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Neuroimmunological processes in rodent models of inflammation-associated depressive disorders

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"Somewhere, something incredible is waiting to be known."
Carl Sagan, 1934-1996

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AAALAC	association for the assessment and accreditation of laboratory animal care
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BBB	blood-brain barrier
BCG	Bacillus Calmette-Guérin
BDNF	brain-derived neurotrophic factor
BL	baseline
BSA	bovine serum albumin
CCD	charge-coupled device
C/EBP	cytidine-cytidine-adenosine-adenosine-thymidine enhancer binding protein
cm	centimeter
CNS	central nervous system
CRF	corticotrophin-releasing factor
CRP	C-reactive protein
CXCL1	(C-X-C motif) ligand 1
D	day
dT	delta temperature
ε	epsilon
ELISA	enzyme-linked immunosorbent assay
FAM	familiarization day
g	gram
GFAP	glial fibrillary acidic protein
Gfap-luc	FVB/N-Tg(Gfap-luc)-Xen
h	hours
H	height
HPA	hypothalamic-pituitary-adrenal
Iba1	ionized calcium-binding adapter molecule 1
IDO	indoleamine 2,3-dioxygenase
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IL-1β	interleukin-1 beta
IL-1ra	interleukin-1 receptor antagonist
IL-2	interleukin-2
IL-4	interleukin-4
IL- 6	interleukin-6
IL-10	interleukin-10
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
IVC	individually ventilated cage
K3MO	kynurenine 3-monooxygenase
kg	kilogram
L	length
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
mg	milligram
MIP-1α	macrophage inflammatory protein-1 alpha
mL	milliliter

mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-kappa beta
NMDA	N-methyl-D-aspartate
NS	no stress
OFT	open field test
PBS	phosphate-buffered saline
PET	positron emission tomography
PFA	paraformaldehyde
Poly(I:C)	polyinosinic:polycytidylic acid
rmANOVA	repeated measures analysis of variance
RNA	ribonucleic acid
ROI	region of interest
s	seconds
S	stress
SEM	standard error of the mean
SIH	stress-induced hyperthermia
SNRIs	serotonin-norepinephrine reuptake inhibitors
SPT	sucrose preference test
SSRIs	selective serotonin reuptake inhibitors
sTNFRs	soluble tumor necrosis factor-alpha receptors
sr	steradian
SUV	standardized uptake values
TDO	tryptophan 2,3-dioxygenase
TGF-β	transforming growth factor beta
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TSPO	translocator protein
TST	tail suspension test
Veh	vehicle
W	width
W/W	water/water
W/S	water/sucrose

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Introduction and aims

1.1 The immune system

Living organisms are constantly threatened by potentially harmful stimuli. The nature of these stimuli is diverse and comprises both sterile tissue damage and septic challenges such as bacterial and viral infections. Fortunately, all vertebrates possess a complex defense mechanism that protects them from disease. This so-called immune system is comprised of several biological structures and cell types that provide a layered defense with increasing specificity. These processes are generally classified into innate and adaptive immune responses. Innate immunity is mainly executed by the complement system, circulating leukocytes, natural killer cells and tissue resident macrophages. The primary role of innate immunity is to quickly eliminate pathogens in a non-specific manner and to present antigens to cells from the adaptive immune system, such as T and B lymphocytes. These cells in turn initiate adaptive immunity by generating responses that are tailored to specifically eliminate the pathogen or pathogen-infected cells. Moreover, the adaptive immune system creates immunological memory which facilitates faster, more effective responses to previously encountered antigens.

1.1.1 Inflammation

Inflammation is the immune system's first line of defense against tissue damage and occurs rapidly upon detection of harmful challenges. Cells from the innate immune system express pattern recognition receptors that react with specific molecular patterns affiliated with cellular stress or microbial pathogens. Upon recognition of such patterns, these immune cells undergo rapid activation and elicit an inflammatory cascade which aims to protect and defend the body [1]. Central to the initiation of inflammatory responses is a robust release of proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α. This cytokine release occurs in a highly orchestrated fashion and includes several positive and negative feedback mechanisms. For example, IL-1β, IL-6 and TNF-α induce their own expression but also activate the synthesis and release of other proinflammatory cytokines such as interferons [2-4]. Moreover, they trigger the production of cytokines that antagonize their action.

These anti-inflammatory molecules can be specific of a given proinflammatory cytokine, such as IL-1 receptor antagonist (IL-1ra) that binds specifically to IL-1 receptors, or suppress the production of a number of proinflammatory cytokines, such as IL-10 that downregulates the production of IL-1β, IL-6 and TNF-α [5-7]. In addition to pro- and anti-inflammatory cytokines, a wide variety of chemokines are also released during an inflammatory response. These chemokines are small chemotactic cytokines that regulate the migration and infiltration of immune cells, such as leukocytes, into the site of inflammation. An important step in this infiltration process is the upregulation of cell surface molecules on endothelial cells of blood vessels. These molecules facilitate selective extravasation of leukocytes into the afflicted tissue site, where they get activated by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells [8]. Eventually, the mobilization and interaction of several cell types and signaling molecules leads to neutralization of the inflammatory trigger and initiation of processes that ultimately lead to tissue repair. Depending on the degree or extent of the inflammatory stimulus, inflammatory responses can go systemic, thereby leading to the release of cytokines into the circulation and the production of acute phase proteins in the liver [9]. Under normal circumstances, inflammation is a local and acute, self-limiting process that serves to remove any source of danger without causing excessive harm to the host. However, maladaptive inflammatory responses may continue unabated and eventually result in chronic low-grade inflammation that fails to resolve [10]. Signs of such sustained inflammation have been demonstrated in several diseases, including cardiovascular disorders, autoimmunity, diabetes, asthma and neurodegenerative and psychiatric disorders.

1.1.2 Innate immunity in the central nervous system

The brain has long been thought to be an immune-privileged organ. Though immune processes within the central nervous system (CNS) are not identical to those occurring in the periphery, it is now clear that the brain has resident immune cells that are capable of inducing a wide range of inflammatory responses, collectively termed neuroinflammation. The main effector cells of innate immunity in the brain are microglia, which are the tissue specific

macrophages of the CNS. In the healthy brain, microglia display a ramified appearance, characterized by a small soma with fine cellular processes. This ramified morphology is associated with a quiescent phenotype [11]. However, even in this presumed "resting state", microglia are highly active, providing trophic support to neurons, rearranging synapses and continually surveying the local microenvironment [12]. Upon detection of even the slightest homeostatic disturbance, microglia undergo rapid transition to an "activated state". During this activation process, microglia go through multiple morphological, molecular and functional changes, which include upregulation of specific cell surface markers and the release of cytokines and other inflammatory molecules. Over the past years it has become clear that microglia show many states of activation, depending on the nature of brain injury and the stage of the inflammatory response [1, 13, 14].

Apart from microglia, astrocytes also play a fundamental role in innate immune processes in the brain. These astrocytes are specialized glial cells that are ubiquitous throughout all regions of the CNS [15]. Under physiological circumstances, astrocytes are involved in a wide variety of crucial brain processes, including the regulation of ion and neurotransmitter homeostasis, modulation of synaptic activity, provision of metabolic support to neurons, control of cerebral blood flow and regulation of blood-brain barrier (BBB) permeability [16-20]. As is the case for microglia, astrocytes also respond to impaired homeostasis by undergoing various molecular, morphological and functional changes. [21]. These changes are considered to be crucial in the regulation of neuroinflammation and have been shown to facilitate neural repair and protection [21, 22]. Although astrocytes and microglia are the best characterized mediators of neuroinflammation, they are not the only cell types that are involved in this highly orchestrated process. Other brain cells, such as perivascular macrophages, endothelial cells, oligodendrocytes and neurons are also known to produce chemokines and cytokines and express their receptors [23]. Normally, neuroinflammation serves the physiological purpose of restoring brain homeostasis. However, impaired or protracted neuroinflammatory processes can potentially be harmful and glial cell abnormalities are commonly found in patients suffering from prevalent neurodegenerative and psychiatric disorders [19, 20, 24].

1.1.3 Neuroimmune communication

It is well established that reciprocal communication occurs between the immune system and the brain. The clearest example of such immune-to-brain communication is sickness, a highly orchestrated strategy of the body to fight infection. This motivational state is characterized by a fever response, neuroendocrine changes and a behavioral component that allows the body to save energy and combat the pathogen under optimal conditions [25]. It has become clear that sickness is mediated by proinflammatory cytokines produced and released in the periphery. However, to exert central effects these relatively large and hydrophilic molecules need to pass the lipophilic BBB to access the brain. Although saturable transport systems are identified that actively traverse specific cytokines across the BBB [26], these mechanisms are likely not sufficient to induce a rapid and robust increase in brain cytokines following peripheral infection [27]. Indeed, several other pathways been have identified through which peripheral immune molecules can enter the CNS. These pathways are generally divided into humoral, neural and cellular branches (figure 1.1) [28]. Apart from passing the brain endothelium through active transport mechanisms, cytokines can also passively cross the BBB. This occurs at structures such as the choroid plexus and circumventricular organs, where the vasculature lacks tight junctions between capillary endothelial cells and therefore have a leaky BBB [27, 29]. Moreover, circulating cytokines bind specific cytokine receptors expressed by endothelial cells and perivascular macrophages lining the brain vasculature. This leads to activation of these cells and subsequently stimulates the release of more cytokines and other inflammatory mediators across the brain parenchyma [30]. Another route of neuroimmune communication occurs through the vagus nerve. In this neural pathway, peripheral cytokines bind their receptor on afferent nerve fibers, which directly activates specific brain targets without interference of the BBB [31]. Finally, it is demonstrated that activated immune cells from the periphery can be recruited into the CNS and hence contribute to an inflammatory tone in the brain. Central to this cellular pathway of immune-to-brain communication is the release of chemokines such as monocyte chemoattractant protein-1 (MCP-1), which are released from cerebral microglia triggered by proinflammatory cytokines [3].

Once in the brain, cytokines are sensed by a network of several cell types which in turn produce more immune mediators to amplify the cytokine signal. Eventually the interaction of cytokines with specific brain targets leads to behavioral changes that characterize sickness. To restore homeostasis, proinflammatory cytokines in the brain also activate pathways that provide negative feedback to the immune system and dampen the inflammatory episode [32]. These anti-inflammatory responses include activation of the so-called cholinergic anti-inflammatory pathway, and stimulation of the hypothalamic-pituitary-adrenal (HPA) axis. The immunosuppressive functions of the cholinergic anti-inflammatory pathway are mediated through vagal release of acetylcholine, which activates the α7 subunit of the nicotine acetylcholine receptor expressed by macrophages and other cytokine secreting cells. This ultimately leads to suppression of proinflammatory cytokine production [33]. Stimulation of the HPA axis, on the other hand, reduces the inflammatory response through release of glucocorticoids into the circulation. Various immune cells throughout the body express receptors for glucocorticoids and upon interaction with their ligand these recptors trigger several anti-inflammatory mechanisms, including suppression of proinflammatory cytokine release, activation of anti-inflammatory cytokine production and inhibition of effector cell function [34-36].

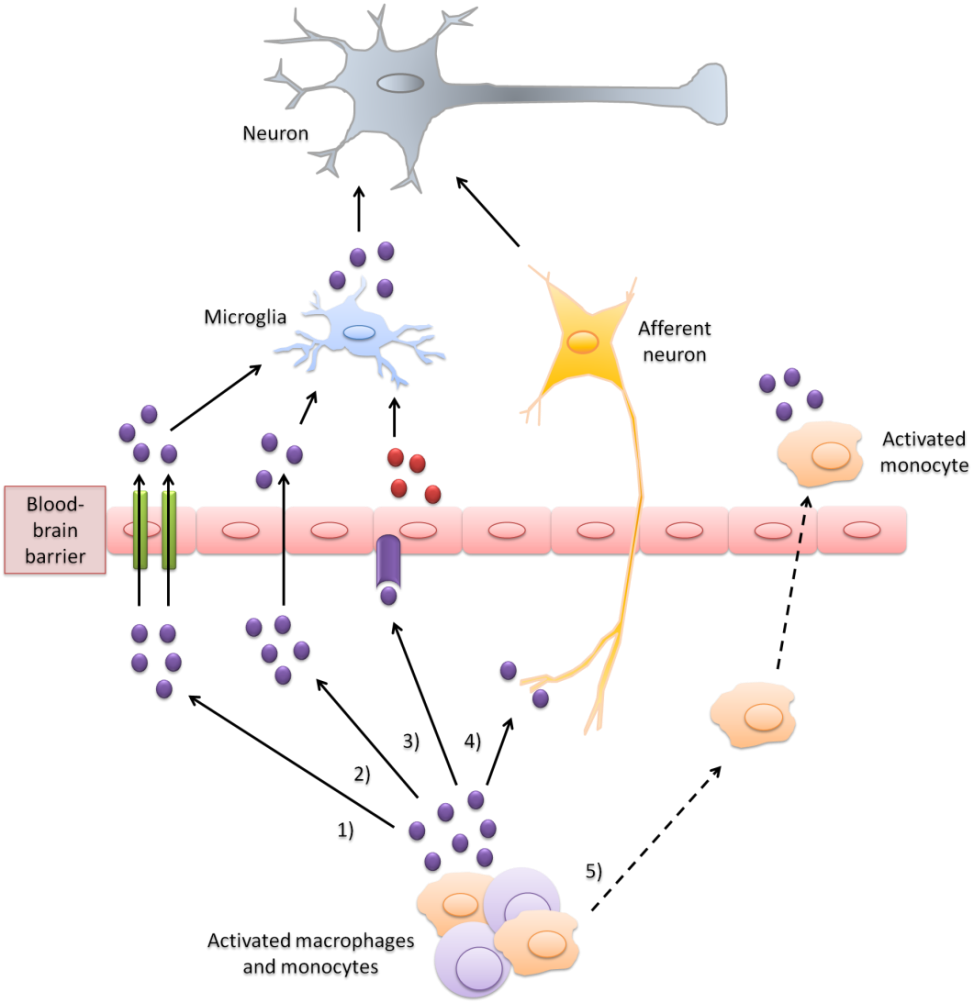

Figure 1.1 Immune-to-brain communication pathways. Cytokines released in the periphery can access the brain through humoral, neural and cellular routes. The humoral route includes active transport (1) and passive diffusion (2) of cytokines across the blood-brain barrier. Moreover, cytokines interact with their receptor expressed on endothelial cells and stimulate these cells to secrete more immune mediators into the brain parenchyma (3). Brain entry through the neural route involves binding of cytokines on receptors expressed by nerve fibers of afferent neurons, which transfer the cytokine signal directly to specific brain areas (4). Finally, although less characterized, a cellular route has been described where cytokines are released from activated monocytes that migrate from the periphery into the perivascular space (5). Once in the brain, cytokines are recognized by a network of several cell types which in turn produce more immune mediators to amplify the cytokine signal. Eventually, cytokines will interact with specific brain targets and induce behavioral alterations. Adapted from Seruga et al. 2008 [37].

1.1.4 The inflammatory nature of psychological stress

Psychological stress is part of everyday life and we are constantly exposed to many types of stressful events. These stressful experiences range from minor affairs such as giving a presentation at work to life changing events such as the loss of a job or ongoing relationship difficulties. Based on the nature and duration of the stressful event, it can cause either short-lasting discomfort or chronic uncontrollable emotional pressure.

Research has indicated that acute and chronic psychological stress induces pronounced changes in innate and adaptive immune responses [38]. Corroborating with this idea, several studies demonstrated that psychological stress induces a proinflammatory tone in the body. For example, stressful academic examination increased the release of proinflammatory cytokines from stimulated blood cells of healthy students [39, 40]. Moreover, exposure to the Trier Social Stress Test, which includes a public speaking and mental arithmetic stressor, increased activity of the inflammatory transcription factor NF-κB in blood cells of healthy volunteers [41]. Using the same test, it was shown that adults with a history of childhood maltreatment exhibit heightened IL-6 plasma levels in response to acute stress challenges [42]. Exposure to work-related stress such as high job demands, organizational downsizing and unemployment caused a measurable reduction in NK cell activity and increased inflammatory markers [43]. Other chronic stressors, such as lower socioeconomic status or feeling lonely for a prolonged period of time, were also associated with elevated inflammatory responses [44-48]. Finally, elevated circulating levels of the inflammatory markers C-reactive protein (CRP) and TNF-α were found in Alzheimer patient caregivers [49].

In addition to these measures of increased peripheral immune activation following stress in humans, experimental work in laboratory animals indicted that psychological stress also leads to a proinflammatory response in the brain. Indeed, several rodent studies using various stress paradigms have indicated that psychological stress increased the release of inflammatory molecules in the CNS [50-55] and stimulated the activation and proliferation of glial cells [56-58]. However, these responses seem to be highly dependent on the type of laboratory stress model used [59] and conflicting results are often reported even when similar paradigms were used.

