD (11–14)

Our most interesting finding was the opposite glial response to stress between groups, as demonstrated by ^{11}C-PBR28 PET and brain cytokines levels. Baseline measurements showed higher ^{11}C-PBR28 uptake (indicative of glial activation) in the cerebellum, cingulate cortex, FCA, MPFC, and OBFC of SS rats than in SN rats. This suggests that exposure to RSD during adolescence primed glial cells (53; 54), inducing

an increased pro-inflammatory profile during ageing. Increasing glial activation during healthy ageing has already been demonstrated both in rodents (55) and humans (56), but priming of microglia by RSD appears to exacerbate the neuroinflammatory profile during ageing. After exposure of aged SN rats to RSD, an increase in tracer uptake was found in the cingulate cortex, MPFC, and OBFC, which persisted until the end of the study. This data is in accordance with glial activation following RSD demonstrated in adolescent rats (22). These results are also in line with recent clinical findings that indicate increased TSPO expression in the prefrontal cortex and cingulate cortex of MDD patients (24; 57). ^{11}C-PBR28 uptake in the cingulate cortex of SN rats correlated with IL-1β levels, suggesting an important role of the cingulate cortex in the induction of depressive-behaviour after exposure to stressful events (58). Interestingly, SS rats demonstrated persistently decreased ^{11}C-PBR28 uptake in response to the recurrence of RSD in several brain regions such as the entorhinal cortex, FCA, hippocampus, hypothalamus, insular cortex, MPFC, OBFC, and striatum. This reversed glial response to a recurrence of stressful stimuli might be considered as either an adaptive or maladaptive response to recurrent stress, highlighting the need for further research to unveil such phenomena. As an adaptive approach, blunting of glial response might be considered as a protective mechanism against hyperactivity of the immune system (59). Decreased microglial activation upon repeated stimuli has recently been described as a hypo-active tolerant phenotype, characterized by a decreased cytokine response to proinflammatory stimuli (60). In agreement with the described phenotype, our cytokine measurements displayed significantly lower levels of the pro-inflammatory cytokine IL-1β in the cerebellum, frontal cortex, hippocampus and P/T/O cortex of SS rats, as compared to SN rats. The anti-inflammatory cytokine IL-10 was also significantly decreased in SS rats. On the other hand, a maladaptive response refers to the cumulative effects generated after repeated stress exposure (i.e. allostatic overload), leading to an inefficient (neuro)immunological and neuroendocrine response to recurrent RSD (61; 62). Further studies are required to establish the dynamic role of glial cells in these neurobiological responses.

In order to measure the stress reactivity of the HPA axis in SN and SS rats, corticosterone was measured at several time points after RSD. Our measurements demonstrated a differential corticosterone secretion pattern between groups. SN rats showed a significant increase in corticosterone after RSD exposure, which is in agreement with previous studies in adolescent rats that evaluated corticosterone response shortly

after RSD (19; 22; 29). SS rats, on the other hand, had increased corticosterone levels at baseline, which decreased significantly over time. A similar blunted corticosterone response was recently observed in a rat model of chronic unpredictable stress (63). In adult patients with a history of early life stress, blunted cortisol response was also observed after exposure to acute stressors or dexamethasone suppression (64), supporting the hypothesis that previous trauma is able to modulate the neuroendocrine response to subsequent events.

Interestingly, a significant positive correlation was only found between corticosterone levels and ^{11}C-PBR28 uptake in the MPFC and OBFC of SN rats. No such correlations were found in SS rats. No correlations between corticosterone levels and ^{18}F-FDG uptake were observed at all. These results suggest that activation of the HPA axis and thus increased corticosterone levels might be involved in glial activation in response to a novel stress exposure (as in SN rats). The lack of correlation in SS rats might suggest that other pathways than HPA axis activation (not investigated in the present article) are involved in the decreased glial response to a recurrence of a stressful stimuli and the associated anhedonic- and anxiety-like behaviour displayed by SS rats.

The present study has some limitations, mainly due to its longitudinal design. First, PET findings were not confirmed by immunohistochemistry of microglia and/or astrocytes alterations. Instead, quantification of pro- and anti-inflammatory levels of cytokines were used as a proxy for glial activation. Second, tracer uptake was measured as SUV, a simple semi-quantitative measure that allows individual monitoring over time (65). In order to obtain a fully quantitative measure of tracer binding to its receptor (e.g. TSPO), the optimal procedure would be to perform kinetic modelling of ^{11}C-PBR28 kinetics, but this would require a terminal procedure with arterial blood sampling, since no reference region devoid of TSPO is available within the brain. Due to the longitudinal nature of the study, such methodology was not feasible. However, SUV measurements of ^{11}C-PBR28 uptake were strongly correlated with the volume of distribution (V_T) in previous studies (25; 66), suggesting that the SUV can be used to quantify ^{11}C-PBR28 uptake in order to simplify the imaging procedure while retaining reliable quantitative information.

In conclusion, we have demonstrated for the first time a dampened glial activation after a recurrence of psychosocial stress in aged rats, in conjunction with more severe depressive- and anxiety-like behavior. The immune response in stress-sensitized rats was not correlated with corticosterone levels, pointing towards an uninvestigated pathway that

might either play a protective role that preserves the brain from further detrimental stimuli, or a maladaptive response to the recurrence of stressful stimuli.

Acknowledgements

The authors would like to thank Luiza Reali Nazario for the support in the novel object recognition analysis and Natalia M. Peñaranda Fajardo for technical assistance in the biochemical analysis.

Disclosure / Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Paula Kopschina Feltes was a fellowship recipient of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The Authors declare that there is no conflict of interest.

References

1. Santarelli S, Lesuis SL, Wang X-D, Wagner K V., Hartmann J, Labermaier C, *et al.* (2014): Evidence supporting the match/mismatch hypothesis of psychiatric disorders. *Eur Neuropsychopharmacol*. 24: 907–918.
2. de Boer SF, Buwalda B, Koolhaas JM (2017): Untangling the neurobiology of coping styles in rodents: Towards neural mechanisms underlying individual differences in disease susceptibility. *Neurosci Biobehav Rev*. 74: 401–422.
3. Williams LM, Debattista C, Duchemin A-M, Schatzberg AF, Nemeroff CB (2016): Childhood trauma predicts antidepressant response in adults with major depression: data from the randomized international study to predict optimized treatment for depression. *Transl Psychiatry*. 6: e799.
4. Yirmiya R, Rimmerman N, Reshef R (2015): Depression as a Microglial Disease. *Trends Neurosci*. 38: 637–658.
5. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctôt KL (2010): A Meta-Analysis of Cytokines in Major Depression. *Biol Psychiatry*. 67: 446–457.
6. Kopschina Feltes P, Doorduin J, Klein HC, Juárez-Orozco LE, Dierckx RA, Moriguchi-Jeckel CM, de Vries EF (2017): Anti-inflammatory treatment for major depressive disorder: implications for patients with an elevated immune profile and non-responders to standard antidepressant therapy. *J Psychopharmacol*. 31: 1149–1165.
7. Sheth C, McGlade E, Yurgelun-Todd D (2017): Chronic Stress in Adolescents and Its Neurobiological and Psychopathological Consequences: An RDoC Perspective. *Chronic Stress*. 1: 1–22.
8. Perlman WR, Webster MJ, Herman MM, Kleinman JE, Weickert CS (2007): Age-related differences in glucocorticoid receptor mRNA levels in the human brain. *Neurobiol Aging*. 28: 447–458.
9. Buwalda B, Stubbendorff C, Zickert N, Koolhaas JM (2013): Adolescent social stress does not necessarily lead to a compromised adaptive capacity during adulthood: A study on the consequences of social stress in rats. *Neuroscience*. 249: 258–270.
10. Sung K-K, Jang D-P, Lee S, Kim M, Lee S-Y, Kim Y-B, *et al.* (2009): Neural responses in rat brain during acute immobilization stress: a [F-18]FDG micro PET imaging study.

Neuroimage. 44: 1074–1080.
11. Su L, Cai Y, Xu Y, Dutt A, Shi S, Bramon E (2014): Cerebral metabolism in major depressive disorder: a voxel-based meta-analysis of positron emission tomography studies. *BMC Psychiatry*. 14: 1–7.
12. Saxena S, Brody AL, Ho ML, Alborzian S, Ho MK, Maidment KM, *et al.* (2001): Cerebral metabolism in major depression and obsessive-compulsive disorder occurring separately and concurrently. *Biol Psychiatry*. 50: 159–170.
13. Martinot J, Hardy P, Feline A (1990): Left prefrontal glucose hypometabolism in the depressed state: a confirmation. *Am J Psychiatry*. 147: 1313–1317.
14. Biver F, Goldman S, Delvenne V, Luxen A, Demaertelaer V, Hubain P, *et al.* (1994): Frontal and Parietal Metabolic Disturbances in Unipolar Depression. *Biol Psychiatry*. 36: 381–388.
15. Anders S, Tanaka M, Kinney DK (2013): Depression as an evolutionary strategy for defense against infection. *Brain Behav Immun*. 31: 9–22.
16. Koolhaas JM, Coppens CM, de Boer SF, Buwalda B, Meerlo P, Timmermans PJA (2013): The Resident-intruder Paradigm: A Standardized Test for Aggression, Violence and Social Stress. *J Vis Exp*. 77: 1–7.
17. Beery AK, Kaufer D (2015): Stress, social behavior, and resilience: Insights from rodents. *Neurobiol Stress*. 1: 116–127.
18. McKim DB, Weber MD, Niraula A, Sawicki CM, Liu X, Jarrett BL, *et al.* (2017): Microglial recruitment of IL-1β-producing monocytes to brain endothelium causes stress-induced anxiety. *Mol Psychiatry*. E-pub ahead of print. DOI: 10.1038/mp.2017.64.
19. Ramirez K, Fornaguera-Trías J, Sheridan JF (2016): Stress-Induced Microglia Activation and Monocyte Trafficking to the Brain Underlie the Development of Anxiety and Depression. *Brain Imaging Behav Neurosci*. pp 155–172.
20. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, *et al.* (2011): β-Adrenergic Receptor Antagonism Prevents Anxiety-Like Behavior and Microglial Reactivity Induced by Repeated Social Defeat. *J Neurosci*. 31: 6277–6288.
21. Wohleb ES, McKim DB, Shea DT, Powell ND, Tarr AJ, Sheridan JF, Godbout JP (2014): Re-establishment of Anxiety in Stress-Sensitized Mice Is Caused by Monocyte Trafficking from the Spleen to the Brain. *Biol Psychiatry*. 75: 970–981.
22. Kopschina Feltes P, de Vries EF, Juarez-Orozco LE, Kurtys E, Dierckx RA, Moriguchi-Jeckel CM, Doorduin J (2017): Repeated social defeat induces transient glial activation and brain hypometabolism: A positron emission tomography imaging study. *J Cereb Blood Flow Metab*. E-pub ahead of print. DOI: 10.1177/0271678X17747189.
23. Wohleb ES, Franklin T, Iwata M, Duman RS (2016): Integrating neuroimmune systems in the neurobiology of depression. *Nat Rev Neurosci*. 17: 497–511.
24. Setiawan E, Wilson AA, Mizrahi R, Rusjan PM, Miler L, Rajkowska G, *et al.* (2015): Role of Translocator Protein Density, a Marker of Neuroinflammation, in the Brain During Major Depressive Episodes. *JAMA Psychiatry*. 72: E1–E8.
25. Parente A, Feltes PK, Vallez Garcia D, Sijbesma JWA, Moriguchi Jeckel CM, Dierckx RAJO, *et al.* (2016): Pharmacokinetic Analysis of 11C-PBR28 in the Rat Model of Herpes Encephalitis: Comparison with (R)-11C-PK11195. *J Nucl Med*. 57: 785–791.
26. Buwalda B, Geerdink M, Vidal J, Koolhaas JM (2011): Social behavior and social stress in adolescence: A focus on animal models. *Neurosci Biobehav Rev*. 35: 1713–1721.
27. Ma X, Jiang D, Jiang W, Wang F, Jia M, Wu J, *et al.* (2011): Social Isolation-Induced Aggression Potentiates Anxiety and Depressive-Like Behavior in Male Mice Subjected to Unpredictable Chronic Mild Stress. *PLoS One*. 6: e20955.
28. Visser AKD, Meerlo P, Ettrup A, Knudsen GM, Bosker FJ, den Boer JA, *et al.* (2014): Acute social defeat does not alter cerebral 5-HT 2A receptor binding in male Wistar rats. *Synapse*. 68: 379–386.
29. Patki G, Solanki N, Atrooz F, Allam F, Salim S (2013): Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. *Brain Res*. 1539: 73–86.
30. Razzoli M, Domenici E, Carboni L, Rantamaki T, Lindholm J, Castrén E, Arban R (2011): A role for BDNF/TrkB signaling in behavioral and physiological consequences of social defeat