Taken together, these findings demonstrate that stress is linked to inflammation. Although a proinflammatory phenotype might be beneficial in case of brief stressors, for example to facilitate wound healing after a predator attack, it is generally accepted that prolonged or uncontrolled continuation of stress responses can disrupt immune function and increase the risk of developing mood disorders.

1.2 Major depressive disorder

Major depressive disorder, or depression, is a devastating psychiatric illness that has a life time prevalence of around 16% [60]. It is predicted that by 2030 depression will be the second leading cause of disability worldwide [61]. Clinical manifestations of depression include a range of symptoms, such as a persistent depressed mood, the inability to experience pleasure from naturally rewarding activities (anhedonia), feelings of worthlessness or excessive guilt, anxiety, slowness (psychomotor retardation) and suicidal ideations. In addition, depressed patients may display vegetative symptoms such as fatigue, changes in appetite, weight loss, sleep disturbances and impaired concentration [62]. Apart from limiting a patient's ability to cope with daily tasks and responsibilities, depression also increases the risk of mortality. Depressed patients, for example, are over 20 times more likely to commit suicide than the general population [63]. Moreover, being diagnosed with depression increases the mortality risk of patients suffering from other medical conditions, but also of people without chronic diseases [64-66].

Several classes of anti-depressive drugs are currently available. However, the therapeutic efficacy of these antidepressants is insufficient. Only a third of depressed patients experience full remission after the first round of treatment and placebo-expectancy effects are typically larger than specific drug effects [67]. In addition to low therapeutical success rates, currently available anti-depressive drugs need to be taken for several weeks before mood-altering effects occur and the development of undesired side effects limits their usefulness even further [68]. Even in case of successful treatment, fifty to eighty percent of patients with depression experience relapse [69]. Eventually, about one third of all depressed patients end up with some degree of treatment resistance.

Despite its high prevalence and socioeconomic impact, very little is known about the pathophysiology of depression. Classic theories on serotonergic dysfunction and cortisol hypersecretion have been studied extensively, but fail to provide sufficient explanations for the etiology of the disease [70]. Though these systems are clearly involved in the pathophysiology of the disorder, it is now generally accepted that depression is a multifactorial disorder that results from a complex interplay between genetic and environmental factors (figure 1.2).

Several lines of evidence suggest that inflammatory processes may also be involved in the pathogenesis of depression, at least in a subset of susceptible individuals.

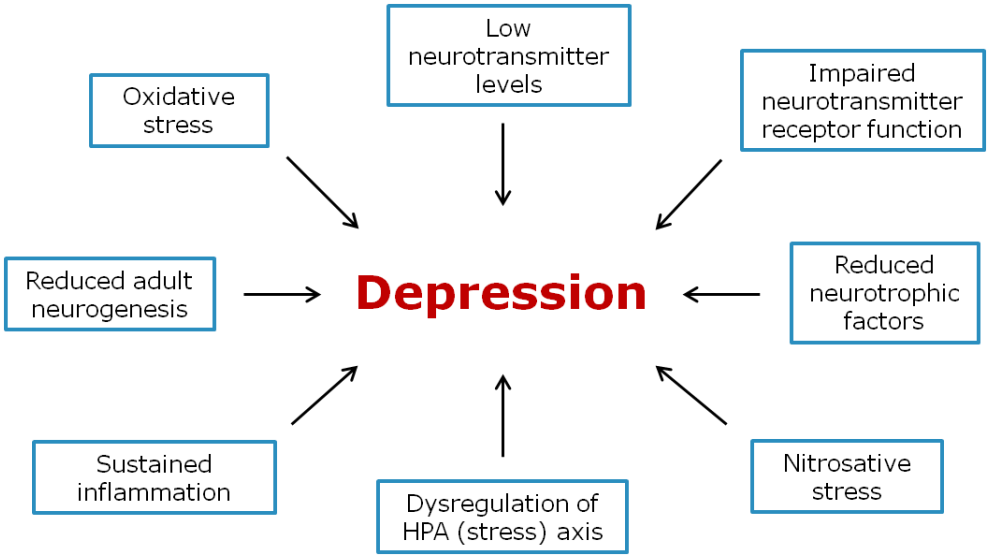

Figure 1.2 Overview of pathogenic processes in depression. Adapted from Chopra et al. 2011 [71].

1.2.1 Inflammation-associated depression

The association between inflammation and depression has been confirmed in various clinical populations [72]. For example, depression frequently occurs as a comorbidity of illnesses characterized by a chronic inflammatory component, including rheumatoid arthritis [73], cancer [74], type 2 diabetes [75] stroke [76], obesity [77] and coronary artery disease [78]. Even in the absence of other medical conditions, depressed patients often show signs of altered immune function. These include impaired cellular immunity and increased markers of inflammation, such as elevated levels of inflammatory cytokines and their receptors in peripheral blood and cerebrospinal fluid, as well as elevations in the circulating concentration of chemokines, acute phase proteins and adhesion molecules [10, 32, 79, 80]. Another line of evidence linking immune activation to depression comes from cancer and hepatitis C patients that receive

therapeutic administration of the proinflammatory cytokines interferon (IFN)-α or IL-2. Several studies showed that up to half of these patients develop psychological symptoms that are very similar to those seen in idiopathic depression [72]. It needs to be noted, though, that in most of these studies the severity of depressive symptoms was compared before and after the onset of cytokine treatment. The lack of a control group consisting of cancer or hepatitis C patients that do not receive cytokine treatment makes it difficult to fully attribute the development of depressive symptoms to the immunostimulating therapy. Indeed, it could be argued that being diagnosed with a medical condition such as hepatitis C or cancer imposes significant psychological distress and is therefore a risk factor for depression per se. However, there are several indications that the immunostimulating therapy does contribute to the development of depressive symptoms. First of all, although prevalence rates of depression are higher in cancer patients than in medically healthy individuals [81], they do not exceed those seen in patients receiving cytokine treatment. Secondly, depression-related neurovegetative symptoms occur rapidly after the onset of cytokine therapy, thereby indicating that they are triggered by immune activation. Thirdly, pretreatment with the antidepressant paroxetine significantly reduces the risk of developing depression in cancer patients undergoing IFN-α therapy [82]. Finally, studies on people receiving typhoid vaccination and volunteers administered with low doses of bacterial endotoxin have demonstrated that immune activation causes a depressed mood in medically healthy subjects [83-86]. Interestingly, the severity of depressive symptoms in these endotoxin-injected volunteers correlated with increases in peripheral blood cytokine concentrations [85].

The behavioral response to immunostimulating therapies appears to be biphasic (figure 1.3). Soon after the initial cytokine administration most individuals develop a neurovegetative syndrome that is characterized by symptoms of fatigue, anorexia, psychomotor slowing and sleep disturbances. These symptoms persist throughout the treatment period and do not respond to antidepressants [87]. After a few days or weeks, however, around half of the patients develop a syndrome in which symptoms such as depressed mood, anxiety, memory disturbance and impaired attention predominate [88]. These symptoms have been shown to ameliorate in response to antidepressants [87]. Taken together, these findings indicate that prolonged or aberrant activation of

the immune system may contribute to the etiology of depression. However, it must be taken into account that not all depressed patients show immune alterations and inflammation-associated depression should be considered a subset, rather than representative of the whole depressed population [89].

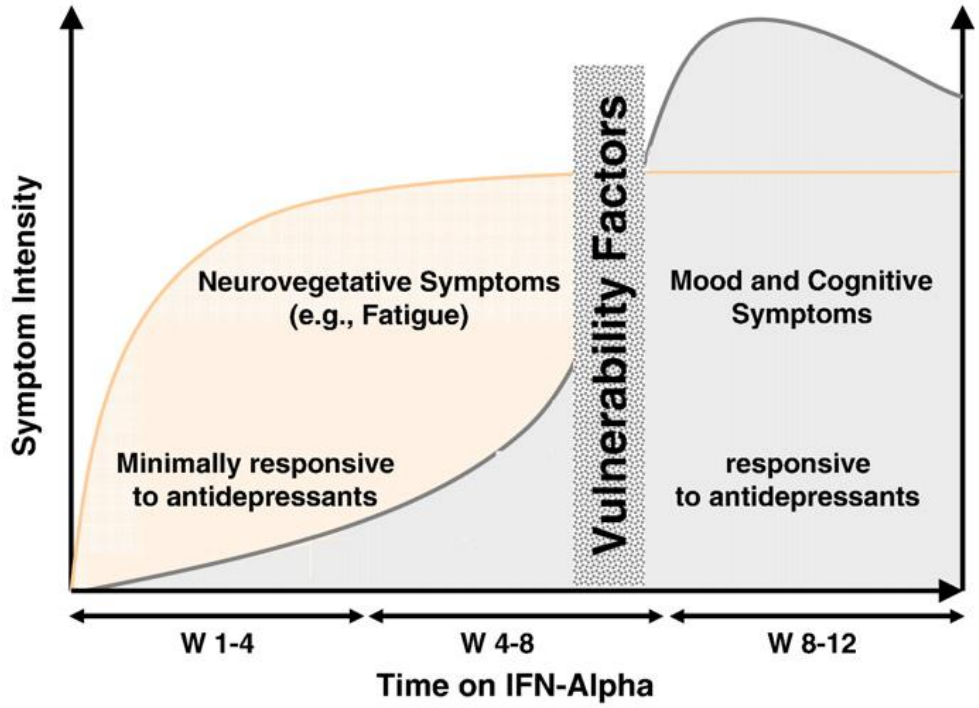

Figure 1.3 Temporal dissociation between the behavioral states induced by proinflammatory cytokine treatment. Therapeutic administration of the inflammatory cytokine interferon-α induces neurovegetative symptoms such as fatigue, anorexia, psychomotor slowing and sleep disturbances. These symptoms occur rapidly after the onset of cytokine treatment and persist throughout the exposure period. After a few days or weeks, up to half of the patients develop mood and cognitive symptoms characterized by depressed mood, anxiety, memory disturbance and impaired attention. These mood and cognitive symptoms ameliorate following antidepressant treatment, whereas neurovegetative symptoms are unresponsive to antidepressants. Reprinted from Capuron et al. 2011 [28].

1.2.2 Modeling inflammation-associated depression in rodents

Fundamental research studying complex neurological disorders such as depression relies on the availability of validated animal models. These models should resemble the human condition in several aspects, including (1) sharing similarity between the behavioral phenotype and the clinical symptomatology (face validity), (2) amelioration or attenuation by antidepressant treatments and conversely unresponsiveness to clinically ineffective therapies (predictive validity), (3) comparable neurobiological underpinnings (construct validity) and (4) triggering by events known to play a role in eliciting the condition in humans (etiological validity) [90-92].

To date, there is no validated animal paradigm that models the entire complex syndrome of depression. Instead, most available animal models focus on certain phenotypic characteristics of the disorder. Despite major scientific efforts over the past decades, the development of more elaborate animal models of depression is hampered in several ways. First of all, depression is a heterogeneous disorder with unknown etiology and many risk factors contributing to disease development. This creates a catch-22 situation as valid animal models are needed to study depression but it is difficult to model an unknown disease construct [92]. In addition, much of the symptomatology of depression such as depressed mood, low self-esteem and suicidal ideology cannot be modeled or addressed in non-humans. Moreover, the high therapeutic inefficacy of currently available antidepressants impedes the use of reference drugs and thereby decreases the predictive capacity of animal models [93].

Based on clinical observations of immune dysregulation in depressed patients, several rodent models of inflammation-associated depression have been generated. One of the most used approaches in these models involves administration of bacterial lipopolysaccharide (LPS), which is a potent activator of the immune system. When introduced into an organism, LPS is recognized by toll-like receptor (TLR) 4 expressed by cells of the innate immune system. The interaction of LPS with this receptor triggers a complex intracellular signaling pathway that results in the activation of nuclear transcription factors such as nuclear factor kappaB (NF-κB), which induces a rapid and robust release of inflammatory cytokines [94]. These cytokines eventually reach the brain and induce sickness, which is a behavioral state characterized by symptoms including lethargy, decreased locomotor activity and appetite, anhedonia, sleep

disturbances and increased sensitivity to pain [25, 95]. There are some indications that LPS-induced sickness is followed by a depressive-like phenotype, in which rodents display behavior similar to clinically relevant symptoms of depression in humans [96-98]. However, the nature and characteristics of LPS-induced sickness behavior can substantially confound measurements of anxiety and depressive-like behavior in commonly used behavioral paradigms (table 1.1). For example, sick rodents display reduced locomotor activity, which can bias measurements of immobility used to estimate behavioral despair in paradigms such as the forced swim and tail suspension test [99]. In addition, sick animals eat and drink substantially less. This potentially confounds measures of sweetened fluid intake in paradigms designed to evaluate anhedonia. A bedside-to-bench rodent model of inflammation-associated depression should thus allow for discrimination between sickness and depressive-like behavior. This can be done by focusing on the temporal dissociation between these behavioral states, which is also seen in patients undergoing cytokine therapy (figure 1.3) [72]. Results from previous rodent studies suggest that a similar biphasic behavioral response occurs in rodents injected with LPS. However, the time window in which potential depressive-like behavior occurs is not very clear and findings often vary across labs. For example, some reports indicate that at 24 h after systemic LPS injection, depressive-like behavior is present without the confounding effects of sickness [96-98]. Other studies, in contrast, describe depressive-like behavior at earlier time points [100, 101], and others still report signs of sickness at 24 h [102-105].

Similar findings have been obtained using different immune challenges such as the viral mimetic polyinosinic:polycytidylic acid (poly(I:C)). Unlike LPS, which is mainly recognized by TLR 4, this synthetic double-stranded ribonucleic acid (RNA) is a ligand for TLR 3 and is therefore used to mimic the acute phase of viral infections [106]. Previous rodent work has demonstrated that systemic administration of poly(I:C) causes sickness in a time-dependent manner [107]. Much like administration of LPS and poly(I:C), both of which induce a "cytokine storm", more specific approaches such as the injection of specific recombinant cytokines also induce the full spectrum of sickness [108, 108].

Table 1.1: Overview of behavioral paradigms to study sickness, anxiety and depressive-like behavior in rodents.

Paradigm	Readout	Behavioral state
Burrowing	Burrowing behavior ↓	Sickness
Open field test	Locomotor activity ↓	Sickness
	Time in center zone ↓	Anxiety
Light/dark test	Locomotor activity ↓	Sickness
	Time in light zone ↓	Anxiety
Elevated plus maze	Locomotor activity ↓	Sickness
	Time in open arm ↓	Anxiety
Social interaction test	Interaction time ↓	Sickness, anxiety
Stress-induced hyperthermia	Basal body temperature ↑	Sickness
	Body temperature change ↑	Anxiety
Novelty-suppressed feeding	Latency to food ↑	Anxiety, depressive-like
Forced swim test	Immobility time ↑	Depressive-like (despair)
Tail suspension test	Immobility time ↑	Depressive-like (despair)
Intracranial self stimulation	Reward response rate ↓	Depressive-like (anhedonia)
Sucrose preference test	Total volume intake ↓	Sickness
	Sucrose preference ↓	Depressive-like (anhedonia)
Urine sniffing test	Sniffing time ↓	Sickness
	Urine preference ↓	Depressive-like (anhedonia)

1.2.3 How do cytokines contribute to depression?

The molecular mechanisms that mediate inflammation-associated depression are only beginning to be understood. However, numerous pathophysiological mechanisms have already been implicated to play a role in the development of idiopathic depression. These include dysfunction of neurotransmitter systems, alterations in neuroendocrine function, impaired neurogenesis and decreased brain levels of growth factors and neuropeptides (figure 1.2). It is well established that cytokines play an important role in normal brain function. Moreover, cytokines have the ability to influence neurocircuitry and neurotransmitter systems to induce behavioral alterations [9]. In line with these findings, several mechanisms have been proposed through which prolonged and/or excessive cytokine release in the brain can contribute to the above mentioned disease mechanisms [109]. For example, cytokines reduce the synthesis of the monoaminergic neurotransmitters serotonin, dopamine and norephinephrine. Moreover, inflammatory cytokines increase the expression and function of the reuptake pumps of these neurotransmitters, thereby reducing their availability even further [9]. Glutamate metabolism is also affected by inflammatory mechanisms, with neuroinflammation leading to increased glutamate release by astrocytes [110]. This elevated glutamate release, combined with reduced glial reuptake, can alter synaptic plasticity, mediate glutamate-induced excitotoxicity and lead to decreased production of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [111, 112]. These findings align with measures of reduced monoaminergic neurotransmission and increased glutamate levels in the brain of depressed patients [113, 114].