stress. *Genes, Brain Behav*. 10: 424–433.
31. Liu L, Zhou X, Zhang Y, Liu Y, Yang L, Pu J, *et al.* (2016): The identification of metabolic disturbances in the prefrontal cortex of the chronic restraint stress rat model of depression. *Behav Brain Res*. 305: 148–156.
32. Dere E, Huston JP, De Souza Silva MA (2007): The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neurosci Biobehav Rev*. 31: 673–704.
33. Elizalde N, Gil-Bea FJ, Ramírez MJ, Aisa B, Lasheras B, Del Rio J, Tordera RM (2008): Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. *Psychopharmacology (Berl)*. 199: 1–14.
34. Hovens IB, van Leeuwen BL, Nyakas C, Heineman E, van der Zee EA, Schoemaker RG (2015): Prior infection exacerbates postoperative cognitive dysfunction in aged rats. *Am J Physiol - Regul Integr Comp Physiol*. 309: R148–R159.
35. Hurley LL, Akinfiresoye L, Kalejaiye O, Tizabi Y (2014): Antidepressant effects of resveratrol in an animal model of depression. *Behav Brain Res*. 268: 1–7.
36. Wong K-P, Sha W, Zhang X, Huang S-C (2011): Effects of Administration Route, Dietary Condition, and Blood Glucose Level on Kinetics and Uptake of 18F-FDG in Mice. *J Nucl Med*. 52: 800–807.
37. Vállez García D, Dierckx RAJO, Doorduin J (2016): Three months follow-up of rat mild traumatic brain injury: a combined [18F]FDG and [11C]PK11195 PET study. *J Neurotrauma*. 33: 1855-1865.
38. Vállez Garcia D, Casteels C, Schwarz AJ, Dierckx RAJO, Koole M, Doorduin J (2015): A Standardized Method for the Construction of Tracer Specific PET and SPECT Rat Brain Templates: Validation and Implementation of a Toolbox. (J.-C. Baron, editor) *PLoS One*. 10: e0122363.
39. Boellaard R, Delgado-Bolton R, Oyen WJ, Giammarile F, Tatsch K, Eschner W, *et al.* (2015): FDG PET/CT: EANM procedure guidelines for tumour imaging: version 2.0. *Eur J Nucl Med Mol Imaging*. 42: 328–354.
40. Weber MD, Godbout JP, Sheridan JF (2017): Repeated Social Defeat, Neuroinflammation, and Behavior: Monocytes Carry the Signal. *Neuropsychopharmacology*. 42: 46–61.
41. Kim Y-K, Won E (2017): The influence of stress on neuroinflammation and alterations in brain structure and function in major depressive disorder. *Behav Brain Res*. 329: 6–11.
42. Phillips JR, Hewedi DH, Eissa AM, Moustafa AA (2015): The Cerebellum and Psychiatric Disorders. *Front Public Heal*. 3: 1–8.
43. Wei K, Xue H, Guan Y, Zuo C, Ge J, Zhang H, *et al.* (2016): Analysis of glucose metabolism of 18F-FDG in major depression patients using PET imaging: Correlation of salivary cortisol and α-amylase. *Neurosci Lett*. 629: 52–57.
44. Hinwood M, Tynan RJ, Day TA, Walker FR (2011): Repeated Social Defeat Selectively Increases FosB Expression and Histone H3 Acetylation in the Infralimbic Medial Prefrontal Cortex. *Cereb Cortex*. 21: 262–271.
45. Yu T, Guo M, Garza J, Rendon S, Sun X-L, Zhang W, Lu X-Y (2011): Cognitive and neural correlates of depression-like behaviour in socially defeated mice: an animal model of depression with cognitive dysfunction. *Int J Neuropsychopharmacol*. 14: 303–317.
46. Marx C, Lex B, Calaminus C, Hauber W, Backes H, Neumaier B, *et al.* (2012): Conflict Processing in the Rat Brain: Behavioral Analysis and Functional μPET Imaging Using [18F]Fluorodeoxyglucose. *Front Behav Neurosci*. 6: 1–12.
47. Lehnert W, Gregoire M-C, Reilhac A, Meikle SR (2012): Characterisation of partial volume effect and region-based correction in small animal positron emission tomography (PET) of the rat brain. *Neuroimage*. 60: 2144–2157.
48. Hardin JW, Hilbe JM (2013): *Generalized Estimating Equations*, Second edition. New York: Chapman & Hall / CRC Press.
49. Streiner DL (2015): Best (but oft-forgotten) practices: The multiple problems of multiplicity-whether and how to correct for many statistical tests. *Am J Clin Nutr*. 102: 721–728.
50. Holm S (1979): A Simple Sequentially Rejective Multiple Test Procedure. *Scand J Stat*. 6: 65–70.

51. McCormick CM, Green MR, Simone JJ (2017): Translational relevance of rodent models of hypothalamic-pituitary-adrenal function and stressors in adolescence. *Neurobiol Stress*. 6: 31–43.
52. McKim DB, Niraula A, Tarr AJ, Wohleb ES, Sheridan JF, Godbout JP (2016): Neuroinflammatory Dynamics Underlie Memory Impairments after Repeated Social Defeat. *J Neurosci*. 36: 2590–2604.
53. Frank MG, Watkins LR, Maier SF (2013): Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. *Brain Behav Immun*. 33: 1–6.
54. Frank MG, Weber MD, Watkins LR, Maier SF (2015): Stress sounds the alarmin: The role of the danger-associated molecular pattern HMGB1 in stress-induced neuroinflammatory priming. *Brain Behav Immun*. 48: 1–7.
55. Liu B, Le KX, Park M, Wang S, Belanger AP, Dubey X, *et al.* (2015): In Vivo Detection of Age- and Disease-Related Increases in Neuroinflammation by 18 F-GE180 TSPO MicroPET Imaging in Wild-Type and Alzheimer's Transgenic Mice. *J Neurosci*. 35: 15716–15730.
56. Schuitemaker A, van der Doef TF, Boellaard R, van der Flier WM, Yaqub M, Windhorst AD, *et al.* (2012): Microglial activation in healthy aging. *Neurobiol Aging*. 33: 1067–1072.
57. Holmes SE, Hinz R, Conen S, Gregory CJ, Matthews JC, Anton-Rodriguez JM, *et al.* (2018): Elevated Translocator Protein in Anterior Cingulate in Major Depression and a Role for Inflammation in Suicidal Thinking: A Positron Emission Tomography Study. *Biol Psychiatry*. 83: 61–69.
58. Lutz P-E, Tanti A, Gasecka A, Barnett-Burns S, Kim JJ, Zhou Y, *et al.* (2017): Association of a History of Child Abuse With Impaired Myelination in the Anterior Cingulate Cortex: Convergent Epigenetic, Transcriptional, and Morphological Evidence. *Am J Psychiatry*. 174: 1185–1194.
59. Schaafsma W, Zhang X, van Zomeren KC, Jacobs S, Georgieva PB, Wolf SA, *et al.* (2015): Long-lasting pro-inflammatory suppression of microglia by LPS-preconditioning is mediated by RelB-dependent epigenetic silencing. *Brain Behav Immun*. 48: 205–221.
60. Eggen BJL, Raj D, Hanisch UK, Boddeke HWGM (2013): Microglial phenotype and adaptation. *J Neuroimmune Pharmacol*. 8: 807–823.
61. McEwen BS (2007): Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. *Physiol Rev*. 87: 873–904.
62. Frank MG, Watkins LR, Maier SF (2013): Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. *Brain Behav Immun*. 33: 1–6.
63. Natarajan R, Forrester L, Chiaia NL, Yamamoto BK (2017): Chronic-Stress-Induced Behavioral Changes Associated with Subregion-Selective Serotonin Cell Death in the Dorsal Raphe. *J Neurosci*. 37: 6214–6223.
64. Yehuda R, Flory JD, Pratchett LC, Buxbaum J, Ising M, Holsboer F (2010): Putative biological mechanisms for the association between early life adversity and the subsequent development of PTSD. *Psychopharmacology (Berl)*. 212: 405–417.
65. Lodge MA (2017): Repeatability of SUV in Oncologic 18 F-FDG PET. *J Nucl Med*. 58: 523–532.
66. Tóth M, Doorduin J, Häggkvist J, Varrone A, Amini N, Halldin C, Gulyás B (2015): Positron Emission Tomography studies with [11C]PBR28 in the Healthy Rodent Brain: Validating SUV as an Outcome Measure of Neuroinflammation. (K. Hashimoto, editor) *PLoS One*. 10: e0125917.

High dopaminergic D2 receptor availability as assessed by ^{11}C-raclopride PET is associated with appetitive aggression in Long Evans rats

Author(s): Paula Kopschina Feltes, Janine Doorduin, Isadora Lopes Alves, Sietse F. de Boer, Rudi AJO Dierckx, Cristina M. Moriguchi-Jeckel, Erik FJ de Vries.

CHAPTER 6

In preparation for submission.

Abstract

Background: Violence and appetitive forms of aggression are serious concerns for modern society**.** Rewarding properties of winning aggressive encounters reinforce aggressive behaviour through instrumental learning, and dopamine (DA) receptors within the nucleus accumbens (NAc) are implicated in these natural rewards of positive behavioural outcomes.

Objective*:* To assess DA D2 receptor availability in the striatum of winning experience-enhanced aggressiveness in Long Evans (LE) rats.

Methods: Male outbred LE rats (n=16, 4 months-old) were screened for aggression levels and their capacity to defeat an intruder rat in the resident-intruder paradigm. Based on the tendency to initiate attacks (attack latency; AL <1 min) and effectiveness to subjugate intruders, rats were assigned to an aggressive (n=10) and a non-aggressive group (n=6). Aggressive rats were further used as residents to successfully defeat intruders with an average of 14±5 aggressive confrontations per rat. At the end of the study, both aggressive and non-aggressive rats underwent 60-min dynamic PET scans with the dopaminergic D2 antagonist ^{11}C-raclopride for quantification of D2 receptor availability in the NAc, nucleus caudates/putamen (CPu) and cerebellum.

Results: During aggression screening, the AL of aggressive rats was 45s, IQR 40-83 s *vs.* 123s, IQR 66-461s in non-aggressive rats, p=0.010). Upon repetitive winning aggressive encounters, the aggressive rats showed a decrease in AL of 1.8s after each victory (p=0.006). ^{11}C-raclopride binding potential (BP_{ND}) was higher in the NAc and in the caudate and putamen (CPu) of aggressive rats, as compared to non-aggressive (1.14, IQR 1.01–1.28 *vs.* 0.83, IQR 0.77–1.03, p=0.007; and 2.26, IQR 2.23–2.40 *vs.* 1.98, IQR 1.66–2.09, p<0.001; respectively). Moreover, the AL of aggressive rats was negatively correlated with the BP_{ND} in the NAc (r_s=-0.720, p=0.019) but not in the CPu.

Conclusions: For the first time we were able to demonstrate through ^{11}C-raclopride PET that aggressive rats exposed to repetitive winning confrontations display higher levels of D2 receptors, when compared to non-aggressive rats. The negative correlation between the AL and BP_{ND} in NAc of aggressive rats suggests that an aggression habit might be developed by the winning reward feeling through stimulation of the dopaminergic system. However, future research is needed to corroborate and further explore our current findings.

Key words: aggression, dopaminergic system, resident-intruder paradigm, PET.

Introduction

It is commonly accepted in biology that aggression is one of the most widespread and functional forms of social behaviour that ultimately contributes to fitness and survival of individuals. Clearly, aggression is the behavioural weapon of choice for essentially all animals and humans to gain and maintain access to desired resources (food, shelter, mates), defend themselves and their offspring from rivals and predators, and establish and secure social status/hierarchical relationships. However, aggressive behaviour can transition from adaptive to maladaptive. A relatively small proportion of individuals may show excessive/inappropriate aggressive behaviours and/or can become extremely violent. This escalated aggression and violence is a major source of death, social stress and ensuing disability, thereby constituting one of the most significant problems for the public health, medical institutions and criminal justice systems worldwide. In order to reduce violent and inappropriate forms of aggressive behaviour, more fundamental knowledge on the determinants of aggression is greatly needed. Much evidence suggests that the interaction between environmental factors and neurochemical substrates is instrumental in escalated and maladaptive forms of aggression (1). Since these interactions are difficult to investigate in humans, experimental laboratory animal models of aggression are necessary.

To date, most laboratory animal studies of aggression are employing the resident-intruder aggression paradigm using highly domesticated rodent species like mice and rats that generally are very placid and docile. In virtually all laboratory inbred/outbred mouse and rat strains, the aggressive behavioural traits have been dramatically compromised due to selection and inbreeding during the course of the domestication process (2). Consequently, in order to promote appreciable levels of aggression in these laboratory animal strains, several procedural manipulations are being employed. One way to increase aggressive tendencies is by providing animals with repeated positive (i.e., winning) aggressive experiences in its home cage. Numerous studies in a wide variety of animal species have convincingly demonstrated that in addition to securing access to resources, the most intriguing consequence of winning aggressive conflicts is the self-reinforcing effect of this type of behaviour. Actually, individuals seek out the opportunity to fight and engaging in aggressive behaviour appears to be a source of pleasure, referred to as “appetitive” aggression (3). The most convincing evidence that successful aggression seems rewarding to animals is that the opportunity to engage in aggressive behaviour can reinforce operant responding for future aggression (see Miczek et al., 2004

for review (4)) and induce conditioned place preference for a location associated with a previously successful aggressive encounter (5).

Not surprisingly, just like other events that function as positive reinforcers such as food, drugs or sex, the mesocorticolimbic dopamine system is closely associated with the rewarding properties of winning fights. Nucleus accumbens (NAc) dopamine is strongly released during anticipation of aggressive episodes (7) and pharmacological antagonism of dopamine D1/D2 receptors in the NAc diminishes the seeking of the opportunity to fight (9; 11). In addition, direct optogenetic activation of ventral tegmental area (VTA) dopamine neurons increases aggression (13), while DA receptor knock-out mice show a reduced aggressive phenotype (15; 17), proving that dopamine function and aggression are causally linked. Furthermore, DA D2/3 receptor binding was elevated in the nucleus accumbens shell and dorsal striatum of dominant rats when compared to subordinate rats and was accompanied by elevated DAT and reduced dopamine content in the nucleus accumbens shell (22). Similarly, socially-housed dominant monkeys that were engaged in aggressive behaviour had increased levels of D2 receptors in the basal ganglia when compared to subordinates as observed with ^{18}F-fluorocleboperide PET imaging (23). This finding was confirmed in dominant female cynomolgus monkeys (24). Together, these studies provide strong evidence for a role of DA receptors in the ventral striatum in mediating winning experience-enhanced aggressiveness.

To date, no study has investigated the link between the dopaminergic system and aggression levels in rodents through PET yet. Therefore, the aim of this study was to evaluate differences in dopaminergic D2 receptor availability between aggressive and non-aggressive Long Evans (LE) rats using ^{11}C-raclopride PET. Aggressive LE rats have been exposed to repetitive winning confrontations leading to escalated and/or appetitive forms of aggressiveness.

Materials and Methods

Experimental animals

Male outbred LE rats (n=16, 16 weeks old, 518 ± 33g; Harlan, Indianapolis, USA) were used as residents in the present study and divided into two groups based on their level of aggressiveness in the resident-intruder test. All animals were kept under a 12:12 hour light:dark cycle, with lights on at 7 a.m. Rats had *ad libitum* access to food and water.

Animal experiments were performed in accordance with the Law on Animal Experiments of the Netherlands. The protocol was approved by the Institutional Animal

Care and Use Committee of the University of Groningen (protocols DEC 6828A and DEC 6828B).

Study design

The study was divided in three parts. In the first part, the male LE rats were screened for aggression for three consecutive days. Each male rat was housed for fourteen days in large cages (80x50x40 cm) together with a tubal-ligated female LE rat in order to stimulate territorial aggression. Before the aggression test, the female was taken out of the cage before a male Wistar rat intruder was placed inside the resident's cage. The attack latency (AL; used as an indicator of an animal's aggressiveness) of the LE rats and the ability to successfully defeat the intruder (i.e. intruder assuming a submissive posture for at least 3 seconds) during a 10-min interaction were recorded. The rats always encountered an unfamiliar opponent. An AL smaller than 1 min (6) during the training period combined with a successful winning confrontation was defined as aggressive behaviour (8). After screening, rats were divided in aggressive and non-aggressive rats. The aggressive rats were used as residents for the second part of the study, a longitudinal repeated social defeat study (RSD, see (10)). The non-aggressive rats were housed with a female until the PET scan, without further interventions. The third part consisted of ^{11}C-raclopride PET imaging of all male LE rats (non-aggressive and aggressive), at least two weeks after the last winning confrontation to minimize any residual effect of acute dopamine release. No age differences were present between groups during the PET scans.