Hyperactivity of the HPA axis is another biological finding commonly observed in depression. This HPA axis is an important part of the neuroendocrine system that controls stress responses and regulates a wide variety of body processes. Activation of the HPA axis starts by secretion of corticotropin-releasing factor (CRF) and vasopressin from the hypothalamus, which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. This promotes the secretion of glucocorticoids from the adrenal cortex, which interact with their receptors in multiple target tissues and induce a negative feedback loop to reduce HPA axis activity [115]. Inflammatory processes influence several steps in this tightly regulated cascade. For instance, proinflammatory cytokines

stimulate the release of CRF, ACTH and glucocorticoids and decrease the expression and function of glucocorticoid receptors [116-118].
Imaging studies have demonstrated that depressed patients have smaller hippocampal volume compared with controls [119]. These findings are associated with low levels of the neurotrophin BDNF and reduced neuronal survival and adult neurogenesis [120, 121]. The notion that cytokines may play a role in these processes comes from preclinical rodent studies indicating that stress-induced decreases in neurogenesis and BDNF expression can be reversed by cytokine antagonism [122-124].

1.2.4 The kynurenine pathway of tryptophan breakdown

Tryptophan is an essential amino acid that is actively transported into the brain, where it serves as a serotonin precursor. Under normal circumstances, the majority of dietary tryptophan (>95%) is degraded in the liver by an enzyme called tryptophan 2,3-dioxygenase (TDO), while tryptophan degradation by the extrahepatic indoleamine 2,3-dioxygenase (IDO) is negligible [99]. However, several immune mediators have been shown to exert drastic effects on the expression and activity of IDO. IFN-γ is the strongest known inducer of IDO [125, 126], but other proinflammatory cytokines such as TNF-α, IL-6 and IL-1β are also known to upregulate IDO expression [127]. Anti-inflammatory cytokines such IL-4, IL-10 and transforming growth factor beta (TGF-β), on the other hand, downregulate the expression of IDO [128-130]. Considering the proinflammatory nature of certain types of depression [70], it is possible that (sustained) overactivity of IDO is one of key mechanisms linking inflammation to depression [70, 131]. Indeed, increased tryptophan breakdown resulting from IDO overexpression has important neuropsychiatric implications. First of all, it influences serotonergic neurotransmission in the brain as tryptophan is the precursor of serotonin and its bioavailability regulates serotonin synthesis [99]. Secondly, enhancing tryptophan breakdown through the so-called "kynurenine pathway" (figure 1.4) increases the amount of kynurenine, which is further degraded along one of two metabolic branches. This leads to the formation of either neurotoxic or neuroprotective metabolites. 3-hydroxykynurenine and quinolinic acid, for instance, participate in the formation of reactive oxygen species leading to neuronal damage. In addition, quinolinic acid activates N-

methyl-D-aspartate (NMDA) receptors which results in neuronal depolarization and possibly excitotoxicity [132, 133]. Kynurenic acid, on the other hand, is a known antagonist of several glutamate receptor subtypes and has neuroprotective properties. These apparently antagonistic pathways are compartmentalized within the brain: microglia preferentially produce quinolinic acid whereas astrocytes produce kynurenic acid [99, 134].

In addition to stimulating IDO, proinflammatory cytokines also increase the expression and activity of the enzyme kynurenine 3-monooxygenase (K3MO) [135]. This implicates that during inflammation, the formation of neurotoxic metabolites (i.e. 3-hydroxykynurenine and subsequently quinolinic acid) is favored over production of the neuroprotective kynurenic acid. Consequently, it is easy to assume that a sustained proinflammatory tone may lead to neurodegeneration and ultimately result in a depressive episode. Indeed, studies on IFN-α-treated hepatitis C patients have shown that IDO activation, as measured by calculating the kynurenine/tryptophan ratio in blood samples, predicts the occurrence of depression [132, 136, 137]. Furthermore, it is demonstrated that IDO is activated in the postmortem anterior cingulate cortex [138] and in the plasma of individuals with bipolar depression [139].

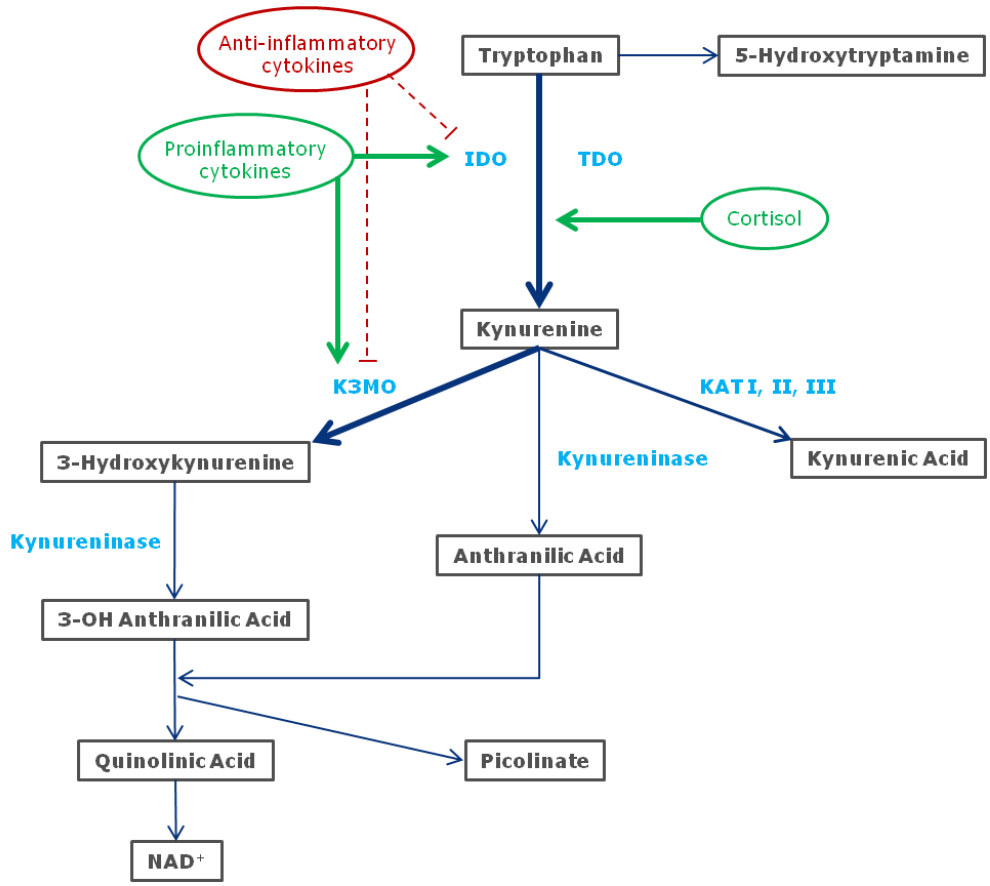

Figure 1.4 Simplified scheme of the kynurenine pathway of tryptophan breakdown during inflammation. Blue arrows show metabolic processes, green arrows indicate stimulation and red, dotted lines signify inhibition. The respective thickness of each arrow indicates the degree or weight of the action or reaction. IDO: indoleamine 2,3 dioxygenase, TDO: tryptophan dioxygenase, K3MO: kynurenine 3-monooxygenase, KAT: kynurenine aminotransferase, NAD^+: nicotinamide adenine dinucleotide. Adapted from Myint et al. 2012 [135].

1.2.5 Tumor necrosis factor alpha: A key cytokine in the development of inflammation-associated depression?

TNF-α is a highly pleiotrophic cytokine that exerts many functions in health and disease. Receptors for TNF-α are localized on neural tissue throughout the brain, including areas that are known to participate in affective and cognitive processing such as the hippocampus and prefrontal cortex [140]. During normal neuronal homeostasis, TNF-α regulates important brain processes such as synapse function, adult neurogenesis, mitochondrial activity and glutamatergic neurotransmission [141]. In addition, TNF-α is an important mediator of immunity as it is one of the master cytokines that precipitate the inflammatory response and the release of downstream immune molecules [141-143]. Considering the large amount of molecular functions attributed to TNF-α and the strong release of this cytokine during inflammation it is not surprising that dysregulation of the TNF-α system leads to a wide variety of affective, cognitive and somatic disturbances.

In line with these findings there are several clinical indications that TNF-α may play a role in the pathogenesis of inflammation-associated depression. In hepatitis C patients that are chronically treated with interferon-α, increased blood levels of TNF-α are correlated with the development of depressive symptoms [144]. Recent meta-analyses confirmed that circulating levels of TNF-α are significantly higher in depressed patients, even in absence of any other medical illness [80, 145]. Moreover, depressed patients show elevated blood levels of soluble TNF-α receptors (sTNFRs), which are more stable and hence more reliable markers of TNF-α activity. The concentration of these sTNFRs correlates to the severity of depression [146-148]. Blood levels of sTNFRs are also found to be higher in heart failure patients that are diagnosed with comorbid depression when compared with nondepressed heart failure patients [149]. A study in patients suffering from bipolar disorder showed that circulating concentrations of sTNFRs correlate to the duration of the disorder [150, 151].

It is shown that circulating levels of TNF-α decrease following antidepressant treatment with venlaxafine and this effect is higher in responders versus non-responders [152]. Although not all antidepressants exert an anti-inflammatory effect, most antidepressant therapies appear to normalize circulating TNF-α levels [153]. Moreover, high plasma levels of TNF-α are associated with treatment resistance to conventional antidepressants [154]. Peripheral

administration of anti-TNF-α antibodies improves depressed mood in patients suffering from psoriasis [155], Crohn's disease [156] and rheumatoid arthritis [157]. TNF-α antagonism was also shown to improve treatment resistant depression in a subgroup of patients with high baseline inflammatory biomarkers [158].

Taken together, these findings indicate that activation of the TNF-α system might be an important process in the development of inflammation-associated depression.

1.3 Aims of the study

Research focused on elucidating the complex biological underpinnings of inflammation-associated depression relies on well validated animal models. Although the body of literature on such animal models has been growing over the past decades, there is no standardized protocol to induce depressive-like behavior in rodents. Current animal models using peripheral immune stimuli to trigger depressive-like phenotype show inconsistency in experimental protocols, such as the use of different inflammatory challenges, doses, time points and functional readouts. Moreover, reports on inflammation-associated changes in behavior are often not corrected for the confounding effects of sickness. Therefore, the aim of this study is to develop and characterize rodent models of inflammation-associated depression. We hypothesize that activation of the immune system in the periphery leads to neuroinflammation and behavioral changes relevant to clinical depression. To study this hypothesis we will first focus on the effect of a systemic immune challenge on molecular changes in the brain, such as glial cell activation and the release of inflammatory cytokines. In addition, we will optimize and combine behavioral paradigms to evaluate the effect of peripheral immune activation on behavioral states relevant to depression. Overall, this study will lead to a broader understanding of the central effects of peripheral immune activation and provide insights that may lead to the development of better rodent models of inflammation-associated depression.

The majority of currently available research techniques to study neuroinflammation require isolation of the brain and therefore sacrificing of the animal. This results in the need to use large cohorts of animals when assessing the time course of glial cell activation. Moreover, even a simple evaluation of glial cell activation using classical histological or biochemical techniques is time-consuming, expensive and does not represent the real-time in vivo situation. Therefore, non-invasive imaging techniques are used in **chapter 2** to investigate the effect of peripheral immune activation on glial cell activation, as a marker of neuroinflammation. Bioluminescence imaging of transgenic reporter mice expressing luciferase under the transcriptional control of the glial fibrillary acidic protein (GFAP) promoter is used to quantify the kinetics of astrocyte activation following systemic immune challenges such as LPS or poly(I:C) administration.

Next, we use positron emission tomography imaging of a radioligand that binds specifically to activated macrophages and microglia to evaluate the effect of LPS injection on activation of these cell types. Finally, we investigate the astrocyte response to repeated systemic LPS administration and test whether sub-chronic stress influences astrocyte activation under unchallenged conditions and following a subsequent LPS challenge.

In **chapter 3** we further characterize the central consequences of peripheral immune activation in mice. First, immune-to-brain communication is evaluated by measuring the effect of systemic LPS administration on glial cell activation using bioluminescence imaging and immunohistochemistry. Moreover, a multiplex immunoassay is used to compare the time-dependent changes in serum and brain levels of several immune molecules. In addition to evaluating LPS-induced immune responses, the second aim in this chapter is to investigate the behavioral effects of peripheral immune activation. This is done by evaluating LPS-injected mice in a panel of behavioral assays that are commonly used to measure sickness, anxiety and depressive-like behavior. Special attention is given to separation of depressive-like behavior from sickness and whether a temporal dissociation can be observed between these behavioral states.

The use of rats allows for taking larger blood samples than is the case with mice. Moreover, the daily fluid intake of rats is higher than that of mice, which allows for more accurate volume measurements in paradigms using sweetened fluid intake as a measure of anhedonia. In **chapter 4**, we evaluate the kinetics of the immunological response to systemic LPS injection in rats by measuring time-dependent changes in serum levels of a panel of analytes, including corticosterone, leptin, pro- and anti-inflammatory cytokines and chemokines. Moreover, the effect of peripheral immune activation on anhedonia is evaluated. To this extent, we optimize a sucrose preference protocol that is able to separate anhedonia from sickness in LPS-challenged rats. Finally, this protocol is used to study whether repeated LPS administration or stress pre-exposure influences the anhedonic response to systemic LPS administration.

Administration of LPS induces a broad inflammatory response that is characterized by a robust release of multiple immune molecules. Based on the clinical data linking TNF-α to human inflammation-associated depression, we hypothesized that peripheral injection of TNF-α might provide a more specific

approach to study depressive-like behavior in mice. In **chapter 5**, we characterize the central consequences of systemic TNF-α injection by quantifying neuroinflammation and behavioral changes over time. First, serum and brain levels of immune mediators are quantified at several time points after systemic TNF-α administration. Next, transgenic bioluminescent Gfap-luc mice are used to evaluate the time course of TNF-α-induced astrocyte activation, as a marker of glial cell activation in vivo. Then, the presence of glial activation is confirmed by immunohistochemistry using a microglial activation marker. Finally, TNF-α-injected mice are tested in a panel of behavioral paradigms to assess whether depressive-like behavior can be separated over time from sickness.

2

Non-invasive imaging of glial cell activation as readout of neuroinflammation

Based on:

Effect of stress and peripheral immune activation on astrocyte activation in transgenic bioluminescent Gfap-luc mice.

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ABSTRACT

Neuroinflammation and the accompanying activation of glial cells is an important feature of many neurodegenerative conditions. It is known that factors such as peripheral infections and stress can influence immune processes in the brain. However, the effect of these stressors on astrocyte activation in vivo remains elusive. In this study, transgenic Gfap-luc mice expressing the luciferase gene under the transcriptional control of the glial fibrillary acidic protein promoter were used to quantify the kinetics of in vivo astrocyte activation following immune challenges relevant to clinical inflammation. It was found that astrocytes respond rapidly to peripheral immune activation elicited by either bacterial LPS or the viral mimetic poly(I:C). By measuring bioluminescence and 18-kDa translocator protein radioligand binding in the same animal it was observed that LPS induces both astrocyte as well as microglial activation at 6 h post-administration. In addition, it was found that the astrocyte response decreased upon repeated systemic LPS injections, indicating development of tolerance to the LPS challenge. Finally, restraining Gfap-luc mice for 1 hour daily on 5 consecutive days did not affect brain bioluminescence, thereby indicating that sub-chronic stress does not influence astrocyte activation under unchallenged conditions. However, stressed animals showed a reduced response to a subsequent systemic LPS injection, suggesting that the immune system is compromised in these animals. Here we demonstrate that Gfap-luc mice can be used to study astrocyte activation in response to stimuli relevant for clinical inflammation and that this approach may provide a more complete characterization of existing and novel models of neuroinflammation.

2.1 Introduction

Astrocytes are specialized glial cells that are ubiquitous throughout all regions of the CNS [15]. Under physiological circumstances, astrocytes are involved in a wide variety of crucial brain processes, including regulation of ion and neurotransmitter homeostasis, modulation of synaptic activity, provision of metabolic support to neurons, control of cerebral blood flow and regulation of BBB permeability (for reviews, see [16-20]). Besides these physiological tasks, astrocytes also play a fundamental role in the innate immune system of the brain, which can be activated following infection of the CNS, brain injury, ischemia, and in autoimmune and neurodegenerative disorders [159]. Historically, most studies investigating glial activation during neuroinflammation have focused on microglia, which are the tissue-specific macrophages of the brain. However, it has been shown that astrocytes also respond to aforementioned insults by undergoing various molecular, morphological and functional changes [21]. These changes are considered to be crucial in the regulation of neuroinflammation and have been shown to facilitate neural repair and protection [21, 22]. Dysfunction or overstimulation of astrocyte activity, on the other hand, can potentially be harmful and astrocyte abnormalities are commonly found in patients suffering from prevalent neurodegenerative and psychiatric disorders [19, 20, 24].