Repeated social defeat (RSD)

The RSD protocol was conducted as previously described (10). The female companion of the LE resident rat was removed from the cage shortly before the defeat test. The resident rat was confronted with an unfamiliar intruder Wistar rat being placed inside the resident's cage for each aggressive confrontation, in order to prevent habituation. In general, the residents quickly explored the intruder and shortly after performing the threatening repertoire (12), proceeded with the overt clinch attack. Both rats were allowed to interact for a period of 10 min or shorter if the resident was able to successfully defeat the intruder, interpreted as the intruder assuming a supine (submissive) position for at least 3 seconds. After submission, the intruder was placed inside a wire mesh cage to avoid physical contact with the resident, still allowing visual, auditory and olfactory

interactions for a total exposure period of 60 min. The RSD experiment always took place between 16:00 and 18:00 p.m.

Tracer synthesis

^{11}C-raclopride was synthetized by alkylation of S-(+)-*O*-desmethyl-raclopride (ABX, Radeberg, Germany) using ^{11}C-methyl iodide as the reagent (14). ^{11}C-methyl iodide was trapped in a solution containing 1mg of S-(+)-*O*-desmethyl-raclopride and 1.4 mg of sodium hydroxide in 300 µl dimethylsulfoxide. The reaction mixture was allowed to react for 4 minutes at 80°C. After the reaction, the product was purified through HPLC using a µBondapak C18 column (7.8mmx 300mm) and acetonitrile/H_3PO4 10mM (30/70) as the eluent (flow 5 ml/min). To remove organic solvents from the product, the HPLC fraction containing the product (retention time of 8 min) was diluted in 100 ml of water and passed through an Oasis HLB 200 mg cartridge. The cartridge was washed twice with 8 ml of water and subsequently eluted with 0.8ml of H_3PO4 1% in ethanol and 8 ml of phosphate buffer (pH 7.2). The product was sterilized with a 0.20 µm Millex LG filter. The radiochemical purity was always >98% and the molar activity at the end of the synthesis was 163 ± 69 GBq/µmol.

Dynamic PET imaging

PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Rats were anesthetized with isoflurane mixed with oxygen (5% for induction, 2% for maintenance) and the tail vein was cannulated for tracer injection. Rats were placed in the camera in prone position with their head in the field of view. A transmission scan was acquired using a ^{57}Co point source for attenuation and scatter correction. ^{11}C-raclopride (21.04 ± 10.55 MBq; 0.18 ± 0.20 nmol, p=0.22) was injected over 1 min using an automatic injection pump at a speed of 1 mL/min, and a 60-min dynamic PET scan was acquired. The body temperature was maintained at 37°C with heating pads, heart rate and blood oxygen saturation were monitored, and eye salve was applied to prevent conjunctival dehydration.

Image reconstruction and analysis

The list-mode data from the 60-min emission scan were reconstructed into 21 frames (6 x 10, 4 x 30, 2 x 60, 1 x 120, 1 x 180, 4 x 300 and 3 x 600 s). Each emission frame was corrected for radioactive decay, scatter, random coincidences and attenuation, and

reconstructed using the two-dimensional ordered-subset expectation maximization (OSEM2D) algorithm (4 iterations and 16 subsets). 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. PET images were automatically co-registered to a functional ^{11}C-raclopride brain template (16), which was spatially aligned with a stereotaxic T2-weighted MRI in Paxinos Space using PMOD 3.6 (PMOD technologies Ltd., Switzerland). Time-activity curves (TACs) were generated for the caudate and putamen (CPu), NAc and cerebellum by applying the corresponding predefined volume of interest (VOIs) (16) to the dynamic data.

Following the well validated approach for ^{11}C-raclopride, the simplified reference tissue (SRTM) model was applied to quantify tracer uptake (18; 19). The instantaneous changes in tracer concentration in each compartment can be described as:

$$\frac{dC_T(t)}{dt} = K_1^T C_P(t) - k_{2a}^T C_T(t)$$
$$\frac{dC_R(t)}{dt} = K_1^R C_P(t) - k_{2a}^R C_R(t)$$

where $C_P(t)$ is the tracer concentration in plasma, $C_T(t)$ and $C_R(t)$ are the concentration in target and reference compartments, K_1^T and K_2^R are the rate constants describing the tracer influx from plasma to the respective compartments, k_2^R is the reference washout rate from the reference to the plasma, k_{2a}^T is the apparent target washout rate constant and t is time (20). The extracted TACs were fitted to the SRTM using the cerebellum as reference region and the non-displaceable binding potential (BP_{ND}) was calculated for the CPu and NAc.

Statistical analysis

Results are reported as median and the 0.25-0.75 interquartile range (IQR). Statistical analysis was performed using IBM SPSS Statistics 23 software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY). Differences between-groups were analysed by the Mann Whitney U test and considered to be significant when $p<0.05$, without correction for multiple comparisons. The correlations between the BP_{ND} of the investigated brain areas and the AL were assessed through the Spearman correlation (r_s) test.

In order to evaluate if changes in the AL (escalation of aggression) were related to the number of winning confrontations, the generalized estimating equations (GEE) model was applied (21) because of repeated measurements and missing data. The AR(1) working correlation matrix was selected according to the quasi-likelihood under the independence model information criterion value. Wald's statistics and associated *p*-values were considered statistically significant if $p<0.05$.

Results

Attack latency

Based on the averaged AL of the first three screening days, it was possible to identify 6 non-aggressive and 10 aggressive rats (AL of non-aggressive rats: 123 s, IQR 66 – 461 s vs. aggressive rats: 45 s, IQR 40 – 83 s, $p=0.01$). The average AL of all winning confrontations of aggressive rats was 20 s, IQR 4 – 75 s. Aggressive rats were exposed to an average of 14±5 winning encounters.

Upon repeated aggression testing and acquiring victorious experiences, the time to initiate aggressive attacks gradually decreased in the aggressive animals. In aggressive rats, a significant correlation between the number of winning confrontations and the AL was observed ($r_s=-0.27$, $p<0.001$), with an average decrease in the AL of 1.8 s for each winning confrontation (Fig. 1-A). No significant correlation between the number of exposures to aggressive confrontations and the AL of the non-aggressive rats was found during the screening session (Fig. 1-B). Moreover, the non-aggressive rats were not able to successfully defeat an intruder opponent, thus not meeting the criteria for aggressive behaviour.

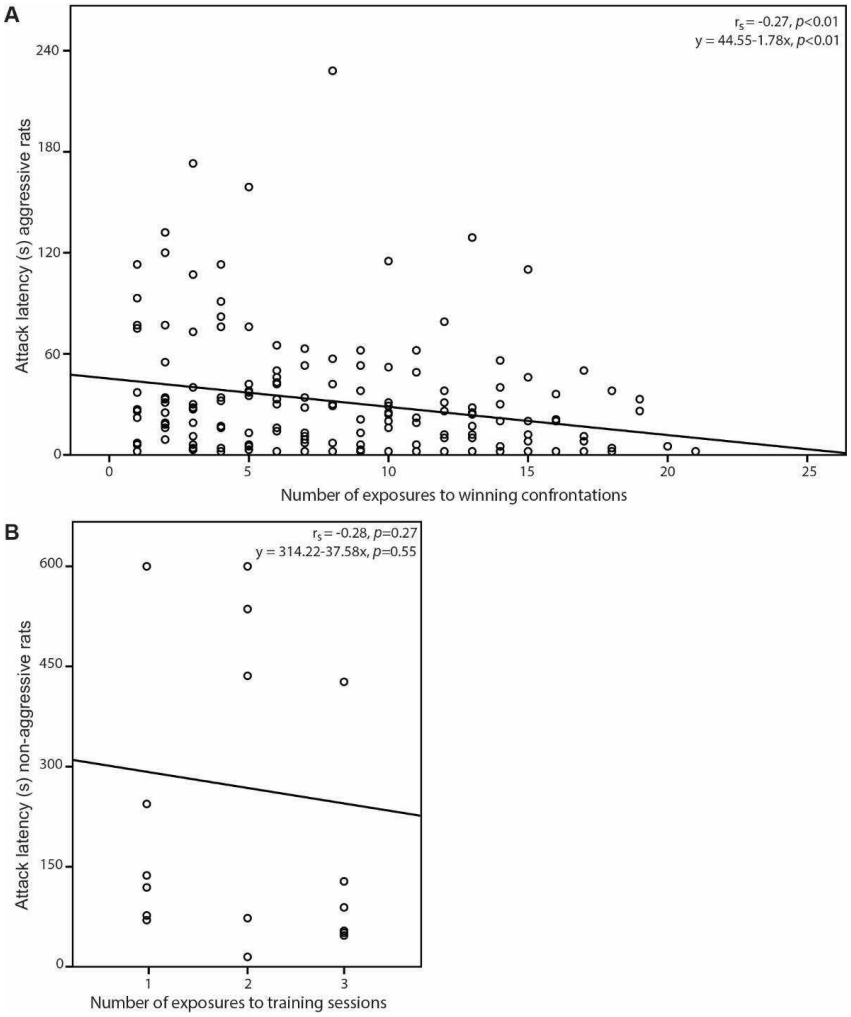


Figure 1 – A: Spearman correlation (r_s) between the number of the repetitive exposures to aggressive social conflicts and the attack latency (AL) in aggressive Long Evans (LE) rats (n=10). **B:** Spearman correlation (r_s) between the number of training sessions and the AL of the non-aggressive rats (n=6).

PET imaging of D2 receptor availability

A representative PET image of ^{11}C-raclopride PET in non-aggressive rats and aggressive rats is displayed in Fig. 2-A. The tracer binding in the investigated brain regions, calculated using the SRTM compartmental model, differed significantly between groups and brain regions (Fig. 2-B). Aggressive LE rats displayed a significantly higher BP_{ND} the NAc than non-aggressive rats (1.14, IQR 1.01 – 1.28 *vs.* 0.83, IQR 0.77 – 1.03, p=0.007). The same pattern was observed for the CPu, with an increased BP_{ND} in

aggressive as compared to non-aggressive rats (2.26, IQR 2.23 – 2.40 *vs.* 1.98, IQR 1.66 – 2.09, $p<0.001$). Time-activity curves of the NAc, CPu and cerebellum of non-aggressive and aggressive LE rats are presented in Fig. 2-C and D.

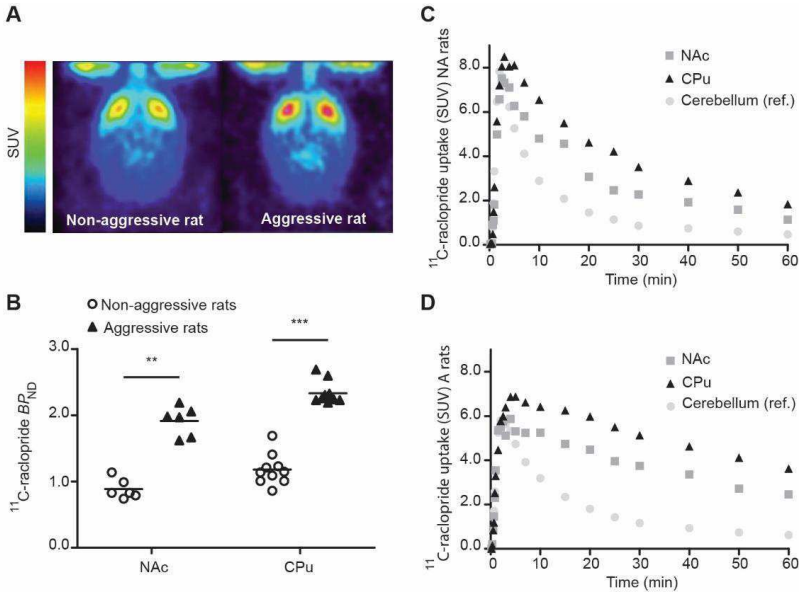


Figure 2 – A: ^{11}C-raclopride representative PET imaging of a non-aggressive rat (NA) and an aggressive rat (A). **B:** Graphical comparison of ^{11}C-raclopride binding potential (BP_{ND}) in the nucleus accumbens (NAc) and caudate and putamen (CPu) between non-aggressive and aggressive Long Evans rats. **C:** Representative ^{11}C-raclopride PET time-activity curves (TACs) of a non-aggressive Long Evans rat, and **D:** of an aggressive Long Evans rat.

Finally, we tested whether individual differences in aggressive temperament are related to D2 receptor availability. Therefore, the AL of the last aggressive exposure was correlated with the BP_{ND} of the NAc and CPu for non-aggressive and aggressive rats. In the NAc (Fig. 3-A), no significant correlation between the BP_{ND} of non-aggressive rats and the AL was found (r_s=0.43, p=0.40). However, a strong and significant negative correlation was observed for aggressive rats (r_s=-0.72, p=0.02). In the CPu (Fig. 3-B), no correlations were found at all for both non-aggressive and aggressive rats (r_s=0.26, p=0.62 and r_s=-0.21, p=0.56; respectively). Also, in order to evaluate if repetitive exposure to winning experiences might alter D2 receptor properties, the three first averaged AL measured during aggressiveness screening were correlated with the BP_{ND} of both NAc and CPu. No significant correlations were found in the NAc for the non-aggressive (r_s= 0.09, p=0.87) and aggressive rats (r_s=-0.24, p=0.51). Similarly, no significant correlations

were found in the CPu for the non-aggressive (r_s= 0.37, p=0.47) and aggressive rats (r_s= 0.15, p=0.68).

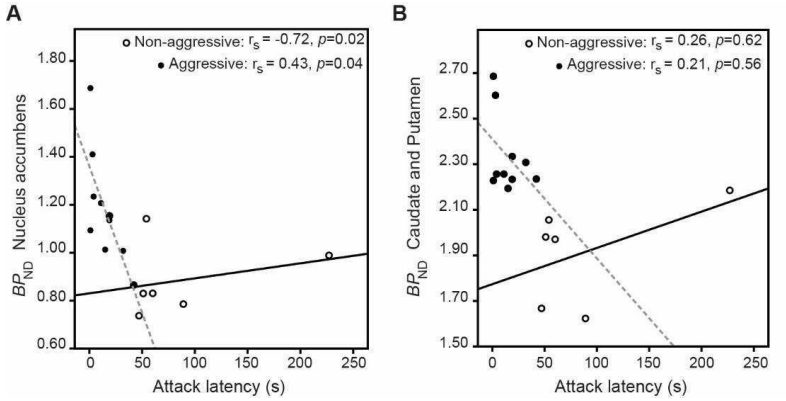


Figure 3: Spearman correlation (r_s) between the last attack latency (AL) and the binding potential (BP_{ND}) of ^{11}C-raclopride PET in the **(A)** nucleus accumbens (NAc) and **(B)** caudate and putamen (CPu) of non-aggressive and aggressive Long Evans (LE) rats.

Discussion

In the present study we have demonstrated through ^{11}C-raclopride PET that aggressive LE rats exposed to repeated winning confrontations display higher levels of D2 receptors in the striatal brain area, when compared to non-aggressive LE rats. This was followed by a decrease in the AL of aggressive LE rats relative to the number of exposures to successful aggressive confrontations.

Our results are in accordance with previously obtained data in rodents and non-human primates. In a study conducted by Jupp et al. (22), higher levels of D2/D3 receptors and dopamine transporter and decreased DA levels were found in the dorsal striatum and NAc in dominant rats than in subordinate rats. Also, in non-human primates, socially housed dominant monkeys that were engaged in aggressive behaviour had increased levels of D2 receptors in the basal ganglia when compared to subordinates, as was observed with ^{18}F-fluoroclebopride PET (23). Nader et al. (24) confirmed this finding in dominant female cynomolgus monkeys. Taking these data together, the higher levels of tracer binding in the aggressive dominant rats in our study indicates increased levels of D2 receptors and/or decreased levels of DA. These results suggest that social dominance status and/or level of aggressiveness should be considered as an important variable underlying individual variation in striatal D2 receptors.

Furthermore, a significant negative correlation between the number of aggressive confrontations and the AL was found. In this context, each exposure to winning confrontations might function as rewarding stimulus. The combination of the decreased AL and the increased tracer binding in the NAc (a brain area extensively associated with addiction (25)) after repetitive victorious aggressive confrontations seems to suggest that repetitive exposure to the rewarding effect of winning a social conflict could develop an "addictive-like" behaviour in conjunction with escalation of aggression. Additionally, an interesting finding arose from the correlation between the three first averaged AL and the BP_{ND} of the investigated brain regions, both in aggressive and non-aggressive rats. In contrast with our findings in the aggressive rats after repeated exposure to winning confrontations, we did not find any significant correlation between the three first averaged AL and brain regions in any group. This might suggest that exposure to repeated winning confrontations might alter the dopaminergic D2 receptor properties, resulting in higher BP_{ND} of ^{11}C-raclopride in the CPu and NAc of aggressive rats, but not in non-aggressive group.

In humans, impulsive violence is the most frequent form of violence with the greatest need for effective and evidence-based treatment (26). In vulnerable individuals, exposure to emotional provocative situations (e.g. drugs of addiction) leads to a weakening of control due to conditioned learning, resulting in impulsivity and compulsivity. Over time, individuals become conditioned to having violent reactions to provocative stimuli so that eventually such behaviour becomes automatic and a compulsive habit (Pavlovian conditioning) (25). This hypothesis of the evolution of violence into a habit or "addiction" might be comparable to how drugs of abuse lead from a single rewarding experience to a compulsive drug-seeking behaviour (26).

The present study has some limitations. Unfortunately, we were not able to conduct immunohistochemical analysis in brain samples in order to discriminate whether the increase in BP_{ND} of ^{11}C-raclopride in the NAc of aggressive rats was due to an increase in the D2 receptor levels or decrease in DA release. Moreover, no challenge with a dopaminergic psychostimulant (e.g. cocaine, amphetamines) was performed with the objective to evaluate vulnerability to DA reinforcers.

In conclusion, we were able to demonstrate increased D2 receptor levels in the NAc of aggressive dominant rats exposed to repetitive winning confrontations. The repetitive and habit-forming nature of aggressive winning of social conflicts might lead to escalated forms of aggression. Further studies are needed to corroborate our findings.

However, novel treatment strategies which targets the dopaminergic system and the restoration of the inhibitory controls might be of interest to decrease violence in society.

Acknowledgments

The authors would like to thank Luiz Eduardo Juárez-Orozco, Jurgen Sijbesma and Ate Boerema for their practical assistance during the conduct of this study.

Disclosure / Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Paula Kopschina Feltes was a fellowship recipient of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The Authors declare that there is no conflict of interest.

References

1. Boer SF De, Caramaschi D, Natarajan D, Koolhaas JM, Sandi C, Polytechnique E (2009): The vicious cycle towards violence : focus on the negative feedback mechanisms of brain serotonin neurotransmission. *Front Behav Neurosci*. 3: 1–6.
2. de Boer SF, van der Vegt BJ, Koolhaas JM (2003): Individual Variation in Aggression of Feral Rodent Strains: A Standard for the Genetics of Aggression and Violence? *Behav Genet*. 33: 485–501.
3. Elbert T, Schauer M, Moran JK (2018): Two pedals drive the bi-cycle of violence: reactive and appetitive aggression. *Curr Opin Psychol*. 19: 135–138.
4. Miczek K, Faccidomo S, Almeida R, Bannai M, Fish E, Debold J (2004): Escalated Aggressive Behavior: New Pharmacotherapeutic Approaches and Opportunities. *Ann N Y Acad Sci*. 1036: 336–355.
5. Golden SA, Heshmati M, Flanigan M, Christoffel DJ, Guise K, Pfau ML, *et al.* (2016): Basal forebrain projections to the lateral habenula modulate aggression reward. *Nature*. 534: 688–692.
6. Visser AKD, Ettrup A, Klein AB, van Waarde A, Bosker FJ, Meerlo P, *et al.* (2015): Similar serotonin-2A receptor binding in rats with different coping styles or levels of aggression. *Synapse*. 69: 226–232.
7. Ferrari P, Van Erp A, Tornatzky W, Miczek K (2003): Accumbal dopamine and serotonin in anticipation of the next aggressive episode in rats. *Eur J Neurosci*. 17: 371–378.
8. Patki G, Atrooz F, Alkadhi I, Solanki N, Salim S (2015): High aggression in rats is associated with elevated stress, anxiety-like behavior, and altered catecholamine content in the brain. *Neurosci Lett*. 584: 308–313.
9. Beiderbeck DI, Reber SO, Havasi A, Bredewold R, Veenema AH, Neumann ID (2012): High and abnormal forms of aggression in rats with extremes in trait anxiety – Involvement of the dopamine system in the nucleus accumbens. *Psychoneuroendocrinology*. 37: 1969–1980.
10. Kopschina Feltes P, de Vries EF, Juarez-Orozco LE, Kurtys E, Dierckx RA, Moriguchi-Jeckel CM, Doorduin J (2017): Repeated social defeat induces transient glial activation and brain hypometabolism: A positron emission tomography imaging study. *J Cereb Blood Flow Metab*. 1–15. E-pub ahead of print. DOI: 10.1177/0271678X17747189.
11. Couppis MH, Kennedy CH (2008): The rewarding effect of aggression is reduced by nucleus

accumbens dopamine receptor antagonism in mice. *Psychopharmacology (Berl)*. 197: 449–456.
12. Koolhaas JM, Coppens CM, de Boer SF, Buwalda B, Meerlo P, Timmermans PJA (2013): The Resident-intruder Paradigm: A Standardized Test for Aggression, Violence and Social Stress. *J Vis Exp*. 77: 1–7.
13. Yu Q, Teixeira C, Mahadevia D, Huang Y-Y, Balsam D, Mann J, *et al.* (2014): Optogenetic stimulation of DAergic VTA neurons increases aggression. *Mol Psychiatry*. 19: 635–635.
14. Lettfuss NY, Fischer K, Sossi V, Pichler BJ, von Ameln-Mayerhofer A (2012): Imaging DA release in a rat model of L-DOPA-induced dyskinesias: A longitudinal in vivo PET investigation of the antidyskinetic effect of MDMA. *Neuroimage*. 63: 423–433.
15. Drago F, Contarino A, Busà L (1999): The expression of neuropeptide-induced excessive grooming behavior in dopamine D1 and D2 receptor-deficient mice. *Eur J Pharmacol*. 365: 125–131.
16. Vállez Garcia D, Casteels C, Schwarz AJ, Dierckx RAJO, Koole M, Doorduin J (2015): A Standardized Method for the Construction of Tracer Specific PET and SPECT Rat Brain Templates: Validation and Implementation of a Toolbox. *PLoS One*. 10: e0122363.
17. Miczek KA, Maxson SC, Fish EW, Faccidomo S (2001): Aggressive behavioral phenotypes in mice. *Behav Brain Res*. 125: 167–181.
18. Lammertsma AA, Hume SP (1996): Simplified Reference Tissue Model for PET Receptor Studies. *Neuroimage*. 4: 153–158.
19. Innis RB, Cunningham VJ, Delforge J, Fujita M, Gjedde A, Gunn RN, *et al.* (2007): Consensus nomenclature for in vivo imaging of reversibly binding radioligands. *J Cereb Blood Flow Metab*. 27: 1533–1539.
20. Alves IL, Willemsen AT, Dierckx RA, da Silva AMM, Koole M (2017): Dual time-point imaging for post-dose binding potential estimation applied to a [11C]raclopride PET dose occupancy study. *J Cereb Blood Flow Metab*. 37: 866–876.
21. Hanley JA (2003): Statistical Analysis of Correlated Data Using Generalized Estimating Equations: An Orientation. *Am J Epidemiol*. 157: 364–375.
22. Jupp B, Murray JE, Jordan ER, Xia J, Fluharty M, Shrestha S, *et al.* (2016): Social dominance in rats: effects on cocaine self-administration, novelty reactivity and dopamine receptor binding and content in the striatum. *Psychopharmacology (Berl)*. 233: 579–589.
23. Morgan D, Grant KA, Gage HD, Mach RH, Kaplan JR, Nader SH, *et al.* (2002): Social dominance in monkeys : dopamine D 2 receptors and cocaine self-administration. *Neuroscience*. 5: 169–174.
24. Nader MA, Nader SH, Czoty PW, Riddick N V., Gage HD, Gould RW, *et al.* (2012): Social Dominance in Female Monkeys: Dopamine Receptor Function and Cocaine Reinforcement. *Biol Psychiatry*. 72: 414–421.
25. Gardner EL (2011): Addiction and Brain Reward and Antireward Pathways. In: Clark M, Treisman G, editors. *Chronic Pain Addict*. (Vol. 30), Basel: KARGER, pp 22–60.
26. Stahl SM (2015): Is impulsive violence an addiction? The Habit Hypothesis. *CNS Spectr*. 20: 165–169.

Discussion and future perspectives

CHAPTER 7

The present thesis aimed to provide evidence linking psychosocial stress with depressive-like behaviour and neurobiological alterations, such as neuroinflammation (i.e. glial activation) and alterations in brain metabolism (i.e. brain activity). Furthermore, we investigated the impact of exposure to a stressful event during adolescence on a recurrent psychosocial stressful event in aged rats. This was assessed through positron emission tomography (PET), a non-invasive technique which allows in vivo imaging of functional processes in the brain. Psychosocial stress was achieved by means of the well-validated rodent model of social defeat (also named resident-intruder paradigm). Furthermore, we addressed the underlying mechanism regarding the other side of psychosocial stress - increased aggression of the resident (dominant rat) upon repeated winning exposures.

This chapter briefly discusses the relation between the results described in the thesis and future directions. Also, it addresses the potential translational impact of this work for research and clinical practice.

Inflammatory hypothesis of depression and possible anti-inflammatory treatment strategies

One of the greatest challenges in psychiatry is to enable effective individualized treatment for patients, considering the different subtypes and symptom profiles of major depressive disorder (MDD). In order to achieve this goal, different treatment strategies may have to be applied to different phenotypes of MDD in order to improve treatment response and achieve remission. Before reaching such point in clinical psychiatry, a thorough knowledge of different underlying processes responsible for the behavioural and physiological manifestations must be achieved, especially in patients with treatment resistant MDD.

In **chapter 2** we discussed the current knowledge on the (neuro)inflammatory hypothesis of depression, a pathway that seems to play an important role in the development and progression of the disease, especially in the subgroup of treatment resistant patients. Important clinical studies performed in depressed patients with or without (and sometimes not assessed) elevated inflammatory profiles who received treatment with non-steroidal anti-inflammatory drugs (NSAIDs) were discussed. The main outcome of the studies was the relief of depressive symptoms, as evaluated through depression severity rating scales, such as the Hamilton Depression Rating Scale (1). Unfortunately, the majority of studies lack proper design and are not suitable for drawing definite conclusions regarding the inclusion of NSAIDs in the treatment of depression

either in the form of monotherapy or augmentative strategy (i.e. usage of agents that are non-standard antidepressants to enhance the therapeutic effect). Future studies should therefore include validated inflammatory biomarkers and correlate them with depression scores. Such biomarkers could be the pro-inflammatory cytokines interleukin-1β (IL-1β), IL-6 and TNF-α, found consistently in the blood of depressive patients with an elevated immune profile (2), or a more traditional biomarker, such as C-reactive protein (CRP) (3; 4). Another biomarker that should ideally be implemented in the clinical trials is the assessment of a marker of inflammation in the brain, such as the translocator protein (TSPO). PET may be able to provide such information, but major drawbacks of this approach are the high costs associated with PET scans and the limited availability of the technique, especially in countries in development. Until the present moment, no ideal PET tracer for the assessment of neuroinflammation is available for clinical imaging (Chapter 4) and therefore substantial research in this area is still required. Only when substantial proof of efficacy of an anti-inflammatory treatment approach and adequate tools for neuroinflammatory biomarker assessment are available, therapeutic guidelines might be updated and a patient tailored treatment strategy could be applied.

PET as a tool to investigate psychosocial stress-induced glial activation and alterations in brain metabolism

Inspired by the (neuro)inflammatory hypothesis of depression and taking into account the fact that social stress is a prominent risk factor for the development of MDD, a proof-of-concept study was designed (**Chapter 3**). The aim of the study was to evaluate in rats if psychosocial stress in the form of repeated social defeat (RSD) (5) was able to induce glial activation and alterations in brain metabolism measurable through PET. Depressive- and anxiety-like behaviour, corticosterone levels and brain pro-inflammatory cytokines were assessed to support the imaging results. The persistence of neurobiological and behavioural alterations was assessed 1 (short-term), 3 and 6 months (long-term) after the RSD paradigm.

In accordance with our hypothesis, five consecutive days of RSD induced glial activation (measured through ^{11}C-PK11195), decreased brain metabolism (^{18}F-FDG) and caused depressive- and anxiety-like behaviour in defeated male rats. However, these alterations were only transient and measurable in the short-term evaluation. Since neuroendocrine a0nd glial cells work together in order to restore homeostasis (6),

recovery of these systems to basal levels can be expected once the stressful stimuli is terminated.