Results from previous rodent studies indicate that neuroinflammation can be triggered by activation of the immune system in the periphery. This process has been studied extensively in rodents by challenging them with LPS. LPS is a major component of the cell wall of Gram-negative bacteria and elicits a powerful immune reaction when introduced into an organism. Systemic administration of LPS induces a temporal inflammatory response in the brain, characterized in part by the release of proinflammatory cytokines and the activation of glial cells [160-163]. Another immunostimulant frequently used to study acute inflammatory responses is the viral mimetic poly(I:C). Unlike LPS, which is mainly recognized by TLR 4, this synthetic double-stranded RNA is a ligand for TLR 3 and is therefore used to mimic the acute phase of viral infection [106]. Previous studies have shown that peripheral administration of poly(I:C) activates immune processes in the CNS in a time-dependent manner [107].

However, the kinetics of glial activation following peripheral poly(I:C) administration has not yet been described in vivo.

Stress is another factor known to affect the immune system in humans and animals. Depending on its nature and duration, stress can either promote or decrease immune function. It is generally accepted that acute, moderate stress enhances certain aspects of the immune system [164-168], while chronic or more severe stress is immunosuppressive [165, 166, 169]. Previous rodent studies suggest that stress leads to a proinflammatory tone in the brain, characterized by the release of proinflammatory cytokines [50-55] and the activation and proliferation of glial cells [56-58]. However, this response seems to be highly dependent on the type of laboratory stress model used [59] and conflicting results are often reported even when similar paradigms were used. In addition, stress has also been suggested to prime the immune system, thereby eliciting an exaggerated response to a subsequent inflammatory stimulus [170]. For example, experimental stress has been shown to sensitize the immunological response to LPS in rodents [171-175]. In contrast, other publications suggest that stress actually suppresses the inflammatory response to LPS [176-180]. To our current knowledge, no study has consistently looked at the effects of stress and subsequent peripheral immune activation on astrocyte activation in living subjects. Instead, most studies focusing on neuroinflammation used research techniques that require sacrifice of the animal, thus making it impossible to study astrocyte activation in the same animal over time. Recently, transgenic Gfap-luc mice have been generated, in which luciferase is expressed under the transcriptional control of the GFAP promoter [181]. GFAP is an intermediate filament protein that is predominantly expressed by astrocytes, and is upregulated when astrocytes are activated [182]. These transgenic Gfap-luc mice thus allow noninvasive bioluminescence imaging of astrocyte activation in living mice over time. We previously used these mice to study the time course of astrocyte activation after a peripheral LPS administration, thereby showing that astrocytes respond rapidly to a single systemic LPS injection and that this response is a good surrogate marker of neuroinflammation [160].

As the triggers and time course of astrocyte activation are not well described in vivo, the goal of the present study was to extend the use of Gfap-luc mice by studying the kinetics of astrocyte activation in response to different stimuli relevant for clinical inflammation. First, we compared the dose-dependency and

kinetics of astrocyte activation following peripheral administration of LPS or poly(I:C). Next, we further characterized LPS-induced glial cell activation by positron emission tomography (PET) imaging of the translocator protein (TSPO), which is a primary marker of microglial activation. Then, we evaluated the astrocyte response to repeated peripheral LPS injections. Finally, we tested whether sub-chronic stress influences astrocyte activation under unchallenged conditions and following a subsequent LPS challenge.

2.2 Materials and methods

2.2.1 Animals and compounds

All animal care and use was performed in accordance with the Guide for the Care and Use of Laboratory Animals (NRC) and approved by the Institutional Ethical Committee on Animal Experimentation. Experiments were conducted in 10- to 14-week-old male Gfap-luc transgenic mice (FVB/N-Tg(Gfap-luc)-Xen) purchased from Taconic Laboratories (Hudson, NY, USA). All animals were housed in groups of 4-6 under a normal 12:12 h light-dark cycle (lights on at 06:00 AM with a 30 min dim and rise phase). Food and water were available ad libitum.

LPS from Escherichia coli (serotype 055:B5) and poly(I:C) were purchased from Sigma-Aldrich. Compounds were freshly dissolved in sterile saline prior to injection.

2.2.2 In vivo bioluminescence imaging

To detect the bioluminescent signal, Gfap-luc mice were anesthetized by inhalation of 2% isoflurane in 1 l/min oxygen, shaved on the head and injected with 126 mg/kg D-luciferin (Promega, product ID E1601) in the tail vein. Three minutes later the animals were scanned with a charge-coupled device (CCD) camera (IVIS Imaging System® 200 Series, PerkinElmer) mounted on a dark box. The imaging signal was measured in physical units of surface radiance (photons/s/cm^2/steradian [sr]) using Living Image® 3.2 software (PerkinElmer). Photon emission from the brain was measured in a region of interest (ROI) that was kept constant across mice in all experiments. Bioluminescence coming from the ears was considered to be basal GFAP activity and was excluded from the ROI.

2.2.3 PET imaging of TSPO in vivo

Gfap-luc mice were anesthetized by inhalation of 2% isoflurane in 1 l/min oxygen, then injected with approximately 400 uCi [F-18]PBR06, a PET ligand which binds to TSPO [183]. Ten minutes after injection, mice were placed on the

imaging bed of the PET scanner (Siemens Inveon), and scanned for 30 min. Images were reconstructed using a 2D OSEM algorithm, and ROIs placed over the whole brain using PMOD software. In addition, ROIs were drawn on peripheral organs, including kidneys, adrenal glands, lungs, and spleen. Standardized uptake values (SUV) were calculated by normalizing PET counts to injected dose and body weight. PET data were obtained immediately after bioluminescence imaging at baseline, and at 6 h, 24 h, 48 h and 72 h post-LPS administration.

2.2.4 Experimental procedures

To test the effect of peripheral immune activation on the time course of astrocyte activation in vivo, Gfap-luc mice were injected intraperitoneal (i.p.) with either bacterial LPS (0, 0.16 or 0.63 mg/kg) or the viral mimetic poly(I:C) (0, 2.5 or 10 mg/kg) (n = 8 per group). This dose range of LPS and poly(I:C) was selected based on previous indications of neuroinflammation following i.p. administration of similar concentrations [107, 160]. Body weight and bioluminescence was measured before (baseline) and at specific time points (2 h, 6 h, 24 h, 48 h, 72 h and 96 h) after administration of the immune challenge. Animals were returned to their home cage in between measurements.

To assess the effect of subsequent immune challenges on astrocyte activation, Gfap-luc mice were weighed and injected i.p. with either vehicle or 0.63 mg/kg LPS daily for 5 consecutive days (n = 8 for vehicle, n = 23 for LPS). Bioluminescence was measured before the first LPS injection (baseline) and at 6 h after every LPS injection. This time point was chosen based on our first experiment in which LPS-induced astrocyte activation peaked at 6 h post-administration.

To evaluate the effect of stress and a subsequent immune challenge on astrocyte activation in vivo, Gfap-luc mice were randomized to 4 experimental groups, i.e. no stress + vehicle, no stress + LPS, stress + vehicle and stress + LPS (n = 8 per group). Animals in the stress groups were weighed prior to restraint and subjected daily to 1 h of physical restraint in a transparent mouse restrainer (Bel-Art Scienceware, product ID 464010000) on 5 consecutive days. All stress sessions were performed between 08:00 and 09:00 AM. To control for possible effects of handling stress, mice from the no stress groups were

weighed, handled and put back in their home cage. The effect of repeated restraint stress on astrocyte activation was determined by measuring bioluminescence before the 5 days restraint stress period (baseline) and at 2 h after the last session of restraint stress. At 24 h after the onset of the last restraint stress session, all mice were weighed and injected i.p. with either vehicle or 0.63 mg/kg LPS. At 2 h, 6 h, 24 h, 48 h, 72 h and 96 h after LPS administration mice were weighed and bioluminescence was measured again.

2.2.5 B Statistical Analysis

Data were analyzed using SPSS Statistics software (Version 20 for Windows, IBM Inc). Analysis of variance (ANOVA) or repeated measure ANOVA (rmANOVA) was performed to assess the statistical significance of differences between treatment groups. A Greenhouse-Geisser correction epsilon (ε) was used for repeated measures analysis to correct for potential violation of the sphericity assumption [184]. This correction multiplies both the numerator and the denominator degrees of freedom by epsilon and the significance of the F-ratio is evaluated with the new degrees of freedom, resulting in a more conservative statistical test. To account for the skewness of the data distribution, bioluminescence observations were log-transformed prior to analysis. Differences in TSPO radioligand binding between treatment groups was analyzed by separate ANOVAs for each organ. When significant, post-hoc comparisons were made by using an independent samples t-test with a Bonferroni-corrected p-value. Significance was accepted for the rmANOVAs, ANOVAs and post-hoc comparisons when $p < 0.05$. For consistency between the analysis and the visualization of bioluminescence data, the group means and its SEM were back-transformed and visually presented on a logarithmic scale. TSPO radioligand binding and body weight data are expressed as mean ± SEM.

2.3 Results

2.3.1 Peripheral immune challenges increase Gfap-luc brain bioluminescence in a dose- and time-dependent manner

To quantify astrocyte activation in response to peripheral immune activation over time, Gfap-luc mice were i.p. injected with different doses of either LPS or poly(I:C) and brain bioluminescence was measured at specific time points. Factorial rmANOVA of the change in body weight after systemic administration of the bacterial endotoxin LPS showed a main effect of time ($F(3,36 = 15.5$, $p < 0.001$, $\varepsilon = 0.58$) and dose ($F(2,21) = 8.7$, $p < 0.01$, $\varepsilon = 0.58$). Post-hoc analysis revealed that LPS reduced weight in a time- and dose-dependent manner (figure 2.1 A). At 24 h after administration all LPS-treated mice had lost more weight than vehicle-injected controls. The weight change of mice administered with 0.16 mg/kg LPS had returned to control values at 48 h post-injection while it took up to 96 h for 0.63 mg/kg LPS-treated mice to recover. Factorial rmANOVA of photons emitted per second in the brain region of interest revealed a significant time x dose interaction for LPS ($F(12,96) = 24.9$, $p < 0.001$, $\varepsilon = 0.69$). Post-hoc analysis showed that at 6 h post-LPS, a strong bioluminescent signal was present in mice treated with 0.63 mg/kg, while at this time point a more moderate but still significant signal was evoked in the 0.16 mg/kg LPS group (figure 2.1 B). For both groups, there was still a significant increase in brain bioluminescence at 24 h. Mice treated with 0.16 mg/kg LPS reached control values at 48 h post-LPS, but this took up to 72 h for animals from the 0.63 mg/kg LPS group.

Systemic administration of the viral mimetic poly(I:C) did not induce changes in body weight at the doses used in this study (figure 2.1 C). However, the mice did lose about 0.5 g during the first test day. This was also seen in vehicle-injected mice in the LPS experiment and probably resulted from the experimental procedure to measure bioluminescence. Factorial rmANOVA indicated that there was a main effect of poly(I:C) dose ($F(2,18) = 6.2$, $p < 0.01$, $\varepsilon = 0.70$), and a trend for time ($F(6,108) = 2.2$, $p = 0.08$, $\varepsilon = 0.70$) on brain bioluminescence. Exploratory post-hoc analysis suggested that both poly(I:C) doses caused a mild increase of brain bioluminescence at 6 h, 24 h and 48 h post-administration as compared to vehicle-injected mice (figure 2.1 D).

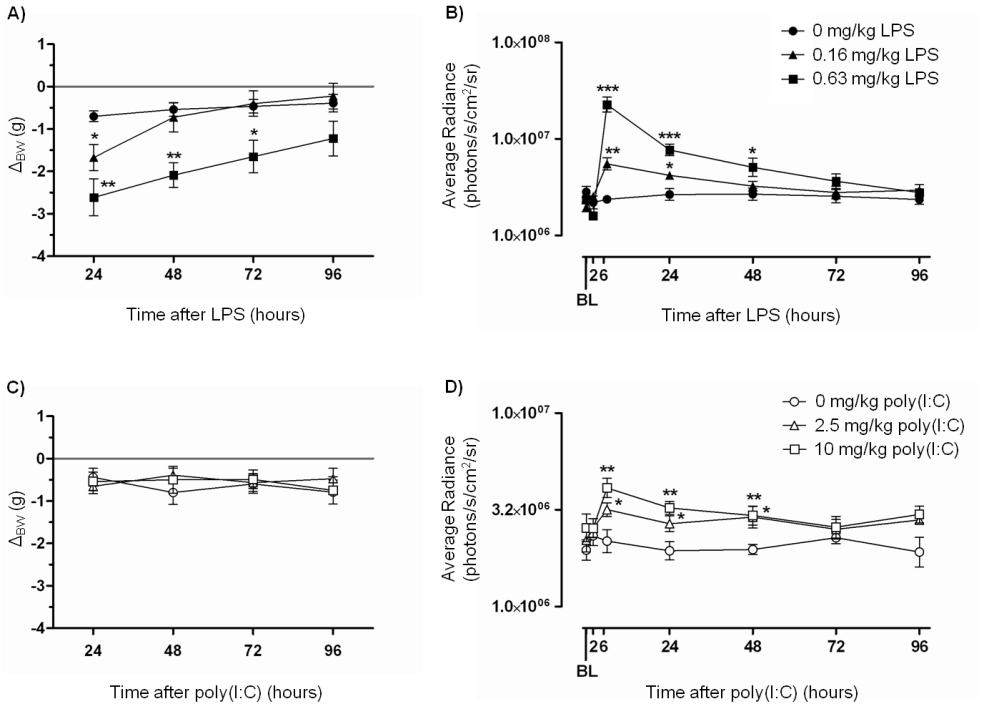

Figure 2.1 Peripheral immune activation activates astrocytes in a dose- and time-dependent manner. Systemic administration of bacterial LPS decreased body weight (A) and increased brain bioluminescence in Gfap-luc mice (B). Systemic administration of the viral mimetic poly(I:C), on the other hand, did not change body weight (C), but did augment brain bioluminescence (D). Please note the different y-axis scaling in (B) and (D). Graphs are plotted as mean ± SEM (n = 8 per group). Data were analyzed by rmANOVA followed by independent samples t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle.

2.3.2 LPS increases TSPO radioligand binding in multiple organs

Peripheral LPS injection caused a stronger bioluminescent signal in the brain of Gfap-luc mice than i.p. administration of poly(I:C) at the selected doses (figure 2.1). Therefore, it was decided to further characterize systemic LPS injection as a model of glial cell activation by measuring TSPO radioligand binding immediately after bioluminescence imaging of LPS-treated Gfap-luc mice. At 6 h post-LPS, bioluminescence increased mainly in the brain while TSPO radioligand binding also augmented in other organs (figure 2.2 A). Pairwise comparison of the TSPO radioligand SUV demonstrated that at 6 h post-LPS the TSPO PET signal was increased in the adrenal glands ($F(1,14) = 10.8$, $p < 0.01$), brain ($F(1,14) = 86.6$, $p < 0.001$) and lungs ($F(1,14) = 57.0$, $p < 0.001$), but not in the kidneys ($F(1,14) = 0.4$, $p = 0.53$) or spleen ($F(1,14) = 0.05$, $p = 0.83$) (figure 2.2 B). As seen in figure 2.2 C, both the bioluminescent signal as well as TSPO radioligand binding were increased in the brain of LPS-injected mice at 6 h post-administration. No differences in TSPO radioligand binding were found between treatment groups at 24 h, 48 h or 72 h post-administration (data not shown).

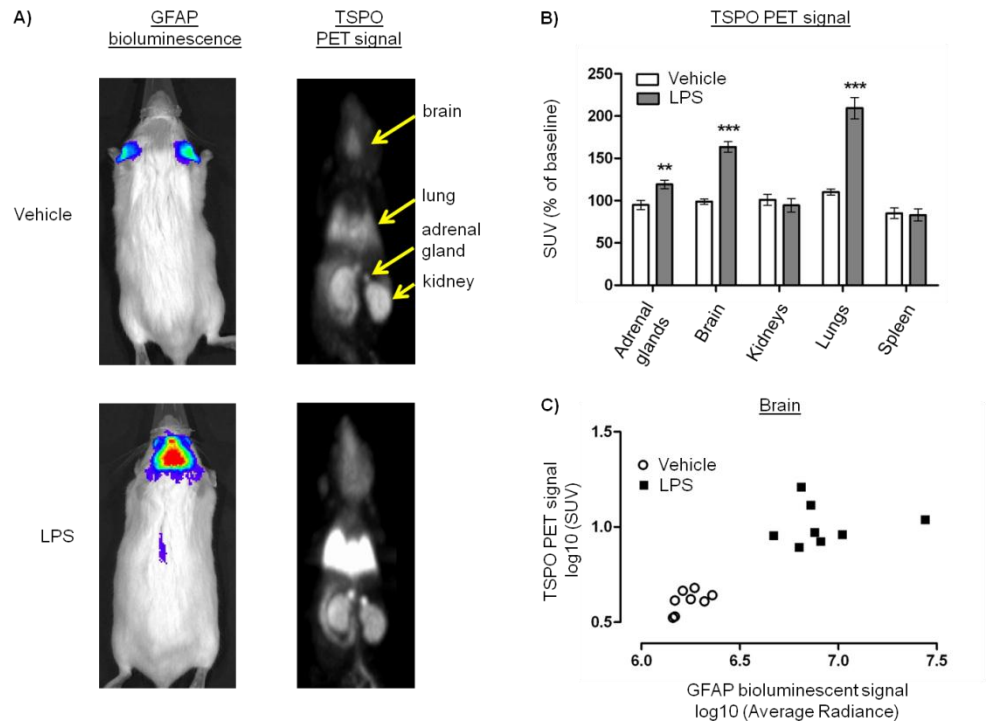

Figure 2.2 Systemic LPS administration increases the TSPO PET signal in several organs. Gfap-luc transgenic mice were injected with LPS (0.63 mg/kg, i.p.) and TSPO radioligand binding was measured immediately following bioluminescence scanning. At 6 h after injection, LPS increased brain bioluminescence and TSPO binding in the adrenal glands, brain and lungs, but not in the kidneys or spleen. Representative images of bioluminescence and maximum intensity projections of TSPO radioligand binding in vehicle- and LPS-injected mice (A), quantified TSPO radioligand binding in different organs (B). As seen in (C), LPS increased bioluminescence and TSPO radioligand binding in the brain, thereby suggesting both astrocyte as well as microglial activation at 6 h post-administration. TSPO PET signal is plotted as mean ± SEM (n = 8 per group) and was analyzed by pairwise comparison. ** $p < 0.01$, *** $p < 0.001$ compared to vehicle.

2.3.3 The astrocyte response to LPS decreases upon repeated dosing

Inflammatory processes associated with neurodegenerative disorders are thought to be of chronic nature. However, it becomes clear from the previous experiments that neuroinflammation evoked by an acute dose of LPS is short-lasting. Hence, it was investigated whether astrocyte activation persists longer after multiple systemic LPS challenges. To do so, mice were injected i.p. with 0.63 mg/kg LPS on 5 consecutive days and brain bioluminescence was measured at 6 h after each LPS challenge. This is the time point at which the astrocyte response to peripherally administered LPS peaked in the previous experiments. Factorial rmANOVA showed a significant main effect of time ($F(4,46) = 4.9$, $p < 0.05$, $\varepsilon = 0.40$) and LPS ($F(1,29) = 124.8$, $p < 0.001$, $\varepsilon = 0.40$) on change in body weight. Systemic LPS administration reduced weight significantly on all days of the experiment (figure 2.3 A). There was a time x LPS interaction for bioluminescence in the brain ($F(5,125) = 21.6$, $p < 0.001$, $\varepsilon = 0.56$). Post-hoc analysis revealed that the first two LPS challenges increased brain bioluminescence to the same extent, while the bioluminescent signal increased less for each of the subsequent LPS challenges, demonstrating potential induction of tolerance to LPS (figure 2.3 B).

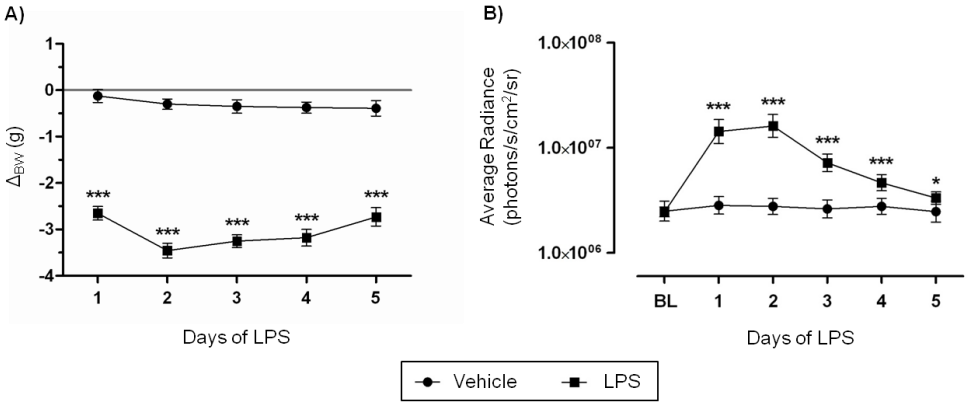

Figure 2.3 The astrocyte response to LPS decreases upon repeated dosing. Daily LPS injections (0.63 mg/kg, i.p.) for 5 consecutive days decreased body weight (A) and increased brain bioluminescence in Gfap-luc mice (B). However, the magnitude of the LPS-induced increase in bioluminescence diminished gradually after the second LPS challenge, indicating the induction of LPS tolerance. Graphs are plotted as mean ± SEM (n = 8 for vehicle group, n = 23 for LPS group). Data were analyzed by rmANOVA followed by independent samples t-test. * $p < 0.05$, *** $p < 0.001$ compared to vehicle.

2.3.4 Stress blunts the astrocyte response to subsequent LPS

Stress is known to both induce and influence neuroinflammation. In this experiment, animals were exposed to 1 h of restraint stress daily for 5 consecutive days. Factorial rmANOVA on change in body weight showed a time x stress interaction ($F(4,116) = 3.7$, $p < 0.05$, $\varepsilon = 0.55$). Restraint stress significantly reduced weight from the first until the last day of the stress protocol (figure 2.4 A). To test whether stress influenced astrocyte activation, brain bioluminescence was quantified 1 h after ending the last stress session. Measuring the bioluminescent signal at this time point allowed to potentially pick up acute and/or more chronic effects of restraint stress on astrocyte activation in the Gfap-luc mouse model. However, restraint stress had no effect on brain bioluminescence (figure 2.4 B). One day after the last stress session, stressed and non-stressed mice were injected with either vehicle or 0.63 mg/kg LPS to investigate whether prior exposure to restraint stress influences the astrocyte response to an acute LPS challenge. Factorial rmANOVA on the change in body weight showed a significant time x stress ($F(4,108) = 7.0$, $p < 0.001$, $\varepsilon = 0.65$) and time x LPS interaction ($F(4,108) = 56.7$, $p < 0.001$, $\varepsilon = 0.65$). Post-hoc analysis revealed that LPS reduced the weight of both stressed and non-stressed animals at 1 day after administration, as compared to their respective controls (figure 2.4 A, right). This decrease in body weight reached control values in stressed animals on day 2, while it remained significant in non-stressed mice until day 3. There was a significant time x stress x LPS interaction ($F(2,54) = 7.6$, $p < 0.001$, $\varepsilon = 0.97$) for brain bioluminescence. Post-hoc analysis showed that at 6 h post-administration LPS increased brain bioluminescence in non-stressed and stressed mice (figure 2.4 B, middle). However, this LPS-induced increase of brain bioluminescence was significantly lower in stressed mice, as compared to non-stressed mice. At 24 h post-LPS administration brain bioluminescence was still increased in LPS-treated mice, but there was no longer a significant difference between stressed and non-stressed mice (figure 2.4 B, right). No significant effect of stress on brain bioluminescence was detected at any of the other time points (data not shown).

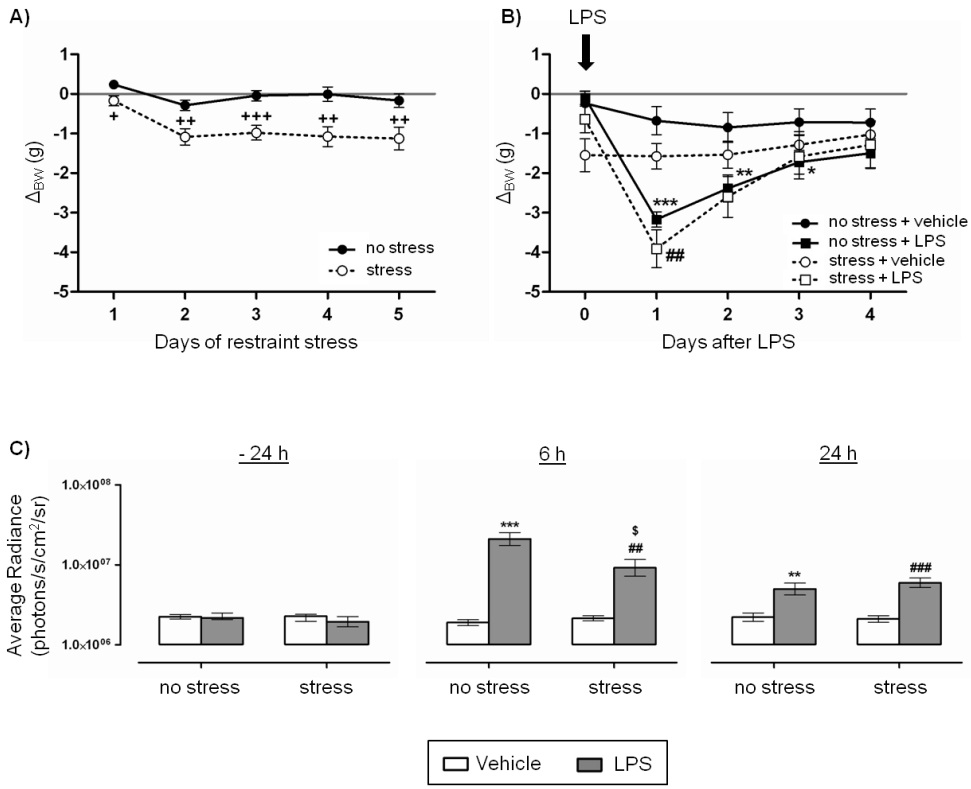

Figure 2.4: Stress blunts the astrocyte response to subsequent LPS. Daily restraint stress for 1 hour on 5 consecutive days reduced body weight (A) but did not influence brain bioluminescence (C, left). Peripheral LPS administration (0.63 mg/kg, i.p.) decreased weight in a time-dependent manner in both non-stressed and stressed mice (B). At 6 h post-administration, LPS increased brain bioluminescence in all challenged mice, but this effect was significantly lower in stressed mice (C, middle). Brain bioluminescence was still elevated in LPS-treated mice at 24 h post-administration but there was no difference anymore between stressed and non-stressed animals (C, right). Graphs are plotted as mean ± SEM (n = 16 per group in (A), n = 8 per group in (B) and (C)). Data were analyzed by rmANOVA followed by independent samples t-test. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ compared to no stress; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to no stress + vehicle, ## $p < 0.01$ compared to stress + vehicle; $ $p < 0.05$ compared to no stress + LPS.

2.4 Discussion

Astrocytes are the most abundant cell type in the CNS. Apart from playing a pivotal role in physiological processes that are required for normal brain function, they are also involved in innate immunity in the CNS. However, the triggers and time course of astrocyte activation are not well described in vivo. In this study, a transgenic bioluminescent mouse model was used to study the kinetics of astrocyte activation following stressors relevant to clinical inflammation.

Systemic administration of bacterial LPS to Gfap-luc mice induced a robust and reliable response in all experiments. At 0.63 mg/kg, LPS reduced body weight by approximately 10% during the first 24 h after administration. This LPS-induced decrease in weight is in accordance with previous rodent data [185, 186], and reflects a strong sickness response in the animal. In line with the weight data, a single peripheral LPS injection induced a time- and dose-dependent increase in brain bioluminescence in the Gfap-luc mice. This increased brain signal peaked at 6 h, faded over time and was abolished by 72 h post-treatment at all LPS doses tested. As discussed previously [160], the upregulation of GFAP at 6 h post systemic LPS injection indicates that astrocytes respond rapidly to a peripheral immune challenge. This astrocyte response is strong in intensity but short-lasting. Similar results were obtained following systemic administration of poly(I:C), which is a synthetic analogue of double-stranded RNA commonly used to induce innate antiviral responses. As was the case for LPS, peripheral administration of poly(I:C) induced a dose-dependent increase in brain bioluminescence during the first 48 h post-administration. This time profile is in accordance with sickness behavior and brain levels of pro-inflammatory cytokines reported in poly(I:C)-challenged mice [107, 187]. In our model, peripherally administered poly(I:C) had a rather small effect on glial activation when compared to LPS. This raised the impression that systemically administered poly(I:C) affects astrocyte activation through indirect pathways. However, in our hands, poly(I:C) did not affect body weight, thereby suggesting that the doses and/or poly(I:C) strand used was not virulent enough to elicit a stronger response. Astrocytes contribute to the structural and functional integrity of the BBB [188]. Unlike LPS, which is known to make the BBB more leaky [161], i.p. administration of poly(I:C) has been shown to make the BBB

less leaky, thereby protecting mice from the deleterious effects of viral encephalitis [189]. Although we used doses that were previously shown to cause sickness [107], it is still possible that the effects of poly(I:C) on the BBB prevent peripheral stimuli to cross it, thereby reducing astrocyte activation. This may in part explain the moderate astrocyte response to poly(I:C).

Since LPS elicited a strong astrocyte response, glial cell activation was further characterized in LPS-treated Gfap-luc mice using a different technique and focusing on another cell type. This was done by measuring TSPO radioligand binding immediately after bioluminescence imaging at several time points following LPS administration. This radioligand binds to the 18 kDa translocator protein, which is a transmembrane protein located mainly in the outer mitochondrial membrane [190]. TSPO has high level of expression in organs such as adrenal glands, kidneys, lungs and spleen [191, 192], but is also found at lower levels in the brain where it is mainly expressed by microglial cells [190, 193, 194]. Under inflammatory conditions, the density of TSPO increases substantially and binding of TSPO specific radioligands is often used to assess the activation of macrophages and microglia in inflammatory animal models [193, 195, 196] and humans [197-200]. From our radioligand data it becomes clear that LPS elevated TSPO binding in the adrenal glands, brain and lungs at 6 h after injection. These findings are in accordance with previous publications also showing increased TSPO binding in the lung [196, 201] and brain [193] following LPS administration. Although macrophages are known to be present in the spleen [202] and kidney [203], no effect of LPS was found on TSPO binding in these organs. This may indicate that tissue-specific macrophages respond differently to a peripheral immune stimulus. However, due to the lack of earlier time points in our study it cannot be ruled out that TSPO was upregulated before the 6 h time point. It is known, for example, that the microglial response to an inflammatory stimulus precedes astrocyte activation and that astrocytes have a more protracted period of activation than microglia [204]. This might also explain why LPS only increased TSPO binding in the brain at 6 h, while astrocyte activation, as measured by bioluminescence imaging, lasted up to 48 h.

In daily life, humans and animals rarely encounter a single acute systemic inflammatory stimulus. Instead they are exposed to more prolonged inflammatory insults such as pathogens that replicate in vivo or conditions that are accompanied by chronic inflammation. Therefore, several groups sought to

induce a longer lasting pro-inflammatory tone by administering LPS repeatedly. However, chronic administration of LPS is known to lead to immune tolerance, a state in which the immune response to subsequent inflammatory challenges is reduced. This type of immune regulation is thought to protect the host against excessive tissue damage and the manifestation of pathological states [205]. Recent experiments showed that microglia exhibited a strong tolerance in response to repeated LPS exposure, whereas astrocytes only showed partial tolerance [206]. However, those experiments were conducted in vitro and little is known about the effect of chronic LPS exposure on astrocyte activation in vivo. Therefore we administered LPS to Gfap-luc mice on 5 consecutive days and measured brain bioluminescence at 6 h after every injection. Mice that were challenged daily with LPS clearly lost weight after the first two injections, but did not lose additional weight in response to subsequent LPS administrations. However, these mice did recover slower than acutely challenged mice, indicating that there was still a biological response to subsequent LPS injections. The bioluminescence data are in line with these findings, as the first two LPS injections induced strong astrocyte activation while subsequent LPS challenges elicited weaker bioluminescent responses. This indicates the development of LPS tolerance and reduced astrocyte activation in subsequently challenged animals. These findings are in contrast with previously published data, which showed that 6 daily i.p. injections of LPS led to a higher brain GFAP immunoreactivity than when LPS was only administered once [163]. However, the exaggerated GFAP staining in repeatedly challenged mice was reported in the hippocampus and hypothalamus, while we quantified GFAP upregulation in the whole brain. Therefore, it cannot be excluded that regional differences in astrocyte activation also exist in our model. Moreover, in the study by Borges et al., GFAP protein levels were measured by immunohistochemistry. This technique does not allow for discrimination between activation (GFAP messenger ribonucleic acid (mRNA) expression) and reactive astrogliosis that has already taken place (e.g. GFAP in glial scars). The bioluminescence model used in our study, in contrast, does allow quantification of astrocyte activation after every immune stimulus, and is not confounded by GFAP protein present due to previous inflammatory insults. One limitation of the Gfap-luc bioluminescence model, however, is the fact that it cannot be used to identify the pathways underlying reduced astrocyte activation to subsequent LPS challenges. It is possible that the astrocytes

develop tolerance themselves, or that they receive less activating stimuli (i.e. pro-inflammatory cytokines) from other cell types that have become tolerant.