Studies with depressed patients measuring glial activation and brain metabolism with PET in the clinical setting are in accordance with our preclinical RSD findings. Setiawan et al. investigated patients in a major depressive episode (MDE) secondary to MDD using the TSPO radioligand, ^{18}F-FEPPA. Increased tracer uptake in brain areas, such as the prefrontal cortex, anterior cingulate cortex and insula, was found in the MDE group, as compared to healthy controls. Importantly, tracer uptake correlated with depression severity, providing evidence of glial activation during a MDE (7). Hannestad et al. reported negative results when investigating patients with mild to moderate depression, using ^{11}C-PBR28 (8). An important factor that might have contributed to this result is that elevated CRP was an exclusion criterion for patients, thus excluding the MDD patients with an elevated inflammatory profile. Considering the diversity in MDD profiles, it seems plausible that glial activation is not present in all depressed patients, but only in a subgroup. In order to corroborate this hypothesis, future research should include PET imaging of TSPO expression in depressive patients with elevated peripheral inflammatory biomarkers, depressive patients with normal inflammatory biomarker levels and healthy controls. Another interesting approach would be to perform PET scans in depressive patients with treatment resistant depression. Regarding ^{18}F-FDG, the decreased brain metabolism found in the defeated rats is in agreement with the consistent decreased brain metabolism in depressed patients (9–11).

In the past, the RSD model was predominantly performed in male rats due to the resident's high levels of aggression towards an intruder. Considering that the incidence of depression is higher in women (12), with increased vulnerability to depression during the perimenopausal period (13), this was regarded as a major limitation of the model. Recent studies attempted to perform social defeat with older, lactating females as residents to elicit aggressive behaviour towards a naïve female intruder (14; 15). In contrast to male RSD, the lactating females do not show overt physical attacks against the intruder, but only threating behaviour. Despite the difference in procedure, the RSD paradigm in females was capable of increasing corticosterone levels and altering monoamine levels in the brain of the intruders (14). Whether RSD among females is also able to induce behavioural and neurobiological alterations such as glial activation and alterations in brain metabolism is yet to be determined.

Corticosterone levels are a paramount measurement to validate RSD as a rodent model for depressive-like behaviour, since it has been consistently reported that corticosterone levels are increased after RSD exposure (16). The HPA axis response is important to differentiate depression from post-traumatic stress disorder (PTSD) in animal models, since both disorders show behavioural overlap. Patients with depression typically display increased levels of plasma cortisol (17), whereas PTSD is associated with significantly lower concentrations of cortisol in plasma and urine (18). Therefore, it is hypothesized that PTSD leads to enhanced negative glucocorticoid feedback and hypocortisolism, a finding that may be highly specific for PTSD and, consequently, of major utility in the critical evaluation of experimental paradigms (19). The induction of a PTSD-like syndrome in animals should include a brief and very intense stressor, in contrast to more chronic and mild stressors in animal models of depression (20).

Even though the translation of preclinical studies to the clinic is difficult, especially in animal models of mood disorders such as depression, the agreement between our preclinical results and the available data from clinical research indicate that RSD is a good animal model to mimic the subgroup of depressed patients with elevated inflammatory profile, in combination with a psychosocial stress background. Moreover, studying social stress in the form of RSD in developmental stages could be an attractive tool to evaluate the short- and long-term impact of early-life adversities, such as peer-victimization (i.e. bullying) in adolescents, modelling physical abuse and social subordination (21).

The quest for a more suitable PET tracer for neuroinflammation

For many years, ^{11}C-PK11195 has been the tracer most commonly used for the assessment of glial activation. However, it was already demonstrated that ^{11}C-PK11195 has its limitations, such as poor signal-to-noise ratio and high non-specific binding, making this tracer not sensitive enough for detection of mild elevations in TSPO expression (22; 23). Considering these limitations, second generation TSPO PET ligands, such as ^{11}C-PBR28, have been developed and applied in animal and clinical studies (8; 24). Second generation TSPO tracers have improved signal-to-noise ratio and a higher affinity for TSPO as compared to ^{11}C-PK11195 (24). Nevertheless, these new compounds are sensitive to the human TSPO single-nucleotide polymorphism (rs6971) (25), which divide individuals in three groups: high-affinity binders (HABs; 49% of the Western population), mixed-affinity binders (MABs; 42%) and low-affinity binders (LABs; 9%)

(25), meaning that almost 10% of the Western population cannot undergo brain PET scans with second generation TSPO tracers (26). An additional test for genotyping patients is required prior to the scan and complicated statistical analyses are required to account for the differences in binding affinity between HABs and MABs. This polymorphism was not detected in rodents so far and second generation TSPO tracers are therefore still attractive for studies evaluating glial activation in the preclinical setting.

In **Chapter 4**, ^{11}C-PBR28 was validated and compared to ^{11}C-PK11195 in the rat model of herpes encephalitis (HSE). ^{11}C-PBR28 demonstrated superior imaging characteristics over ^{11}C-PK11195, resulting in the detection of more affected brain areas. Moreover, the parameters binding potential (BP_{ND}) and volume of distribution (V_T) obtained with full kinetic modelling, showed a good correlation with ^{11}C-PBR28 uptake, expressed as SUV. This enables simplified data analysis without the need of repeated blood sampling in future preclinical longitudinal studies.

Although good results can be obtained for preclinical PET imaging of TSPO with the second-generation tracers, further developments to visualize alterations in the neuroinflammatory cascade are expected. Neuroinflammation is a complex phenomenon that includes activation of microglia and astrocytes (i.e. glial cells), production of both pro- and anti-inflammatory cytokines, tissue damage and repair (27). Since neuroinflammation has detrimental and beneficial effects, knowledge of the relative contribution of each could provide information to selectively intervene in specific inflammatory processes, modifying the possible detrimental outcome that might lead to tissue damage and neurodegeneration (27), while stimulating the neurotrophic effects that lead to tissue repair. Thus, PET tracers that are able to distinguish between pro- and anti-inflammatory phenotypes of glial cells would be desired. Moreover, other targets involved in inflammation could represent new possibilities for PET imaging. Currently, PET ligands targeting for example the purinergic P2X7 receptor (28–30) and the cannabinoid receptor type 2 (CB2) (31; 32) are being evaluated in animal models of neuroinflammation.

Neurobiological and behavioural profiles following a recurrence of psychosocial stress in stress-naïve and stress-sensitized rats: impact of a previous adolescent stress exposure

Considerable evidence obtained from clinical and epidemiological research demonstrates that early-life adversity significantly increases the risk for psychiatric conditions and suicide. However, the neurobiological processes underlying this increased vulnerability

remain unclear. Long-term sensitization of both the hypothalamic-pituitary-adrenal axis (33; 34) and glial cells (35) might occur after a first exposure to psychosocial stress.

In order to evaluate the effects of a previous exposure to psychosocial stress in adolescence (in the form of RSD) on a recurrence of the stressful stimuli later in life, control and defeated rats from Chapter 3 were re-evaluated at the age of 14 months. Control rats were exposed for the first time to RSD (stress-naïve group), whereas previously defeated rats were re-subjected to the protocol (stress-sensitive group) (**Chapter 5)**. Behavioural (sucrose preference and open field test), endocrine (corticosterone), inflammatory (pro- and anti-inflammatory), cognitive (novel object recognition) and neurobiological (glial activation and glucose metabolism) alterations were assessed. Instead of using ^{11}C-PK11195, we used the previously validated second generation TSPO tracer ^{11}C-PBR28 to evaluate glial activation.

Stress-naïve (SN) rats demonstrated increasing levels of corticosterone after RSD, coupled with anxiety-like behaviour, glial activation, decrease in brain metabolism and increase in both pro- and anti-inflammatory cytokines. These effects of RSD were in accordance to results observed in Chapter 3. Surprisingly, SN rats did not show anhedonia-like behaviour, suggesting a more resilient coping style to stressful events at older age as compared to adolescence (35). Stress-sensitized (SS) rats displayed an increased neuroinflammatory (i.e. activation of glial cells) and endocrine profile even before the re-exposure to RSD, indicating that psychosocial stress during adolescence sensitizes the immune and neuroendocrine system to future stimuli. After RSD, SS rats displayed depressive- and anxiety-like behaviour, accompanied by a blunted corticosterone and glial response, decreased brain glucose metabolism and diminished levels of pro- and anti-inflammatory cytokines. Two hypotheses can be formulated based on these results: 1) the decreased (neuro)inflammatory and endocrine response to a recurrence of RSD represents a neuroprotective mechanism, halting the production of pro-inflammatory mediators that might induce further damage to the brain; 2) an inadequate (neuro)inflammatory response to a subsequent RSD, due to the cumulative effects or "costs" generated during the repeated stress exposure (36), leading to a breakdown of specific homeostatic systems (i.e. allostatic overload) (37). The design of the study in this thesis did not allow discrimination between these hypotheses and therefore, further research addressing the mechanisms orchestrating the response to recurrent psychosocial stress is warranted. Possibly other pathways than the neuroendocrine and neuroinflammatory mechanism, are responsible for differences in

behaviour between groups. Since the brain is a complex network, the interplay between neurotransmitter alterations, (neuro)inflammation, hormonal changes and epigenetic modifications (38) requires further investigation.

The other side of the resident-intruder paradigm: investigation of the reward-associated effect of repetitive winning confrontations in the brain of dominant rats

The stress-induced behavioural alterations generated in the intruder rat after repetitive defeat by the dominant rat are regularly explored as a model of depression. However, the neurobiological effects of repetitive winning conflicts in dominant (resident) rats have been significantly less investigated. In this context, a higher social rank or social status was associated with increased levels of D2 dopaminergic receptors both in primates and humans (39). Social rank in hierarchy has been linked with several behavioural characteristics such as aggression and impulsivity (39). Since the dopaminergic system has been extensively linked to the rewarding properties in the brain, it is plausible that rewarding benefits after winning aggressive confrontations might lead to alterations in the dopaminergic receptors. Seeking the rewarding feeling of defeating an intruder might be linked to further escalation of aggressiveness in dominant rats in the resident-intruder paradigm. Aggression is also present as a symptom in patients with psychiatric diseases (40) and represents a great burden to society. Therefore, research investigating the neurobiological mechanisms behind aggression is highly needed, as it would provide insights that could enable improved treatment strategies.

In **chapter 6**, we aimed to investigate if the levels of dopaminergic D2 receptors were altered in aggressive rats exposed to repeated winning confrontations, as compared to non-aggressive rats. D2 receptor levels were measured through ^{11}C-raclopride PET, using the nucleus accumbens (NAc) and caudate e putamen (CPu) as regions of interest (ROIs). In both brain regions, increased D2 receptor availability was found in aggressive dominant rats as compared to non-aggressive rats. Interestingly, binding of the tracer in the NAc, a region highly associated with addiction, was negatively correlated with the AL of dominant rats. Also, the AL was negatively correlated with the number of winning confrontations, suggesting that each exposure to winning confrontations could indeed function as rewarding stimuli.

Increased D2 receptor levels in striatal areas of the brain were also found in dominant monkeys (41) and humans with higher social / hierarchical status (42), as was assessed through PET. However, it is still unknown if higher uptake of PET tracers were

associated with increased D2 receptor expression and/or decreased dopamine release. Dopamine levels could be addressed through the combination of ^{18}F-FDOPA PET and microdialysis in future pre-clinical research. An interesting clinical population to undergo further evaluation would be martial arts aggressive fighters and violent perpetrators in order to investigate if repeated physical aggression in humans is associated with dopaminergic system alterations.

Final remarks

In conclusion, functional imaging techniques such as PET may greatly contribute to a better understanding of the underlying mechanisms in MDD and aggression both in animals and humans. Insight provided by this technique could stratify patients based on altered biomarkers and thus, improve targeted treatment strategies. PET offers the opportunity to non-invasively investigate functional alterations inside the brain. With an increasing number of clinical trials making use of this diagnostic and follow-up tool, both patients and physicians would highly benefit from the outcomes. Moreover, the continuous pursuit of optimal tracers to visualize targets of interest and optimization of PET acquisition techniques is of great importance for future advances in psychiatry and related areas.

References

1. Hamilton M (1960): A rating scale for depression. *J Neurol Neurosurg Psychiatry*. 23: 56–62.
2. Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctôt KL (2010): A Meta-Analysis of Cytokines in Major Depression. *Biol Psychiatry*. 67: 446–457.
3. Miller AH, Haroon E, Raison CL, Felger JC (2013): Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. *Depress Anxiety*. 30: 297–306.
4. Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, *et al.* (2013): A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. *JAMA psychiatry*. 70: 31–41.
5. Koolhaas JM, Coppens CM, de Boer SF, Buwalda B, Meerlo P, Timmermans PJA (2013): The Resident-intruder Paradigm: A Standardized Test for Aggression, Violence and Social Stress. *J Vis Exp*. 77: 1–7.
6. Anders S, Tanaka M, Kinney DK (2013): Depression as an evolutionary strategy for defense against infection. *Brain Behav Immun*. 31: 9–22.
7. Setiawan E, Wilson AA, Mizrahi R, Rusjan PM, Miler L, Rajkowska G, *et al.* (2015): Role of Translocator Protein Density, a Marker of Neuroinflammation, in the Brain During Major Depressive Episodes. *JAMA Psychiatry*. 72: E1–E8.
8. Hannestad J, DellaGioia N, Gallezot J, Lim K, Nabulsi N, Esterlis I, *et al.* (2013): The neuroinflammation marker translocator protein is not elevated in individuals with mild-to-moderate depression: A [11C]PBR28 PET study. *Brain Behav Immun*. 33: 131–138.