One of the models commonly used to study the effects of psychological stress on immune function in rodents is restraint stress. Earlier studies have shown that when applied acutely (single 2 h session) restraint stress enhances immune function [165], while chronic exposure (longer and/or repeated sessions) induces immune suppression [207, 208]. Other groups have shown signs of glial activation in mice that underwent repeated restraint stress, thereby suggesting immune activation in the brain of these mice [54, 58]. Most of these studies have focused on microglia, while other models showed that astrocytes also display metabolic [209] and structural changes [210] in response to psychological stress. As the astrocyte response to sub-chronic stress is not well described in vivo, we decided to test the effect of repeated restraint stress on astrocyte activation in Gfap-luc mice that were exposed daily to 1 h of restraint stress on 5 consecutive days. The fact that repeated daily exposure to restraint stress decreased body weight indicates that our stress protocol was effective. However, at 1 h after the last stress session no difference in brain bioluminescence could be found between stressed and control animals. This suggests that our stress model did not affect GFAP expression and are in contrast with other studies in which repeated restraint stress was found to increase [54] or decrease [211, 212] GFAP levels. However, differences in stress protocols and the fact that these studies focused on specific brain regions, while we measured the bioluminescent signal in the whole brain, make it difficult to compare results.

Some studies suggest a synergistic relationship between stress and inflammation, in which stress can prime the immune system and thereby elicit an exaggerated response to a subsequent inflammatory stimulus [170]. Indeed, stress has been shown to sensitize the inflammatory response to LPS in rodents [171-175]. In contrast, results from an equal amount of other publications suggest that stress actually suppresses the immunological response to LPS [176-180]. To elucidate the effect of stress on astrocyte activation following peripheral immune activation, we measured brain bioluminescence in stressed Gfap-luc mice that were subsequently challenged with a systemic LPS injection. Results from this experiment showed that repeated restraint stress led to a significant reduction in LPS-induced astrocyte activation. Interestingly, this

effect was only seen at 6 h post-LPS while brain bioluminescence had returned to non-stressed levels at 24 h. This indicates that stress does not completely eliminate the astrocyte response to a peripheral immune stimulus, but merely blunts its peak. Such an immunosuppressive effect could have detrimental consequences in the normally tightly regulated inflammatory response to infections or other pathological conditions. It is possible, for instance, that chronic stress lowers the immune response below the threshold of what is required to clear an inflammatory challenge. This could, in part, explain the negative influence stress has on infection and disease outcome [169, 213, 214].

In conclusion, by using a specific transgenic bioluminescent mouse model we were able to non-invasively study the effect of various challenges and stress exposure on the in vivo activation of astrocytes over time. The present set of experiments indicates that astrocytes respond rapidly, but temporarily, to peripheral immune activation elicited by bacterial LPS and to a lesser extent by the viral mimetic poly(I:C). Moreover, it was shown that subsequent LPS challenges elicit weaker astrocyte responses, suggesting the development of tolerance. Finally, it was demonstrated that sub-chronic stress does not influence astrocyte activation under unchallenged conditions, but blunts the astrocyte response to subsequent LPS administration. With an increasing amount of functions attributed to astrocytes during health and disease, studies such as those presented here are important to understand how astrocytes are activated in vivo. Moreover, characterizing the triggers and time profile of astrocyte activation using Gfap-luc mice may be helpful to evaluate the therapeutic utility of altering neuroinflammation in mouse models of other disorders.

3

Characterization of the central nervous system response to peripheral LPS administration in mice

Based on:

Systemic immune activation leads to neuroinflammation and sickness behavior in mice.

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ABSTRACT

Substantial evidence indicates an association between clinical depression and altered immune function. Systemic administration of bacterial LPS is commonly used to study inflammation-associated behavioral changes in rodents. In these experiments we tested the hypothesis that peripheral immune activation leads to neuroinflammation and depressive-like behavior in mice. We report that systemic administration of LPS induced astrocyte activation in transgenic Gfap-luc mice and increased immunoreactivity against the microglial marker ionized calcium-binding adapter molecule 1 in the dentate gyrus of wild-type mice. Furthermore, LPS treatment caused a strong but transient increase in cytokine levels in the serum and brain. In addition to studying LPS-induced neuroinflammation, we tested whether sickness could be separated from depressive-like behavior by evaluating LPS-treated mice in a panel of behavioral paradigms. Our behavioral data indicate that systemic LPS administration caused sickness and mild depressive-like behavior. However, due to the overlapping time course and mild effects on depression-related behavior per se, it was not possible to separate sickness from depressive-like behavior in the present rodent model.

3.1 Introduction

Clinical depression is a devastating, recurrent psychiatric illness that has a lifetime prevalence of 16% [60]. By the year 2030, depression is predicted to become the second leading cause of disability worldwide [61]. Despite its high prevalence and considerable socioeconomic impact, very little is known about the pathophysiology of the disorder. Increasing numbers of studies support the idea that depression is a multifactorial disease with both genetic and environmental factors contributing to disease development [215]. Inflammatory processes may also play a role in the etiology of depression, at least in a subset of susceptible individuals. It has been reported that depressed patients commonly display alterations in their immune system, including impaired cellular immunity and increased levels of pro-inflammatory cytokines (for reviews and meta-analyses see [10, 79, 80, 216]). Furthermore, depression frequently occurs as a comorbidity of conditions that are characterized by a sustained, systemic inflammation such as rheumatoid arthritis [73, 217], coronary heart disease [78, 218], stroke [76], type 2 diabetes [75] and obesity [77]. Another indication that inflammation and depression are linked comes from clinical observations in which therapeutic administration of the proinflammatory cytokines interleukin-2 and interferon-α to cancer or hepatitis C patients resulted in depression in up to half of these patients [32, 219, 220].

Bacterial LPS is a potent activator of the immune system. Numerous reports have shown that systemic administration of LPS in animals leads to sickness, a behavioral state characterized by symptoms including lethargy, decreased locomotor activity and appetite, anhedonia (the inability to experience pleasure from naturally rewarding activities), sleep disturbances and increased sensitivity to pain [25, 95]. Several of these symptoms are thought to be very similar to clinically relevant symptoms of depression in humans [99, 221]. Therefore systemic administration of LPS is frequently used to study inflammation-associated depression in rodents. Some rodent studies report that 24 h after systemic LPS injection, depressive-like behavior is present without the confounding effects of sickness [96-98]. However, these findings are not consistent across the literature, with some studies describing depressive-like behavior at earlier time points [100, 101], and others still reporting signs of sickness at 24 h [102-105]. Moreover, studies focusing on LPS-induced

depressive-like behavior often vary in LPS dose, LPS serotype, application route and assays used, which makes it difficult to compare results between research groups. In addition, many of these studies only use a single dose of LPS and/or a few time points, thus making it impossible to assess time- and dose-dependent changes in neuroinflammation and behavior.

The present study aimed at evaluating central effects of peripheral immune activation by combining multiple techniques to quantify neuroinflammation and behavioral changes at several time points after systemic LPS administration. First, transgenic Gfap-luc mice were used to assess the kinetics of LPS-induced astrocyte activation, as marker of neuroinflammation. After confirming the presence of neuroinflammation by immunohistochemistry using the microglial marker ionized calcium-binding adapter molecule 1 (Iba1), serum and brain levels of immune mediators were measured at time points corresponding to glial activation. Finally, LPS-treated mice were tested in a panel of behavioral paradigms to evaluate whether depressive-like behavior could be separated over time from sickness.

3.2 Materials and methods

3.2.1 Animals and LPS

Male NMRI mice were obtained from Charles River Laboratories (France), male wild-type FVB mice from Janvier (France), and Gfap-luc transgenic mice (FVB/N-Tg(Gfap-luc)-Xen) were purchased from Taconic Laboratories (USA). These last animals express the firefly luciferase gene under the control of a 12 kb murine glial fibrillary acidic protein (GFAP) promoter [181] and are commonly used to noninvasively measure astrocyte activation in the same animal over time [181, 222-226]. Unless mentioned otherwise animals were housed in groups of 4 in individually ventilated cages (IVC; L x W x H: 36 x 20 x 13 cm; Tecniplast, Italy) under a normal 12:12 h light-dark cycle (lights on at 06:00 AM with a 30 min dim and rise phase). Procedure rooms were maintained at a temperature of 22 ± 2 °C and a humidity of 54 ± 2%. Food and water were available ad libitum. All experimental protocols were approved by the Institutional Ethical Committee on Animal Experimentation, in compliance with Belgian law (Royal Decree on the protection of laboratory animals dd. April 6, 2010), and conducted in facilities accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).
LPS from Escherichia coli (serotype 055:B5) was purchased from Sigma-Aldrich and freshly dissolved in sterile saline prior to injection.

3.2.2 In vivo bioluminescence imaging

Astrocyte activation in 10-week-old male Gfap-luc mice was monitored before (baseline) and at specific time points (2 h, 6 h, 24 h, 48 h, 72 h and 96 h) after i.p. administration of either 0, 0.16 or 0.63 mg/kg LPS. Results from a pilot experiment showed that a dose of 2.5 mg/kg LPS in combination with the experimental procedure to measure bioluminescence was lethal in Gfap-luc mice. Therefore, it was decided to use 0.63 mg/kg LPS as the highest dose in this experiment.
To detect the bioluminescent signal, mice were anesthetized by inhalation of 2% isoflurane, shaved on the head and injected with 126 mg/kg D-luciferin (Promega, product ID E1601) in the tail vein. Three minutes later the animals

were scanned with a CCD camera (IVIS Imaging System® 200 Series, Xenogen) mounted on a dark box. The imaging signal was measured in physical units of surface radiance (photons/s/cm^2/steradian [sr]) using Living Image® 3.2 software (Xenogen). Photon emission from the brain was counted using a ROI that was kept constant within the experiment. Bioluminescence coming from the ear was considered to be basal GFAP activity and was excluded from the ROI.

3.2.3 Immunohistochemistry

10-week-old male FVB mice were injected i.p. with vehicle or 0.63 mg/kg LPS and tissue was collected for immunohistochemical staining 24 h later. Mice were anesthetised with 60 mg/kg sodium pentobarbital (Nembutal), and transcardially perfused with 25 mL heparinised 0.9% saline followed by 50 mL 4% paraformaldehyde (PFA) in 1x phosphate-buffered saline (PBS) (pH 7.4, 4 °C). Brains were dissected and post-fixed in 4% PFA overnight at 4 °C, before being washed twice in PBS and stored in PBS/0.1% NaN3 at 4 °C. Free-floating coronal brain sections of 100 µm thickness were cut at the level of the hippocampus (Interaural 1.50 mm, Bregma -2.30 mm, Paxino & Watson, 2001) using a Leica VT1000S vibratome (Leica Microsystems) and were subsequently stored in PBS/0.1% NaN3 at 4 °C until use.

For the immunofluorescent staining of Iba1 protein, sections were washed 3 x 5 min in PBS before being incubated in blocking buffer (5% goat serum, 0.3% Triton X 100 and 0.1% bovine serum albumin (BSA) in PBS) for 3 h. Subsequently, sections were incubated overnight at 4 °C with a rabbit polyclonal anti-Iba1 (1:500, Wako) primary antibody in blocking buffer. The following day, sections were washed in 3 x 5 min PBS before being incubated in PBS-BSA containing the secondary fluorescent antibodies Alexa 555 goat anti-rabbit (1:500, Invitrogen), for 2 h at room temperature in the dark. After 3 x 5 min washes in PBS, sections were mounted onto glass slides using a glycerol based mounting medium containing DABCO (100 mg/mL), and stored in the dark.

A confocal scanning Zeiss Axiovert 100M microscope was used to obtain fluorescent images. Single images were captured using a Zeiss Plan-Neofluar 10x (numerical aperture 0.30) lens. For each animal, two brain sections were analyzed and fluorescent images containing immunopositive cells at the level of the hippocampal dentate gyrus were captured from the 555 nanometer

wavelength. Image analysis software from Zeiss (LSM 510) was used in order to detect changes in the quantity of Iba1 staining levels. Thresholding was used to distinguish positive cells from background. A boundary was drawn around the dentate gyrus of the hippocampus to exclude other regions from quantification. The output of the analysis was 'number of pixels'.

3.2.4 Cytokine measurements

Based on the time course of neuroinflammation seen in Gfap-luc mice, it was decided to measure cytokine levels in serum and brain at 2 h, 6 h and 24 h after LPS administration. For this purpose, 10-week-old male NMRI mice were injected i.p. with 0, 0.63 or 2.5 mg/kg LPS. To reduce animal usage, the 0.16 mg/kg LPS dose was left out as this dose only caused mild GFAP upregulation in the Gfap-luc mice.

At the relevant time points, mice were killed by decapitation and serum and brain samples were collected. Serum samples were obtained by collecting truncal blood in Vacutainer SST II Advance blood tubes (BD Biosciences, product ID 367955). After being kept for 30 minutes at room temperature, the blood samples were centrifuged (1300 g, 10 min, room temperature), aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C until further use. Within two minutes after decapitation, the brain was isolated from the skull and the hemispheres were separated. They were then weighed, transferred to TallprepTM Matrix D tubes (MP Biomedicals, product ID 116973025), flash frozen in liquid nitrogen and stored at -80 °C until further processing.

A slightly modified protocol adapted from Erickson et al. 2001 [161] was used to extract total protein from brain samples. Briefly, frozen hemispheres were immersed in a 5x volume of extraction buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA) containing a protease inhibitor (Roche, product ID 11873580001) and phosphatase inhibitor cocktail (Roche, product ID 4906837001) and the tissue was homogenized by shaking with a bench top homogenizer (FastPrep-24, MP Biochemicals) for 25 sec. The homogenate was then centrifuged (1000 g, 10 min, 4 °C) and supernatant was removed to be centrifuged a second time (20000 g, 40 min, 4 °C). Finally, the protein content of each sample was determined using a bicinchoninic acid assay (Sigma Aldrich), with bovine serum albumin (Sigma Aldrich, product ID A4503) as a standard.

Concentrations of IFN-γ, IL-1β, IL-6, IL-10, MCP-1 and TNF-α were simultaneously determined in serum and brain samples using a mouse cytokine/chemokine magnetic bead panel kit from Merck Millipore. This assay is based on Luminex™ technology in which capture antibodies specific for individual cytokines are coated with magnetic beads that have a distinct emitting fluorescence pattern. All steps in the assay were conducted according to the manufacturer's instructions. A Bio-Plex 200 System (Bio-Rad) was used to measure the fluorescent signal and the data was analyzed using Bio-Plex Manager 5.0 software (Bio-Rad) with five-parameter logistic regression curve fitting. Cytokine and chemokine concentrations in brain samples were then normalized to the total protein concentration determined for each sample. Cytokines levels below detection limit were assigned a value equal to the lowest detectable value of that cytokine. Cytokine values outside of the average ± 3 times standard deviation range were considered outliers and were excluded from all calculations. This happened for less than 2% of all measured cytokines.

3.2.5 Behavioral tests

Behavioral tests were conducted on 10-week-old male NMRI mice. The open field test (OFT), tail suspension test (TST) and forced swim test (FST) setups were custom-made in-house. In all of these paradigms, groups of naive mice (n = 10/group) were injected i.p. with 0, 0.31, 0.63 or 1.25 mg/kg LPS and tested at either 2 h, 6 h or 24 h post-LPS administration. This dose range of LPS was selected based on results from our previous experiments. The lowest dose of LPS (0.31 mg/kg) was chosen because 0.16 mg/kg LPS only resulted in a mild increase of bioluminescence in the Gfap-luc mice, and it was speculated that a stronger signal was needed to induce behavioral effects. The highest dose of LPS was set to 1.25 mg/kg because 2.5 mg/kg LPS was lethal in the Gfap-luc mice.
The OFT setup consisted of 4 individual arenas (L x W x H: 40 x 40 x 40 cm). Each arena was lit from the top by a lamp producing a light intensity of 800 lux at the bottom. The four arenas allowed testing of four mice at once, while they were separated by non-transparent walls. A video camera with an infrared filter was fixed into the ceiling of each arena, in a way that it covered the entire surface area of that arena. Infrared illumination was provided below the floor of the arenas so mice could be detected and tracked under optimal conditions.

Exactly 2 sec after the detection of each individual mouse, tracking of movement was started and performed for 10 min using Noldus Ethovision, version 6.1 (Noldus Information Technology, The Netherlands) with software set up to detect immobility time and distance moved. In this test, exploration behavior of the animal was used to measure locomotor activity.