9. Saxena S, Brody AL, Ho ML, Alborzian S, Ho MK, Maidment KM, *et al.* (2001): Cerebral metabolism in major depression and obsessive-compulsive disorder occurring separately and concurrently. *Biol Psychiatry*. 50: 159–170.
10. Martinot J, Hardy P, Feline A (1990): Left prefrontal glucose hypometabolism in the depressed state: a confirmation. *Am J Psychiatry*. 147: 1313–1317.
11. Biver F, Goldman S, Delvenne V, Luxen A, Demaertelaer V, Hubain P, *et al.* (1994): Frontal and Parietal Metabolic Disturbances in Unipolar Depression. *Biol Psychiatry*. 36: 381–388.
12. Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, *et al.* (2003): The Epidemiology of Major Depressive Disorder. *JAMA*. 289: 3095–3105.
13. Katz-Bearnot S (2010): Menopause, Depression, and Loss of Sexual Desire: A Psychodynamic Contribution. *J Am Acad Psychoanal Dyn Psychiatry*. 38: 99–116.
14. Jacobson-Pick S, Audet M-C, McQuaid RJ, Kalvapalle R, Anisman H (2013): Social Agonistic Distress in Male and Female Mice: Changes of Behavior and Brain Monoamine Functioning in Relation to Acute and Chronic Challenges. *PLoS One*. 8: e60133.
15. Holly EN, Shimamoto A, DeBold JF, Miczek KA (2012): Sex differences in behavioral and neural cross-sensitization and escalated cocaine taking as a result of episodic social defeat stress in rats. *Psychopharmacology (Berl)*. 224: 179–188.
16. Patki G, Solanki N, Atrooz F, Allam F, Salim S (2013): Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. *Brain Res*. 1539: 73–86.
17. Gold PW, Goodwin FK, Chrousos GP (1988): Clinical and Biochemical Manifestations of Depression. *N Engl J Med*. 319: 348–353.
18. Yehuda R (2005): Neuroendocrine Aspects of PTSD. *Anxiety and Anxiolytic Drugs*. Berlin/Heidelberg: Springer-Verlag, pp 371–403.
19. Schöner J, Heinz A, Endres M, Gertz K, Kronenberg G (2017): Post-traumatic stress disorder and beyond: an overview of rodent stress models. *J Cell Mol Med*. 21: 2248–2256.
20. Flandreau EI, Toth M (2017): Animal Models of PTSD: A Critical Review. *Brain Imaging Behav Neurosci*. pp 289–320.
21. Buwalda B, Geerdink M, Vidal J, Koolhaas JM (2011): Social behavior and social stress in adolescence: A focus on animal models. *Neurosci Biobehav Rev*. 35: 1713–1721.
22. van der Doef TF, Doorduin J, van Berckel BNM, Cervenka S (2015): Assessing brain immune activation in psychiatric disorders: clinical and preclinical PET imaging studies of the 18-kDa translocator protein. *Clin Transl Imaging*. 3: 449–460.
23. Chauveau F, Boutin H, Van Camp N, Dollé F, Tavitian B (2008): Nuclear imaging of neuroinflammation: a comprehensive review of [11C]PK11195 challengers. *Eur J Nucl Med Mol Imaging*. 35: 2304–19.
24. Parente A, Feltes PK, Vallez Garcia D, Sijbesma JWA, Moriguchi Jeckel CM, Dierckx RAJO, *et al.* (2016): Pharmacokinetic Analysis of 11C-PBR28 in the Rat Model of Herpes Encephalitis: Comparison with (R)-11C-PK11195. *J Nucl Med*. 57: 785–791.
25. Kreisl WC, Jenko KJ, Hines CS, Lyoo CH, Corona W, Morse CL, *et al.* (2013): A Genetic Polymorphism for Translocator Protein 18 Kda Affects both *in Vitro* and *in Vivo* Radioligand Binding in Human Brain to this Putative Biomarker of Neuroinflammation. *J Cereb Blood Flow Metab*. 33: 53–58.
26. Owen DRJ, Gunn RN, Rabiner E a, Bennacef I, Fujita M, Kreisl WC, *et al.* (2011): Mixed-Affinity Binding in Humans with 18-kDa Translocator Protein Ligands. *J Nucl Med*. 52: 24–32.
27. Varrone A, Lammertsma AA (2015): Imaging of neuroinflammation: TSPO and beyond. *Clin Transl Imaging*. 3: 389–390.
28. Janssen B, Vugts DJ, Funke U, Spaans A, Schuit RC, Kooijman E, *et al.* (2014): Synthesis and initial preclinical evaluation of the P2X7 receptor antagonist [11C]A-740003 as a novel tracer of neuroinflammation. *J Label Compd Radiopharm*. 57: 509–516.
29. Ory D, Celen S, Gijsbers R, Van Den Haute C, Postnov A, Koole M, *et al.* (2016): Preclinical Evaluation of a P2X7 Receptor-Selective Radiotracer: PET Studies in a Rat Model with Local Overexpression of the Human P2X7 Receptor and in Nonhuman Primates. *J Nucl Med*. 57: 1436–1441.

30. Territo PR, Meyer JA, Peters JS, Riley AA, McCarthy BP, Gao M, *et al.* (2017): Characterization of 11 C-GSK1482160 for Targeting the P2X7 Receptor as a Biomarker for Neuroinflammation. *J Nucl Med*. 58: 458–465.
31. Hosoya T, Fukumoto D, Kakiuchi T, Nishiyama S, Yamamoto S, Ohba H, *et al.* (2017): In vivo TSPO and cannabinoid receptor type 2 availability early in post-stroke neuroinflammation in rats: a positron emission tomography study. *J Neuroinflammation*. 14: 69.
32. Slavik R, Herde AM, Bieri D, Weber M, Schibli R, Krämer SD, *et al.* (2015): Synthesis, radiolabeling and evaluation of novel 4-oxo-quinoline derivatives as PET tracers for imaging cannabinoid type 2 receptor. *Eur J Med Chem*. 92: 554–564.
33. Post M (1992): Transduction of Psychosocial Stress Into the Neurobiology of Recurrent Affective Disorder. *Am J Psychiatry*. 149: 999–1010.
34. Monroe SM, Harkness KL (2005): Life stress, the "kindling" hypothesis, and the recurrence of depression: Considerations from a life stress perspective. *Psychol Rev*. 112: 417–445.
35. Frank MG, Watkins LR, Maier SF (2013): Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. *Brain Behav Immun*. 33: 1–6.
36. Radley J, Morilak D, Viau V, Campeau S (2015): Chronic stress and brain plasticity: Mechanisms underlying adaptive and maladaptive changes and implications for stress-related CNS disorders. *Neurosci Biobehav Rev*. 58: 79–91.
37. McEwen BS (2007): Physiology and Neurobiology of Stress and Adaptation: Central Role of the Brain. *Physiol Rev*. 87: 873–904.
38. Lutz P-E, Tanti A, Gasecka A, Barnett-Burns S, Kim JJ, Zhou Y, *et al.* (2017): Association of a History of Child Abuse With Impaired Myelination in the Anterior Cingulate Cortex: Convergent Epigenetic, Transcriptional, and Morphological Evidence. *Am J Psychiatry*. 174: 1185–1194.
39. Yamaguchi Y, Lee Y-A, Kato A, Jas E, Goto Y (2017): The Roles of Dopamine D2 Receptor in the Social Hierarchy of Rodents and Primates. *Sci Rep*. 7: 43348.
40. van Schalkwyk GI, Beyer C, Johnson J, Deal M, Bloch MH (2018): Antipsychotics for aggression in adults: A meta-analysis. *Prog Neuro-Psychopharmacology Biol Psychiatry*. 81: 452–458.
41. Morgan D, Grant KA, Gage HD, Mach RH, Kaplan JR, Nader SH, *et al.* (2002): Social dominance in monkeys : dopamine D2 receptors and cocaine self-administration. *Neuroscience*. 5: 169–174.
42. Martinez D, Orlowska D, Narendran R, Slifstein M, Liu F, Kumar D, *et al.* (2010): Dopamine Type 2/3 Receptor Availability in the Striatum and Social Status in Human Volunteers. *Biol Psychiatry*. 67: 275–278.

Summary

CHAPTER 8

Major Depressive Disorder (MDD) is considered by the World Health Organization one of the most burdensome diseases in the world, with a lifetime prevalence of approximately 16%. By 2020, it is estimated to be the second leading cause of disability, secondary only to ischemic heart diseases. Not only the economic impact is problematic, but also the high associated mortality rates (suicide). Of special concern are the 30-50% of the patients who do not respond to treatment with conventional antidepressants, impacting the quality of life and increasing the vulnerability to further depressive episodes. The high rates of ineffective treatment point out to unknown mechanisms that play a role in the development and progression of this debilitating psychiatric disease, warranting further research in order to improve the patient's quality of life, with the ultimate goal of achieving remission.

For almost three decades, the neuroinflammatory hypothesis of depression has been explored and evidence has been found indicating that inflammatory processes and brain-immune interactions are involved in the pathogenesis of MDD. In **chapter 2**, we summarized the most recent data regarding the (neuro)inflammatory hypothesis of MDD (the main focus of this thesis). Moreover, we reviewed preclinical and clinical data available regarding anti-inflammatory treatments for MDD, in the form of monotherapy or augmentative strategies to conventional antidepressants. Furthermore, we discussed the anti-inflammatory properties of some antidepressants. Even though the results obtained so far are promising, the lack of proper study design makes it difficult to draw firm conclusions and to establish a solid foundation for future clinical guideline modifications.

Previous research has provided data that stress (either in physical or psychosocial form) is a major risk factor for the development of depression. Almost 25% of the patients exposed to highly stressful situations might develop MDD. In response to stressful situations, the hypothalamic-pituitary-adrenal (HPA) system releases glucocorticoids (i.e. cortisol in humans and corticosterone in animals) to regulate inflammatory responses as a consequence of stress system activation. However, prolonged stressful situations might induce neuroimmune, neuroendocrine and behavioural alterations, leading to MDD. In the proof-of-concept study designed in **chapter 3,** we investigated how repeated exposure to psychosocial stressful conditions in the form of the repeated social defeat (RSD) was able to induce neuroinflammation and alterations in brain metabolism (brain activity) in adolescent defeated rats. One of our main goals was to evaluate if those alterations could be visualized and quantified through positron emission tomography (PET), since this

technique allows in vivo visualization of tissue function and investigation of possible mechanisms underlying disease. For investigation of neuroinflammation, we used the ^{11}C-PK11195 PET tracer. ^{11}C-PK11195 has been widely used for imaging and quantification of translocator protein (TSPO) overexpression in the brain's immune cells – mainly microglia and to a lesser extent, astrocytes (glial cells). Evaluation of brain activity was performed through the glucose analogue, 2'-[^{18}F]fluoro-2'-deoxyglucose (^{18}F-FDG). In addition, we investigated behavioural and physiological biomarkers in response to RSD, shortly after RSD (1 month) and 3 and 6 months afterwards. In summary, defeated rats showed transient depressive- and anxiety-like behaviour, increased corticosterone and brain pro-inflammatory cytokine IL-1β levels, as well as glial activation and brain hypometabolism in the first month after RSD. During the 3- and 6-month follow-up, no between-group differences in any investigated parameter were found. PET imaging demonstrated to be a useful tool for the detection of RSD-induced brain alterations, which included transient glial activation and reduced brain glucose metabolism in rats. These imaging findings were associated with stress-induced behavioural changes and provide support for the hypothesis that neuroinflammation could be a contributing factor in the development of depression.

Even though ^{11}C-PK11195 is still widely used for TSPO PET imaging, second generation tracers have already been developed and proved to have superior imaging properties than ^{11}C-PK11195, such as improved signal-to-noise ratio and higher affinity for TSPO. ^{11}C-PBR28 is a second-generation tracer for TSPO imaging, which in the past has already been used in the clinics and without a full pharmacokinetic analysis in animal models of neuroinflammation. For that reason, in **chapter 4**, we evaluated ^{11}C-PBR28 as a tool for detection and quantification of neuroinflammation in the animal model of herpes encephalitis (HSE) and compared the results with those obtained with ^{11}C-PK11195 in the same animal model. Image-derived analysis such as volume-of-interest and voxel-based analysis demonstrated that ^{11}C-PBR28 is capable of detecting more brain regions affected by HSE than ^{11}C-PK11195, and the results were corroborated by the pharmacokinetic analysis (considered the gold standard of quantitative PET analysis). These results suggest that further preclinical studies would benefit from using ^{11}C-PBR28 as TSPO tracer instead of ^{11}C-PK11195, specially for mild-to-moderate animal models of neuroinflammation.

Early-life trauma and adversities in developmental stages of life are predisposing factors for developing psychiatric conditions, including MDD, at any point in life. For

that reason, we sought to investigate how a recurrence of RSD affects the neurobiological and behavioural profile of aged rats in **chapter 5**. Rats used in chapter 3 were allowed to age during 12 months under monitored conditions. At 14-months old, stress-naïve (SN; controls at adolescence) and stress-sensitized rats (SS; RSD-exposed rats at adolescence) were subjected to a 5-day RSD protocol, with neuroinflammation (i.e. glial activation) and brain activity being evaluated with the previously validated tracer ^{11}C-PBR28 and ^{18}F-FDG. Moreover, behavioural outputs, corticosterone and anti- and pro-inflammatory cytokine levels were measured at the end of the protocol. SN aged rats demonstrated a similar response as adolescent rats exposed to RSD – i.e. increased glial activation, decreased brain activity, elevated corticosterone levels and increased levels of both anti- and pro-inflammatory brain cytokines. Behaviourally, SN rats demonstrated anxiety-like behaviour. On the other hand, SS rats differed already at baseline measurements from SN rats. SS rats demonstrated increased ^{11}C-PBR28 uptake at baseline in several brain regions (indicative of glial activation), suggesting that a prior exposure to stressful conditions exacerbates glial activation during ageing. Interestingly, after the recurrence of RSD, SS rats demonstrated a decrease of ^{11}C-PBR28 uptake overtime, blunted corticosterone response followed by decreased levels of IL-1β and IL-10, as compared to SN rats. Behaviourally, SS rats showed both anxiety- and depressive-like behaviour. The neurobiological, endocrine and behavioural discrepancies observed between groups in this study cannot be explained with the current design. We hypothesize that SS rats might develop an adaptive and thus protective mechanism to cope with stress and decrease further brain damage; or these results points to a maladaptive response, demonstrating inability of SS rats to cope with stress overload. Nonetheless, other mechanisms might be involved in the alterations provoked by early-life adversities, such epigenetics, and further research is warranted to investigate the present results.

Repeated social defeat, also termed the resident-intruder paradigm, is a well-known psychosocial stress animal model capable of inducing depressive-like behaviour in defeated rats. In order to defeat a rat, a trained aggressive dominant rat is required. Thus, RSD animal model allows the investigation of both defeated and aggressive rats used in the paradigm. As the resident rats were subjected to repeated winning confrontations (in chapter 3 and 5), a unique opportunity was presented to investigate how these exposures could induce neurobiological alterations. Since we observed a reduced time to attack the intruder (i.e. attack latency; AL) as the residents won more confrontations, in **chapter 6,** we hypothesized that repetitive winning could have

rewarding effects. Using brain PET with a dopaminergic D2 receptor antagonist, ^{11}C-raclopride, we compared the dopaminergic D2 levels in striatal areas in both aggressive and non-aggressive Long Evans rats. In summary, we found increased D2 receptor levels in areas such as the nucleus accumbens, an area highly associated with addiction, in aggressive rats which correlated negatively with the AL. Taking it all together, these results suggest that repetitive winning confrontations elicit rewarding and habit-forming effects that can ultimately lead to escalated forms of aggression and violence.

Nederlandse Samenvatting

CHAPTER 9

Depressie wordt door de Wereldgezondheidsorganisatie gezien als een van de meest nijpende ziekten ter wereld, met een levensprevalentie van 16,2%. Naar schatting is depressie, na ischemische hartziekten, in 2020 de één na belangrijkste oorzaak van functiebeperking. Niet alleen de economische impact van depressie is een probleem, maar ook de hoge gerelateerde sterfte door zelfmoord. Grote zorg is ook dat 30-50% van de patiënten met een depressie niet voldoende reageert op behandeling met antidepressiva. Dit leidt tot een lage kwaliteit van leven voor deze patiënten en het maakt ze kwetsbaar voor het ontstaan van nieuwe depressieve periodes. De hoge mate van ineffectieve behandeling wijst er op dat onbekende mechanismen een rol spelen in de ontwikkeling en progressie van deze slopende psychiatrische aandoening. Dit rechtvaardigt het doen van verder onderzoek, zodat de kwaliteit van leven van de patiënten kan worden verbetert, met genezing als ultiem doel.

In de afgelopen 30 jaar is de neuroinflammatie hypothese van depressie onderzocht en is er bewijs gevonden dat interacties tussen het immuun systeem en het brein betrokken zijn bij de pathogenese van depressie. In **hoofdstuk 2** hebben we de meest recente gegevens over de (neuro)inflammatiehypothese van depressie (als focus van dit proefschrift) samengevat. Daarnaast hebben we een overzicht gegeven van de preklinische en klinische data met betrekking tot de behandeling van depressie met anti-inflammatoire medicatie, hetzij als monotherapie hetzij als additionele behandeling bij het gebruik van antidepressiva. Ook hebben we de anti-inflammatoire werking van antidepressiva bediscussieerd. Hoewel de resultaten tot dusver veelbelovend zijn, mist een goed ontwerp van dergelijke studies. Hierdoor ontbreekt de basis om een goede conclusie te trekken en aanbevelingen te doen voor toekomstige veranderingen van het klinisch beleid.

Eerder gedaan onderzoek heeft aangetoond dat stress (fysisch of psychosociaal) een groot risicofactor is voor het ontwikkelen van depressie. Bijna 25% van de patiënten die zeer stressvolle situaties hebben meegemaakt, ontwikkelen een depressie. Als reactie op stressvolle situaties geeft de hypothalamus-hypofyse-bijnier-as glucocorticoïden af (cortisol in mensen en corticosteron in dieren), voor het reguleren van inflammatoire reacties als gevolg van de activatie van het stress systeem. Echter, een te lange blootstelling aan stressvolle situaties kan resulteren in veranderingen in immuun- en endocriene systemen in het brein en tot gedragsveranderingen, wat uiteindelijk leidt tot depressie. In de proof-of-concept studie in **hoofdstuk 3** hebben we onderzocht hoe herhaalde blootstelling aan psychosociale stress, in de vorm van zogenoemde herhaalde

social defeat (RSD, herhaald verslagen worden in een sociale confrontatie)), leidt tot neuroinflammatie en veranderingen in hersenmetabolisme (hersenactiviteit) in jong volwassen ratten. Een van de voornaamste doelen was om te bepalen of deze veranderingen zichtbaar gemaakt en gekwantificeerd konden worden met positron emissie tomografie (PET), omdat deze techniek het toestaat functies van weefsels in vivo te visualiseren en mechanismen die ten grondslag liggen aan ziekten in vivo te onderzoeken. Voor het onderzoeken van neuroinflammatie is gebruik gemaakt van de PET tracer ^{11}C-PK11195. ^{11}C-PK11195 is veel gebruikt voor het afbeelden en kwantificeren van het translocator eiwit (TSPO) dat tot expressie wordt gebracht in de immuuncellen van het brein – voornamelijk microglia cellen en in mindere mate astroglia cellen. De hersenactiviteit werd gemeten met de glucose analoog 2'-[^{18}F]fluoro-2'-deoxyglucose (^{18}F-FDG). Daarnaast zijn gedrag en fysiologische biomarkers in reactie op RSD onderzocht op 1, 3 en 6 maanden na afloop van RSD. Samenvattend, ratten die *social defeat* ondergingen lieten tijdelijk depressief en angstig gedrag zien, hadden verhoogde niveaus van corticosteron in het bloed en de pro-inflammatoire cytokine IL-1β in het brein, activatie van de immuuncellen van het brein en een verlaagd hersenmetabolisme in de eerste maand na *social defeat*. Na 3- en 6-maanden werden geen verschillen tussen de groepen meer gevonden voor de onderzochte parameters. PET beeldvorming bleek een bruikbare methode voor het aantonen van veranderingen in het brein als gevolg van RSD, waaronder tijdelijke activatie van immuuncellen en verlaging van hersenmetabolisme. De resultaten van de beeldvorming waren geassocieerd met gedragsveranderingen als gevolg van de stress en dit leverde aanvullend bewijs voor de hypothese dat neuroinflammatie een bijdragende factor kan zijn in de ontwikkeling van depressie.

Hoewel ^{11}C-PK11195 nog steeds veel wordt gebruikt voor het afbeelden van TSPO met PET, zijn er tweede generatie tracers ontwikkeld die bewezen superieure kwaliteiten hebben voor moleculaire beeldvorming, zoals een betere signaal-ruis verhouding en een hogere affiniteit voor de TSPO. ^{11}C-PBR28 is een tweede generatie tracer voor het afbeelden van TSPO en is al gebruikt voor moleculaire beeldvorming binnen de kliniek en, zonder volledige farmacokinetische analyse, in diermodellen voor neuroinflammatie. Omdat nog geen volledige farmacokinetische analyse is gedaan in proefdieren hebben we in **hoofdstuk 4** ^{11}C-PBR28 geëvalueerd voor het detecteren en kwantificeren van neuroinflammatie in een diermodel voor herpes simplex virus encefalitis (HSE), en vergeleken met ^{11}C-PK11195. Analyse van de PET beelden, zoals

een analyse op basis van vooraf gedefinieerde hersengebieden of op basis van individuele voxels, liet zien dat ^{11}C-PBR28 in staat is om meer door HSE aangedane hersengebieden te detecteren dan ^{11}C-PK11195. Dit werd bevestigd door farmacokinetische analyse (wat wordt beschouwd als de gouden standaard van kwantitatieve PET analyse). De resultaten suggereren dat toekomstige preklinische studies voordeel hebben bij het gebruik van ^{11}C-PBR28 als tracer voor TSPO in plaats van ^{11}C-PK11195, vooral voor diermodellen met milde tot matige neuroinflammatie.

Het meemaken van trauma vroeg in het leven of tegenslagen in ontwikkelingsfasen van het leven, zorgt voor een verhoogde vatbaarheid voor het ontwikkelen van psychiatrische aandoeningen, waaronder depressie, op elk moment in het leven. Daarom hebben we in **hoofdstuk 5** onderzocht hoe een herhaling van RSD de neurobiologie en het gedrag van oud volwassen ratten beïnvloedt. De ratten die werden bestudeerd in hoofdstuk 3 mochten onder gecontroleerde condities ouder worden. Op een leeftijd van 14 maanden werden stress naïeve ratten (SN, de controle ratten op jong volwassen leeftijd) en stress sensitieve ratten (SS, ratten blootgesteld aan *social defeat* op jong volwassen leeftijd) blootgesteld aan *social defeat*. Neuroinflammatie (glia activatie) en hersenactiviteit werden geëvalueerd met de eerder gevalideerde tracer ^{11}C-PBR28 en met ^{18}F-FDG. Daarnaast werden gedrag, corticosteron, en anti- en pro-inflammatoire cytokines gemeten aan het einde van het protocol. SN ratten lieten een zelfde reactie zien op *social defeat* als jong volwassen ratten, d.w.z. een toename in glia activatie, afname in hersenactiviteit, verhoogde corticosteron niveaus, en verhoogde niveaus van anti- en pro-inflammatoire cytokines. SN ratten lieten ook angstig gedrag zien. Voor andere parameters, echter, waren de SS ratten al verschillend bij de baseline meting. SS ratten hadden een verhoogde ^{11}C-PBR28 opname in verschillende hersengebieden (dit wijst op glia activatie) wat suggereert dat eerdere blootstelling aan sociale stress leidt tot een verhoogde glia activatie tijdens het ouder worden. Interessant is dat na de sociale stress de SS ratten een verlaging in de ^{11}C-PBR28 opname over de tijd lieten zien, en een verminderde toename in corticosteron spiegels in combinatie met lagere niveaus van de cytokines IL-1β en IL-10, in vergelijking tot SN ratten. SS ratten lieten wel angstig en depressief-achtig gedrag zien. Deze studie geeft onvoldoende onderbouwing om de verschillen tussen SN en SS ratten in neurobiologie, endocriene reactie en gedrag al te kunnen verklaren. We hypothetiseren echter dat SS ratten een aangepaste en dus neuroprotectief mechanisme hebben ontwikkeld om te kunnen omgaan met de stress en om verdere hersenschade te voorkomen; of dat de resultaten wijzen op

een onaangepaste reactie die laat zien dat de SS ratten niet goed kunnen omgaan met de stress. Echter, andere mechanismen kunnen ook betrokken zijn geweest in de veranderingen als gevolg van de stress op jonge volwassen leeftijd, zoals epigenetische mechanismen, en verder onderzoek is daarom nodig om de resultaten beter te begrijpen.

Social defeat, ook wel het resident-intruder paradigma genoemd, is een algemeen bekend diermodel voor psychosociale stress dat in staat is om depressie-achtig gedrag te voorzaken in de rat die sociaal verslagen is. Om een rat in een confrontatie te laten verliezen is een goed getrainde en aggressieve dominante rat nodig. Het RSD diermodel laat daarom toe zowel de verliezende als de winnende dominante rat te bestuderen. De dominante ratten die in hoofdstuk 3 en 5 werden gebruikt voor veroorzaken van *social defeat*, hebben herhaaldelijk gewonnen in de confrontatie en dit bood een unieke kans voor het bestuderen van het effect van herhaaldelijk winnen van confrontaties op neurobiologische veranderingen. Omdat we zagen dat hoe vaker de dominante rat won hoe korter de tijd tot aanvallen (AL) werd, was de hypothese in **hoofdstuk 6** dat herhaaldelijk winnen een belonend effect had. Met behulp van hersen PET met de dopaminerge D2 receptor antagonist, ^{11}C-raclopride, hebben we de dopaminerge D2 receptor niveaus in striatale gebieden vergeleken tussen agressieve en niet-agressieve Long Evans ratten. Samenvattend vonden we in agressieve ratten een toename in D2 receptor niveaus in gebieden zoals de nucleus accumbens, een gebied sterk geassocieerd met verslaving. Deze toename was negatief gecorreleerd aan de AL. Deze resultaten suggereren dat herhaaldelijk winnen in een confrontatie een belonend effect heeft en aanleiding geeft tot gewoontegedrag, wat uiteindelijk leidt tot uit de excessieve vormen van agressie en geweld.

Acknowledgements

CHAPTER 10

Many people were important in this journey called a "sandwich PhD", either during the first part in the Netherlands, or back in Brazil. In the present chapter, I would like to thank the ones that were crucial for this work to be completed.

To my supervisors

Sometimes things happen in the most fortuitous manner and in the best timing as possible. I would not dare to try to explain how these events happen, since scientific reasoning lacks. However, it all began in July of 2013 when my desire to change employment met the exact timing of a between-Universities collaboration (RUG and PUCRS) agreement. At the same period as my home supervisor Dr. Cristina Moriguchi-Jeckel (PUCRS) was in Groningen for this agreement, we communicated through e-mail and all of a sudden, after an interview with Dr. Erik de Vries and a project proposition, I had scheduled my one-month "Research Voucher" through the Abel Tasman Talent Program Scholarship. **Cristina**, you were essential for all of this to happen. Your encouragement and support were fundamental for my decision to pursue an academic career. Even before that, when I was only a pharmacy student at PUCRS, you believed in me and introduced me to the Nuclear Medicine field. Moreover, you were a constant example of an ethical professional and a pioneer researcher of the area in Brazil.

Already in November of the same year, I arrived in Groningen for my one-month at the Nuclear Medicine department (NGMB). It was when I met in person my future supervisors **Dr. Erik de Vries and Dr. Janine Doorduin**. I can truly describe that one month as a life altering period, when I felt appreciated for being able to work on a hypothesis-driven project of my personal interest, with access to such knowledgeable and approachable researchers. Any doubts and fears I could have regarding moving to the Netherlands and spending the next two years performing research at the department vanished. I believe I had the best complementary set of supervisors to be able to conduct my projects, with Erik being a radiochemist and Janine, a biologist.

Erik – words lack to explain how grateful I am for all the patience and time you spent teaching me about radiochemistry (which I was a complete newbie) and science overall at the beginning of my PhD. Moreover, all the Friday meetings thought me invaluable lessons on being always critic about my data, always questioning my findings and making me seek more knowledge. Certainly, I learned a lot from you and your work ethics (and discovered you have a great taste in music!). I am forever thankful for the potential you saw in me as a future independent researcher and the opportunities you gave

me. Also, I have great recollections of funny moments during our meetings or lab "issues" with the robots, laughing even when the joke was on me. Again: *thank you.* **Janine** – without your experience and great interest in neuropsychiatry and neuroinflammation, our ideas would have continued on paper. Even when I was unsure about our experiments and animal models, you believed in it and most importantly, believed in me as having the competence to conduct them in an independent manner. Despite having a fully booked agenda, I could count on your training, help and assistance at the microPET or any other issue. All the times you went with me to the DEC meetings, and when you accepted to use my unexpected "puppies" for future projects, are fond memories. I learned so much from you and your work, and importantly, about your preclinical work ethics. For all that and much more, I thank you. Having you and Erik as my supervisors always made me unsure about many things (especially when submitting a manuscript first version), which was amazing for my knowledge growth curve. Thank you for being my most difficult and critical readers, always pushing me to be a better researcher as the Friday before.

As for my promotor, **Prof. Rudi Dierckx**, I am very grateful for the opportunity of conducting my PhD at the department. I remember every single meeting we had about my project and for the trust you always had in me. Especially, for the great interest and enthusiasm for the topics, making me feel renewed for the challenges ahead. Thank you as well for creating such an amazing work environment at the department, which for sure was a big success factor for the completion of this work. Additionally, I would like to thank you and Erik for providing the means and support necessary for my next career step.