After single-housing the animals for one day prior to testing, the stress-induced hyperthermia (SIH) paradigm started by measuring the baseline temperature (Temp1). This was done by dipping a rectal probe (Model N9001, Comark Limited, UK) into peanut oil and inserting the probe for 2 cm into the rectum of the mouse while holding the animal in a head-upward position. 15 min later this procedure was repeated (Temp2) to determine the impact of the mild stress procedure of handling and probe insertion on rectal temperature. In both cases, the rectal probe was kept in place for 15-20 sec in order to standardize stress exposure and reach a stable temperature readout. The mild stress of handling and probe insertion causes a hyperthermic response and the difference in temperature before and after stress (dT = Temp2 – Temp1) reflects the SIH response. This SIH response is suppressed by anxiolytic drugs and is evaluated as a measure of anxiety [227].

The TST consisted of six individual chambers (2 rows with 3 columns; each chamber L x W x H: 14 x 14 x 19.5 cm). A 2.5 cm long hook was fixed to the ceiling of each chamber. The paradigm started by wrapping a piece of tape around the distal part of the tail of each mouse (about 2 cm from the tip) and positioning the mouse upside-down when the tape is placed over the hook. The six chambers allowed testing of six mice at once, while they were visually separated by non-transparent walls. A video camera was fixed onto a frame in front of the chambers such that it covered the entire surface of the units. Detection contrast was optimized by using black panels behind the white mice. Exactly 2 sec after detection of each mouse separately, the tracking of movement was started and performed for 6 min. Animals were tracked using Noldus Ethovision, version 6.1 with the software set up to detect immobility time and distance moved (based on center point of gravity of the detected surface). In this test the animal's immobility was evaluated as a measure of 'behavioral despair'.

The FST setup consisted of four independent cylinders (diameter 11 cm) which were automatically flushed and filled with water (10 cm deep, 24-25 °C). The

four cylinders allowed testing of four mice at once, while they were separated by non-transparent walls. A video camera with an infrared filter was fixed onto a frame in front of the cylinders such that it covered the entire surface area of all four units. Behind the cylinders, infrared illumination was provided to allow optimal detection and tracking of the mice. Exactly 2 sec after detection of each individual mouse, the tracking of movement was started and performed for 6 min using Noldus Ethovision 6.1 software. Immobility time and distance moved (based on center point of gravity of the detected surface) were detected and the animal's immobility was evaluated as a measure of 'behavioral despair'.

In the sucrose preference test (SPT), animals were single-housed in special Plexiglas IVC (L x W x H: 35 x 31 x 16 cm; Tecniplast, Italy) fitted with two 250 mL plexiglass drinking bottles (Tecniplast). Each bottle contained either filtered tap water or a sucrose solution (1, 2, 5 or 10%). The experiment consisted of a familiarization and a test phase. During the familiarization phase all animals were presented for 24 h with two water-filled bottles (W/W) on day (D) 1 and D3, or one water- and one sucrose-filled bottle (W/S) on D2 and D4. The bottles were removed between 08:00 and 09:00 AM each day and weighed using Software Wedge for Windows 1.2 (TAL Technologies). Animals were weighed and freshly prepared bottles were put onto the cages. The amount drunk by a mouse was determined by subtracting the weight of the bottle at the start of the observation period and at the end 24 h later (taking fluid density as 1 g/mL). Total fluid intake was taken as the total change in volume from both bottles combined, while the preference for sucrose was calculated as a percentage of consumed sucrose solution of the total fluid intake. A total fluid intake that was greater than the mean + 2 x standard deviation was considered to be an invalid measure that probably resulted from leaking bottles. Invalid measures were replaced by the mean of all the bottles either on the relevant side (for W/W) or for either water or sucrose (for W/S). This happened in less than 1% of all bottle measurements. The test phase of the experiment started 3 days after the familiarization phase by injecting the mice with either vehicle or 0.63 mg/kg i.p. LPS. This dose of LPS was chosen because it had a clear effect on neuroinflammation and sickness behavior in the previous experiments. Immediately after LPS administration the mice were presented with W/S for 24 h. This procedure was repeated for 3 consecutive days. Total intake volume was

evaluated as a primary measure for sickness behavior (reduction versus normal daily intake) while sucrose preference was used as a measure for anhedonia.

3.2.6 Statistical analysis

Data were analyzed using SPSS Statistics software (Version 20 for Windows, IBM Inc). ANOVA or rmANOVA was performed to assess the statistical significance of differences between treatment groups. A Greenhouse-Geisser correction epsilon (ε) was used for repeated measures analysis to correct for potential violation of the sphericity assumption [184]. This correction multiplies both the numerator and the denominator degrees of freedom by epsilon and the significance of the F-ratio is evaluated with the new degrees of freedom, resulting in a more conservative statistical test. When significant, post-hoc comparisons were made by using an independent samples t-test with a Bonferroni-corrected p-value. Significance was accepted for the ANOVAs and post-hoc comparisons when $p < 0.05$. All data are expressed as mean ± SEM.
Bioluminescent signals in the Gfap-luc mouse were analyzed by rmANOVA using dose (3 levels: 0, 0.16 and 0.63 mg/kg LPS) as a between-subjects factor and time (7 levels: BL, 2 h, 6 h, 24 h, 48 h, 72 h, 96 h) as a within-subject factor. Number of pixels in Iba1 positive cells were analyzed by ANOVA using dose (2 levels: 0 and 0.63 mg/kg LPS) as between-subjects factor. Cytokine levels were analyzed by separate ANOVAs for each cytokine with dose (3 levels: 0, 0.63 and 2.5 mg/kg LPS) and time (2 h, 6 h and 24 h) as between-subjects factor. Distance moved in OFT and immobility time in TST and FST were analyzed using separate ANOVAs with dose (4 levels: 0, 0.31, 0.63 and 1.25 mg/kg LPS) as between-subjects factor. For the SIH procedure, both temperatures (Temp1 and Temp2) were analyzed as a repeated measure and dose (4 levels: 0, 0.31, 0.63 and 1.25 mg/kg LPS) as a between-subjects factor. Total volume intake and sucrose preference in both phases of the SPT were separately analyzed using rmANOVA. In the familiarization phase, flavor (2 levels: W/W and W/S) and repeat (2 levels: first test and retest) were used as within-subject factor, and treatment group (5 levels: 1%, 2%, 5% and 10% Sucrose / LPS and 10% Sucrose / Vehicle) as a between-subjects factor. For the test phase, treatment group (5 levels: 1%, 2%, 5% and 10% Sucrose / LPS and 10% Sucrose /

Vehicle) was again used as a between-subjects factor and time (3 levels for total volume intake and sucrose preference: D8, D9, D10) as a within-subject factor.

3.3 Results

3.3.1 LPS induces glial cell activation

Factorial rmANOVA of photons emitted per second in the brain ROI revealed a significant time x dose interaction ($F(12,96) = 15.0$, $p < 0.001$, $\varepsilon = 0.18$). Post-hoc analysis showed that at 6 h post-LPS, a strong and brain specific bioluminescent signal was present in mice treated with 0.63 mg/kg, while at this time point a more moderate but still significant signal was evoked in the 0.16 mg/kg LPS group (figure 3.1). For both groups, there was still a significant increase in brain bioluminescence at 24 h, but no longer at 48 h post-LPS. Bioluminescence coming from the ears did not change during the experiment and was considered to be a background signal.

Because the bioluminescence data revealed a significant LPS-induced GFAP upregulation it was decided to confirm the presence of glial activation by immunohistochemistry using a microglial marker. Therefore, the expression of Iba1 was quantified in the hippocampal dentate gyrus at 24 h after systemic administration of vehicle or 0.63 mg/kg LPS. This brain structure was chosen because it is associated with stress and depression [228-230], and commonly studied in models of LPS-induced neuroinflammation [231, 232]. Although astrocyte activation in the Gfap-luc mouse peaked at 6 h post-LPS, it was decided to quantify Iba1 expression at 24 h as some studies reported depressive-like behavior in the absence of sickness at this time point [97, 98]. Furthermore, astrocyte activation was still increased in LPS-treated mice at 24 h, thereby indicating the relevance of measuring glial activation at this point. Factorial ANOVA indicated a significant effect of dose ($F(1,18) = 23.9$, $p < 0.001$) and post-hoc analysis showed that the pixel number of Iba1 positive cells was significantly higher in mice that received LPS when compared to vehicle-treated mice (figure 3.2).

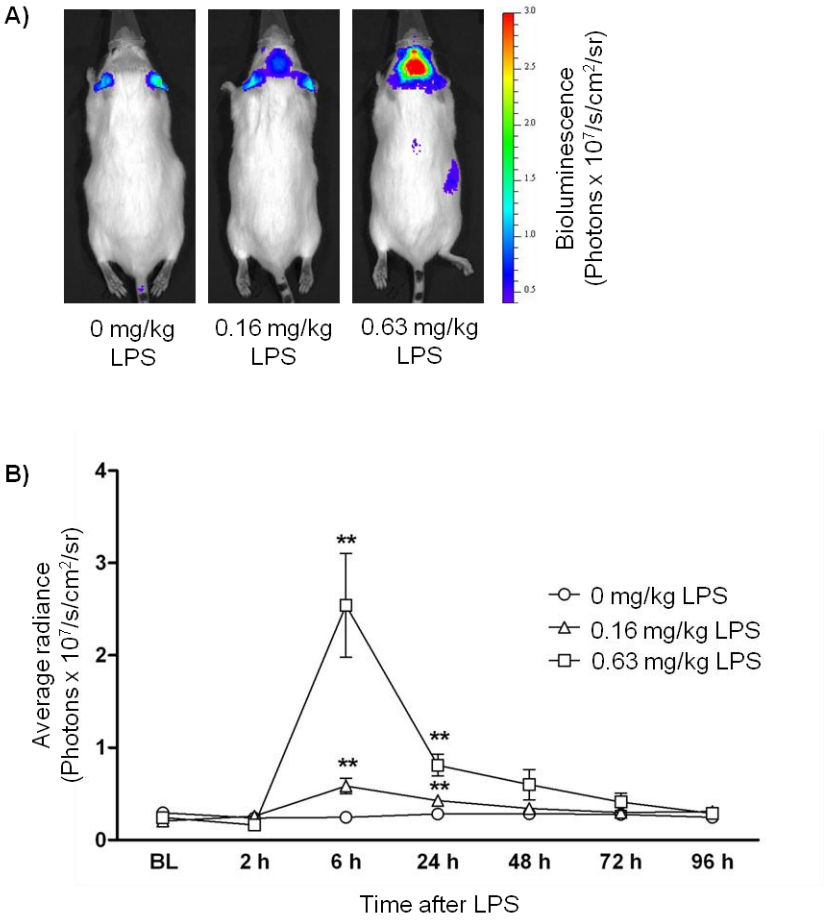

Figure 3.1 LPS activates astrocytes in vivo. Intraperitoneal injection of LPS caused a clear bioluminescent signal in the brain of Gfap-luc mice, as shown on representative images taken at 6 h after injection (A). This signal peaked at 6 h and then gradually waned over time (B) (bioluminescence data adapted from 2.1 B).The color on the image represents the number of photons emitted from the animal per second, as indicated in the color scale on the right. Graphs are plotted as mean ± SEM (n = 8 per group). Data were analyzed by rmANOVA followed by independent samples t-test. ** $p < 0.01$ compared to 0 mg/kg LPS.

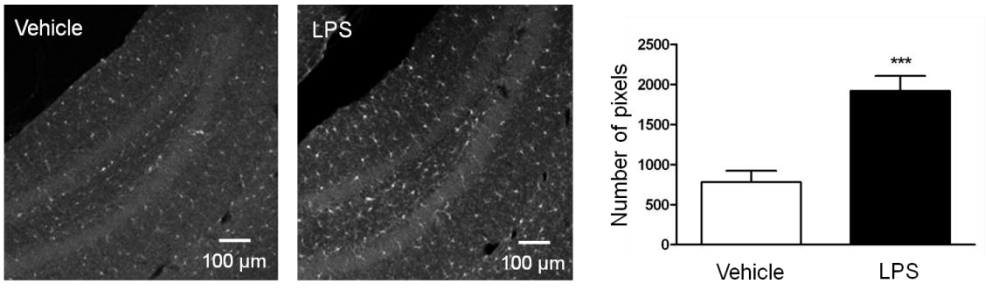

Figure 3.2 LPS increases Iba1 immunoreactivity in the dentate gyrus. LPS (0.63 mg/kg, i.p.) caused a strong upregulation of the microglial activation marker Iba1 in the hippocampal dentate gyrus at 24 h post-administration. Representative images (10x) (left), quantified images of n = 10 per group (right). Graph is plotted as mean + SEM. Data were analyzed by ANOVA followed by independent samples t-test. *** $p < 0.001$ compared to vehicle.

3.3.2 LPS increases serum and brain cytokine levels

For all cytokine levels measured in serum a significant time x dose interaction was found (IL-1β: $F(4,96) = 6.9$, $p < 0.001$; IL-6: $F(4,97) = 40.9$, $p < 0.001$; TNF-α: $F(4,95) = 18.8$, $p < 0.001$; IFN-γ: $F(4,98) = 4.9$, $p < 0.01$; IL-10: $F(4,95) = 14.3$, $p < 0.001$; MCP-1: $F(4,95) = 22.7$, $p < 0.001$). Post-hoc analysis demonstrated that serum cytokine levels in vehicle-treated mice were undetectable or minimal at all time points (figure 3.3, left column). Serum levels of IL-1β, IL-6, TNF-α, IL-10 and MCP-1 increased significantly after administration of 0.63 or 2.5 mg/kg LPS, peaking at 2 h post administration and gradually decreasing over time. Serum IFN-γ levels in LPS-treated animals followed a slightly different time course as the peak for this cytokine was reached at 6 h post-LPS. At 24 h after LPS administration, the serum levels of IL-1β, TNF-α and IFN-γ had returned to baseline values, while IL-6 and MCP-1 were still elevated in 0.63 and 2.5 mg/kg LPS-treated animals, and IL-10 only in 2.5 mg/kg LPS-treated mice.

A significant time x dose interaction was found on brain levels of IL-1β, IL-6, TNF-α and MCP-1 (IL-β: $F(4,98) = 5.6$ $p < 0.05$; IL-6: $F(4,96) = 9.7$, $p < 0.001$; TNF-α: $F(4,97) = 8.2$, $p < 0.001$; MCP-1: $F(4,97) = 24.3$, $p < 0.001$), but no significant effect of time or dose could be detected on IFN-γ or IL-10 brain levels. Comparable to the time course of their release in serum, brain levels of IL-6, TNF-α and MCP-1 peaked at 2 h post-treatment in mice exposed to 0.63 and 2.5 mg/kg LPS (figure 3.3, right column). Apart from MCP-1 levels, which were still elevated in the brains of LPS-treated mice at 24 h, all brain cytokine levels had returned to baseline values at 24 h post-LPS injection. IL-1β was slightly, but significantly, increased at 6 h in the brains of mice that received 2.5 mg/kg LPS, but not at 0.63 mg/kg.