To my colleagues-friends at the NGMB/UMCG

I have such a great recollection of memories of everyone I met, befriended, collaborated with either in our basement offices, radiochemistry or microPET lab. Throughout the whole PhD - even when I was back in Brazil – you've made yourselves present in my life, assuring me that more than friends, I have a family in the Netherlands.

Andrea Parente (my "buddy"), thank you for accepting the task of teaching me how to perform radiochemical synthesis and performing our first project together. Along with **Bram Maas** (a.k.a. "Urso Branco"), learning how to perform ^{11}C synthesis would have been way less fun and for sure not as intensive as it was. **Andrea Monroy**, your funny quotes during hard days were a nice constant to have at the office. Thank you for the friendship and for the informal medical advices.

Anniek, you were my first example on how Dutch people were very honest and straightforward. During the unforgettable period we shared at the girls "gezellig" office, you became an inspiration of a researcher who shared common interests and thought me so much. Our friendship grew quickly, with our laughs, delicious dinners, parties and a sisterhood for life. Thank you for always giving me great insights, being critical and enthusiast about my projects and personal life events. And mostly, for always being there for me, with open arms for a "bear hug".

Caroline Real, the intensive period and the struggles we shared during your postdoc at the department were the foundation of a long-lasting friendship. Thank you for allowing me to be a part of your project and having the patience to teach me stereotaxic surgery. **Carlos**, your uplifting mood and great conversations were a pleasant break from extensive data analysis and writing periods.

David, if it wasn't for your patience and great teaching skills, probably learning to independently analyze my data in two years and a half (and I mean statistical analysis, kinetic modeling, voxel-based and behavioral analysis) would not have been possible. One of the greatest memories I have with you it is about my first manuscript submission to a scientific journal. Even though it was after working hours, you stood at the office with me to help and gave me the confidence I needed to submit it. Also, your sensible and calming words in stressful times were just what I needed to keep on going. As I already told you, if one day I can be half of the professional and person you are, it will be a great achievement. Thank you for your sincere friendship.

Ewelina, you were also a great source of practical skills and help during the beginning of my PhD. You were always available when I needed, being a great collaborator and friend. Thank you for the memories in and out of work and teaching me that I should never try to keep up with a Polish girl when the subject is vodka. **Heli**, thank you for the company at the "girls" office and for your honesty. Also, thank you for being a great travel guide and advisor.

Inês, you always made me feel at home even when I was miles and miles away from it. Thank you for being available to teach me how to do ^{18}F labelling, giving me invaluable advices and for our laughs. **Jason**, thank you for our great talks about life and work, and even for the crossfit class that made me walk with so much pain for days afterwards. **Jürgen**, thank you for being constantly available to help, train me in different techniques and for solving any preclinical issues. Your dedication for the microPET

maintenance and organization were fundamental for my crazy schedule and the great number of scans I had to perform.

Isadora, the Isa of "Paulisa" – I still have no idea how we did not meet at Porto Alegre before the PhD, but I am glad Groningen gave us that opportunity. You were my link to home whenever I was homesick, gave me your shoulder whenever I needed and most importantly, was (and still is) one of my most truthful friends. When I had to leave Groningen and you were at the train platform saying goodbye, it was one of the hardest parts of coming back to Brazil. Besides being without my daily company, I was temporarily leaving my sister behind. Thank you for all the love, laughs, patience with our political conversations and for being a family in the Netherlands.

Luis (a.k.a. Dr. Douche), you were a great source of inspiration to me, with your extensive knowledge and willingness to help. You were a great collaborator and a helpful hand during my experiments, being the one I could count on for the more "difficult" tasks – even if you were already out of the office. Thank you for the critical medical and scientific inputs, and all the time spent teaching me statistics. You made the office cozier by playing the role of an "annoying" and funny "older brother", and a great company for beer nights and festivals.

Sharon, thank you for the laughs and great dinners with your delicious "pink soup". **Verena**, thank you for your kindness, sensibility and for caring. Thank you for trusting me in the beginning of your PhD, asking for advices on chemistry and lab issues. **Xiaoyun**, thank you for all the help and talks at the office after hours. To the ones that came afterwards, **Anna, Bruno, Débora, Guilherme, Kars, Lara and Rodrigo**, thank you for the trust you had in me and for being so receptive. You guys are a great team together and brought a fresh air to our group.

Dr. Hans Klein, thank you for your enthusiastic point of view regarding my data - it always prevented an anhedonic behavior after our meetings/discussions. And thank you for the fun and interesting (short) period we shared an office. **Sarita Evers**, thank you for making things happen at the department and always having a welcoming smile and an open door, even where you were crazy busy. My gratitude to all you have done for me and still does is huge. **Gerda Bakker**, thank you for always taking care of us, our offices and all the help. **Sven** and **Paul**, thank you for the constant friendly smile and jokes around the corridors.

As for the radiochemistry lab, a big thank you for all technicians who performed synthesis for me or helped me with some development. I am forever grateful to **Rolf,**

Bram, Chantal, Michel, Petra and Janet. A big thank you to **Prof. Philip Elsinga**, who was always available for giving me a radiochemistry tip and for including me in new projects.

To all the other with whom I shared moments at the coffee room or the corridors, thank you for being so receptive and friendly to me. I always felt so welcome at the department and for sure you were part of it.

As for the CDP, my projects would not have happened without the help of key personnel. Thank you for the friendly "good morning's" and all the help, **Minke van der Laan** and **Linda Smid-van der Woude**. **Ar Jansen**, thank you for the daily care and dedication to my experimental animals - I learned a lot from you. I would also like to thank the microsurgery team, **Annemieke Smit-van Oostern, Michel Weij** and **Andre Zandvoort** for the training and help with surgical procedures. Moreover, I wish to thank **Miriam van der Meulen**, the animal welfare officer, for valuable discussions on the ethical aspects of my experiments.

For my colleagues, friends, professors and staff at PUCRS

It is needless to say that being the first one to be part of a joint PhD collaboration at PUCRS was a challenge. With some necessary adjustments from both sides, we made it work. Cristina, again you were essential. Without your willingness and courage to face our challenges together, I am not sure how I would have gotten though it all. **Dr. Carla Schwanke**, thank you for being understanding and to do your best to fit the CAPES and GeronBio requirements in my PhD trajectory. **Prof. Newton Terra**, thank you for being so enthusiastic about the research with brain PET we were conducting and for being a great professor. It was always great knowing that I could count with your help whenever needed. **Dr. Ana Maria Marques**, you were always so receptive and made me feel welcome at your research group. Your kind words whenever necessary where always soothing and very important for my daily routine through the second part of my PhD. **Dr. Moisés Bauer** and **Dr. Denise Cantarelli**, thank you for your words of encouragement at my qualification exam. It might not have seemed so, but your positive words gave me strength to keep on going in the final stages of my PhD. To all the other professors, a big thank you for all the knowledge transmitted during our classes. **Samanta** and **Mônica**, thank you for all the assistance throughout the bureaucratic requirements for GeronBio and CAPES.

For my colleagues and friends, I have a special thank you for **Francelise de Freitas** – my right hand and friend when I just returned to Brazil and PUCRS. **Michele Alberton**, it was always great to have your friendly face during the classes and someone to discuss and collaborate in the Nuclear Medicine topic. **Bruna Fernandes**, without you my final semester of classes would not have been the same. I am glad that the PhD brought us together again and that I have a faithful friend in you. Thank you for everything, mainly for making sure I would take care of myself even when I forgot to do so (which was a constant). **Caroline Dartora**, thank you for being so friendly and for sharing the same interests and doubts regarding the future with me. Whenever things felt hard, you always made me laugh about it. **Valéria, Renata, Fabiane, Rodrigo, Lucimauro, Gabriela, Ana Paula (Pillatt and Bornes), Francisco Pascoal, Alceu Panini** and **Gabriel Behr**, thank you for the partnership during our classes and all learning we had together. In the second semester, a big thank you to **Luciana de Almeida** for your always cheerful mood. Thank you to all the others with whom I shared inspiring words and a coffee. At the BraIns, I thank everyone who was receptive with me. In particular, **Wyllians Borelli**, who became a collaborator and a great friend. Thank you for allowing and trusting me to be part of your SuperAgers project. **Cris Weber**, you were always there with such a great smile and positive mood – thank you for the hugs and help with the animal protection charity work in Novo Hamburgo. **Luiza Nazario**, you were always so enthusiastic about everything that it was contagious. Thank you for your friendship. **Dr. Samuel Greggio** and **Dr. Gianina Venturini**, thank you for the trust for our collaborations and for our inspirational talks. **Dr. Gabriele Zanirati**, thank you for the trust and for allowing me to be part of your doctorate project. And of course, to **Dr. Jaderson Costa da Costa**, for the warm welcome and for our inspirational talks that made me feel I could always contribute to the BraIns with ideas.

For my European friends

I was so lucky to have befriended so many special people in Groningen, outside the office as well. **Morgana Teixeira**, even though you are Brazilian, you were the first friend I had when I moved to the Netherlands. Thank you for the partnership we shared, the happy moments and for making Groningen more Brazilian-like.

Nicole Pirozzi, who would have imagined that during Dutch classes I would have met such a special person? From our weekly conversations about our new life in the

Netherlands to a real friendship, I thank you for all the moments we shared together. Keep on being this sweet person that always made itself present even when we were far apart.

Yiwen Xi, the Chinese girl I met during a beer trip through Belgium in 2014 and became a sister to me. I am so lucky to have met you and that we had the opportunity of spending every summer together since 2014. Our friendship transcends the Dutch borders and you can count on me for life.

Erika Iglesias, I will never forget all the support you gave to me, particularly in the summer of 2017. Your honest and caring personality are very special. Thank you for everything, truthfully.

Francesca Inglese, thank you for the very special time we had during your internship in Groningen and for your friendship. Also, for cooking delicious homemade Italian dishes for me.

And for the ones who always took care of my dear boyfriend Daniël Baaijens during my times of absence, **Sjoerd, Marthe, Whitney, Mitch, Martijn**, and all the friendly staff of De Pintelier. I wish to thank **Ricky, Sarah, Jesse, Ezra** and **Eric Harder**, for the constant presence as well.

For my friends back in Brazil – para meus amigos no Brasil

Mesmo que as mudanças de endereço, cidade, estado e país tenham sido graduais, nunca foram fáceis. Felizmente, eu tive a sorte de conhecer vocês durante a minha vida e os mesmos sempre se fizeram presente em todos os momentos. Por isso, gostaria de destacar os amigos **Letícia Salin, Bruna Fonseca, Joseane Fabian, Tábata Lammel e Gabriel Cianeto** – palavras faltam para agradecer tudo que passamos juntos. Eu carrego vocês sempre dentro do meu coração cada vez que vou para algum lugar novo e, mesmo que a distância nos separe, nossa amizade será eterna.

Lê, sem a tua amizade e presença constante durante o período que retornei ao Brasil, não sei o que seria de mim, emocionalmente e fisicamente. Tu sempre cuidaste de mim e nossa amizade de mais de 20 anos mostra o quão especial é o que construímos. Mesmo longe, estaremos para sempre do lado uma da outra. **Bruna**, obrigada pelo ombro, pelas palavras, pela amizade, pela segunda família, enfim, por tudo que me ofereceste e que trocamos desde que nos tornamos amigas. **Josie**, a tua presença é uma constante alegria na minha vida. As memórias dos nossos verões inesquecíveis e brincadeiras estão para sempre guardadas no meu coração. **Tabi**, obrigada por nunca esquecer de mim mesmo quando eu esquecia o que era ter vida social durante o doutorado. **Gabi**, é difícil

descrever o que tu representas para mim. Sem dúvida és uma parte especial de mim e da minha vida. Nossos momentos compartilhados desde o Liberato até hoje, sejam eles de qualquer natureza, foram muito especiais. Tu és iluminado na tua bondade e pureza.

Além dos já mencionados, a segunda parte do meu doutorado não teria sido possível sem as inúmeras contribuições intelectuais do profissional e amigo, **Dr. Paulo Roberto Luchesi Soares –** meu eterno obrigado**.**

For my Family – para minha família

Many times, I felt as if I missed important life moments of our Family members due to my career and dreams. Nevertheless, you were always understanding and cheering for my success. For that support, I thank you and love you more than I could ever express.

The one who played the most important role throughout this whole process was my mom, **Márcia**. Sem o teu apoio constante e as palavras de encorajamento durante os momentos que eu pensei que não ia conseguir seguir em frente, eu não teria chegado onde estou. A trilha nunca foi fácil para nós duas, mas sempre permanecemos unidas e atravessamos todas as dificuldades. A distância sempre foi a maior delas e sem dúvida, se não fosse por ti, eu jamais teria a coragem de alçar vôo e conquistar meus sonhos. *Esta tese é dedicada a ti.*

Ao meu **pai (Luiz), mana (Maria Eduarda), Elaine** e **Fernando**, o meu muito obrigada pelas palavras constantes de apoio e pelo orgulho que sempre demonstraram das minhas conquistas. Sem dúvida isso me ajudou a seguir em frente, mesmo quando eu achei que não seria capaz. Obrigada por entenderem a minha natureza viajante e minha curiosidade pela ciência e o mundo.

Aos meus **avós**, por serem sempre compreensivos e orgulhosos da neta, dos quais sempre vou sentir falta de ter passado mais tempo junto.

Ao meu primo **Luis**, obrigada pela parceria de vida e apoio. Um dos maiores presentes que tu pudeste me dar, juntamente com a **Elis**, foi a **Helena** como afilhada. Espero que de alguma forma eu possa influenciar positivamente a jornada da pequena. Obrigada também pela presença constante, mesmo que via telefone e mensagens.

Aos meus tios e primos, obrigada pelo constante apoio e palavras de encorajamento.

And finally, I would like to thank one of my biggest supporters, my boyfriend **Daniël Baaijens**. I still don't know how you had the courage to date a girl that in theory had a limited period of stay in the Netherlands. However, when we find a special person,

we are able to go through the hardest circumstances together. Thank you for staying with me when I went back to Brazil and for keeping up during the heart-breaking moments we had to say "see you soon" at the airport. Thank you for being present every day, physically, or by phone, to give me a word of encouragement and hope of a great future together. This journey would not have been the same without you and I love you for that. **Irma van Leeuwen**, thank you for the gift that your son is and for accepting me at your family with so much love. You and **Tony** made me feel as if I were with my family, even when in the Netherlands.

Pontifícia Universidade Católica do Rio Grande do Sul
Pró-Reitoria de Graduação
Av. Ipiranga, 6681 - Prédio 1 - 3º. andar
Porto Alegre - RS - Brasil
Fone: (51) 3320-3500 - Fax: (51) 3339-1564
E-mail: prograd@pucrs.br
Site: www.pucrs.br