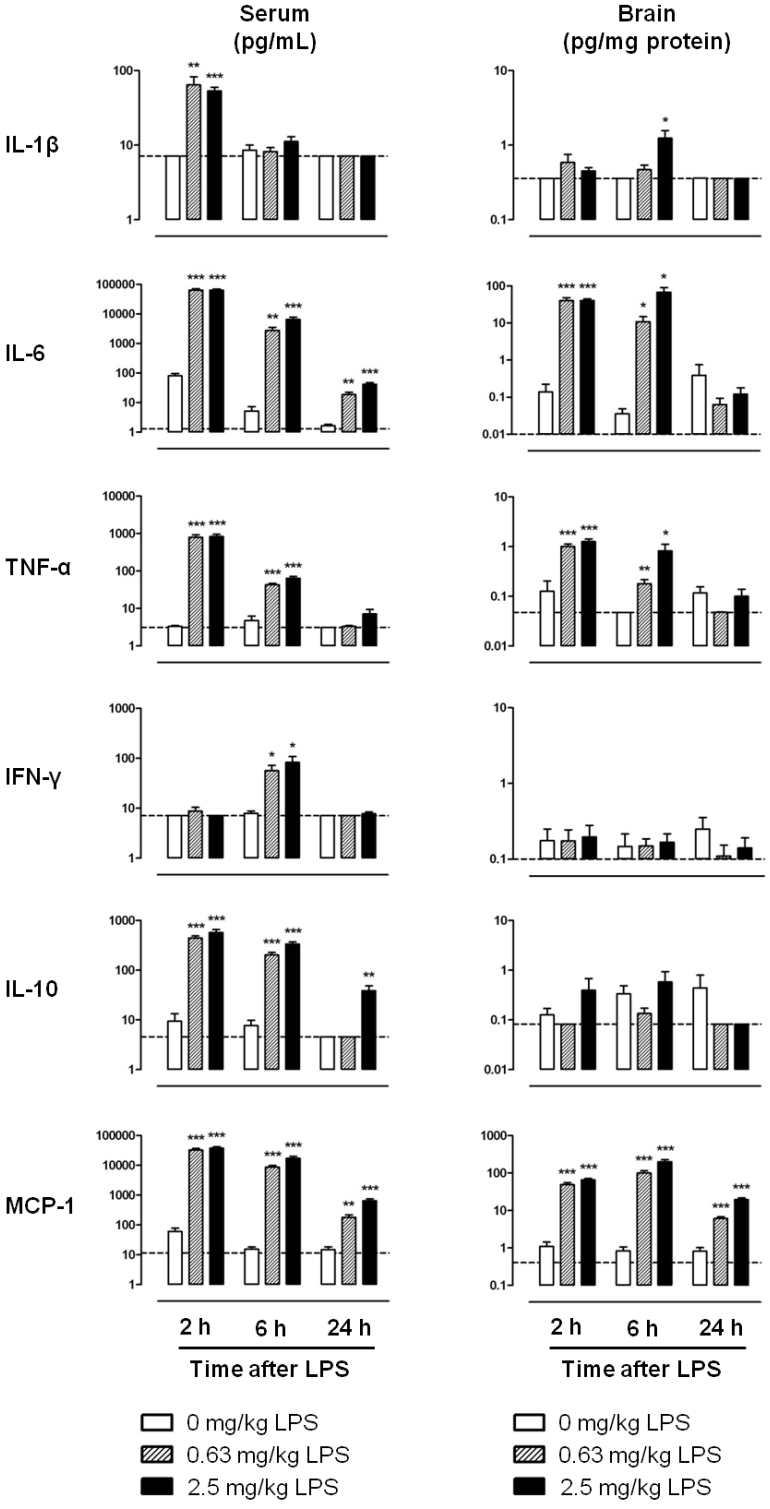
Serum
(pg/mL)
Brain
(pg/mg protein)
IL-1β
IL-6
TNF-α
IFN-γ
IL-10
MCP-1
2 h
6 h
24 h
Time after LPS
0 mg/kg LPS
0.63 mg/kg LPS
2.5 mg/kg LPS

Figure 3.3: LPS induces the release of immune mediators in serum and brain. Comparison of selection of cytokines and one chemokine (MCP-1) in serum (left) and brain (right) after i.p. LPS administration. Dashed lines indicate the detection limit of measured cytokine. Note that serum concentrations are expressed in pg/mL while brain levels are shown in pg/mg protein. Graphs are plotted as mean + SEM (n = 12 per group). Data were analyzed by ANOVA followed by independent samples t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0 mg/kg LPS.

3.3.3 LPS causes sickness but no clear depressive-like behavior

The total distance travelled in the open field test is a general measure for exploration and can be used as a marker of sickness behavior. Factorial ANOVA revealed a significant main effect for the factor dose at all time points tested (2 h: $F(3,36) = 6.6$, $p < 0.01$; 6 h: $F(3,35) = 23.7$, $p < 0.001$; 24 h: $F(3,36) = 4.3$, $p < 0.05$). Post-hoc analysis demonstrated that animals exposed to 0.63 or 1.25 mg/kg LPS showed reduced locomotor activity at 2 h post-treatment (figure 3.4, OFT). At 6 h all doses of LPS led to a reduced distance travelled in the OFT, while at 24 h only mice treated with 0.63 or 1.25 mg/kg LPS showed a significant reduction in exploration when compared to vehicle-treated mice.

The stressed-induced hyperthermia paradigm reflects a physiological response to mild stress exposure and is sensitive to treatment with anxiolytic drugs [227]. The measure for anxiety in this paradigm is the increase in body temperature in response to the mild stress of measuring rectal temperature. rmANOVA revealed a significant interaction for stress x dose at all time points tested (2 h: $F(3,36) = 5.4$, $p < 0.01$; 6 h: $F(3,36) = 14.0$, $p < 0.001$; 24 h: $F(3,36) = 21.3$, $p < 0.001$). Post-hoc analysis demonstrated that at 2 h post-LPS there was a dose-dependent decrease in both Temp1 and Temp2, which was significant for Temp1 at 1.25 mg/kg and for Temp2 in all LPS-treated mice (0.31, 0.63 and 1.25 mg/kg LPS) (figure 3.4, SIH). As LPS lowered both Temp1 and Temp2, there was no SIH effect in any of the LPS-treated mice, while it remained significant in control animals. At 6 h and 24 h post-LPS, Temp1 was significantly increased in LPS-treated mice (0.31, 0.63 and 1.25 mg/kg) but there was no significant difference in Temp2 between LPS-challenged and control mice. At these time points there was a significant SIH effect in all groups.

In the tail suspension test, behavioral despair was evaluated by measuring the time an animal remains immobile after being suspended by the tail. Factorial ANOVA revealed a trend for the factor dose at 6 h post-LPS ($F(3,35) = 2.3$, $p = 0.09$), but no statistical significance was found at 2 h or 24 h. Explorative post-hoc analysis revealed that at 6 h after LPS administration mice treated with 0.63 mg/kg LPS, but not 0.31 or 1.25 mg/kg LPS-treated animals, showed a slightly increased immobility time (figure 3.4, TST).

Behavioral despair in the forced swim paradigm was evaluated by measuring the time a rodent remains immobile after being placed in a water filled cylinder from which it cannot escape. At 6 h post-LPS, a trend was found for the factor dose

($F(3,35) = 2.6$, $p = 0.07$), but no statistical significance was found for any of the other time points. Explorative post-hoc analysis revealed that at 6 h after administration LPS induced a slight increase in immobility time that was significant in the 1.25, but not in the 0.31 or 0.63 mg/kg LPS-treated animals (figure 3.4, FST). At 24 h after LPS injection, animals treated with 0.63 mg/kg remained immobile for a longer period than control animals. However, this increased immobility at 24 h post-injection was not seen in mice exposed to 0.31 or 1.25 mg/kg LPS.

The sucrose preference test, in which the preference of an animal for a sweetened solution versus water is measured, is a commonly used rodent model to evaluate anhedonia. Our experiment consisted of two phases. The purpose of the familiarization phase was to assess normal daily intake volume, familiarize the animals with exposure to sucrose, and determine the effect of different sucrose concentrations on sucrose preference. rmANOVA revealed that for total intake volume during the familiarization phase there was a flavor x repeat x group interaction ($F(4,45) = 5.8$, $p < 0.001$). Furthermore, a main effect of group ($F(4,45) = 20.6$, $p < 0.001$) was found for sucrose preference. Post-hoc analysis demonstrated that total intake volume in the familiarization phase increased significantly when animals were exposed to both sucrose and water (D2 and D4), but only when the animals were retested (D4) with a sucrose concentration of 5 or 10% (figure 3.5, top left panel). The levels of sucrose preference correspond to these findings, as sucrose preference was significantly lower in mice exposed to 1% or 2% sucrose, but not in mice receiving 5% sucrose, when compared to mice exposed to 10% sucrose (figure 3.5, bottom left panel).

In the test phase, the effect of i.p. LPS administration on total intake volume and sucrose preference was assessed over time. rmANOVA revealed that there was a strong time x group interaction for total volume intake ($F(8,90) = 8.5$, $p < 0.001$, $\varepsilon = 0.86$). Post-hoc analysis indicated that in the first 24 h after administration (D8), LPS reduced the total intake volume to less than half of the normal daily water intake, suggesting suppression of drinking as a consequence of sickness (figure 3.5, top right panel). On the second day after LPS administration (D9), the LPS-induced reduction in total volume intake was no longer present in mice exposed to 10% sucrose solution, while it remained in the

mice receiving 1, 2 or 5% sucrose. At D10, the total intake volume of all mice had returned to baseline levels.

For sucrose preference in the test phase, rmANOVA revealed a time x group interaction ($F(8,90) = 4.3$, $p < 0.001$, $\varepsilon = 0.84$). In line with the total intake volume data, post-hoc analysis demonstrated that on D8 the sucrose preference was reduced in all LPS-treated animals (figure 3.5, bottom right panel). In the following days sucrose preference recovered depending on the sucrose concentration as on D9, the sucrose preference for LPS-treated mice receiving 10% sucrose had returned to pre-LPS values while for mice receiving 1, 2 or 5% sucrose this took up to D10.

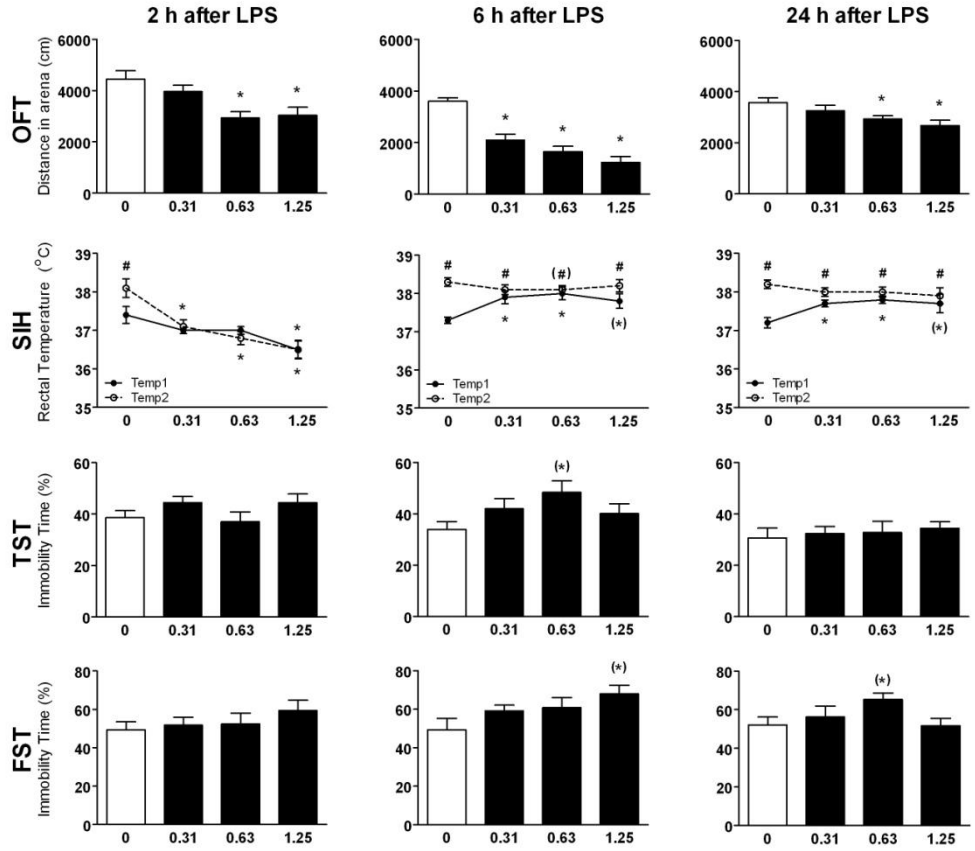

Figure 3.4 LPS causes sickness but not clear depressive-like behavior. Peripheral LPS administration caused a dose- and time-dependent reduction in locomotor activity in the open field test (OFT). However, a single i.p. injection of LPS did not induce clear anxiety or depressive-like behavior in the stress-induced hyperthermia (SIH) test, tail suspension test (TST) or forced swim test (FST). Graphs are plotted as mean + SEM (n = 10 per group). Data were analyzed by multivariate ANOVA followed by independent samples t-test. * $p < 0.05$ compared to 0 mg/kg LPS group, (*) $0.05 > p > 0.1$ compared to 0 mg/kg LPS group, # $p < 0.05$ compared to Temp1 (SIH).

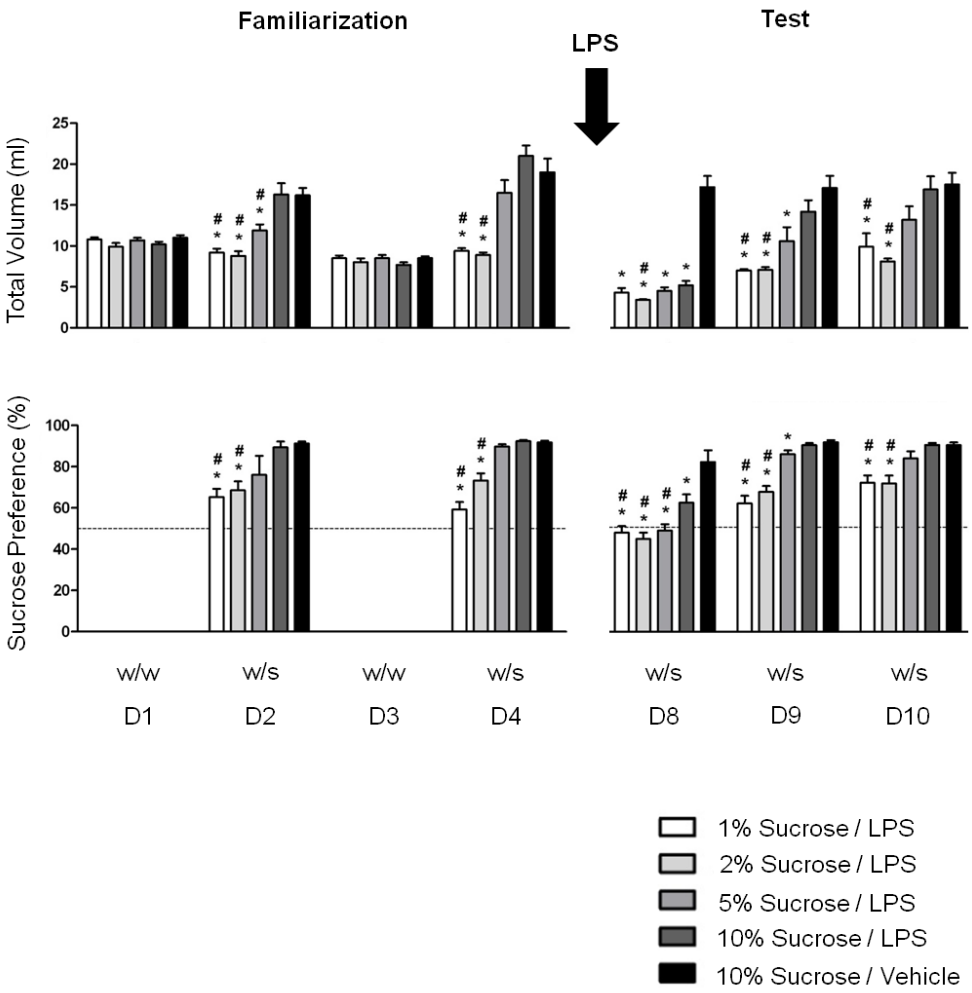

Figure 3.5 LPS decreases fluid intake and sucrose preference in mice. During the familiarization phase of the experiment (left), animals were familiarized to the experimental set up. On day (D) 1 and D3 mice were exposed to 2 bottles of water (W/W), while on D2 and D4 one bottle contained water and the other bottle was filled with a 1, 2, 5 or 10% sucrose solution (W/S). Voluntary consumption of water or sucrose was measured during a period of 24 h for up to 3 days after systemic administration of 0.63 mg/kg LPS (D8-D10). Dashed lines indicate chance level for sucrose preference. Graphs are plotted as mean + SEM (n = 10 per group). Data were analyzed by repeated measures ANOVA followed by independent samples t-test. * $p < 0.05$ compared to 10% Sucrose / Vehicle group, # $p < 0.05$ compared to 10 % Sucrose / LPS group. W/W = water/water; W/S = water/sucrose.

3.4 Discussion

Based on the complexity and heterogeneity of depression it is likely that several interacting systems underlie its pathogenesis. Findings from clinical studies indicate that inflammatory processes are associated with depression, at least in certain clinical subpopulations. For example, subsets of depressed patients show alterations of their peripheral immune system [10, 79, 80, 216] and depression often occurs as a comorbidity in patients suffering from conditions characterized by a sustained, systemic inflammation [73, 75-78, 217, 218]. Moreover, therapeutic stimulation of the immune system leads to depression in up to half of cancer and hepatitis C patients receiving proinflammatory cytokine treatment [219, 220].

Inflammation-associated depression is often studied in rodents by systemic administration of bacterial LPS, which is a potent activator of the immune system. Results from previous rodent studies indicate that systemic application of a single bolus of LPS leads to sickness behavior that peaks at 2 – 6 h, gradually fades over time, and is attenuated at 24 – 48 h after LPS injection (for a review see Dantzer et al 2008 [99]). There are some indications that depressive-like behavior can be separated from sickness at 24 h after systemic LPS administration [96-98]. Contrastingly, other studies showed that LPS-induced signs of sickness are still present at that time [102-105], making it difficult to compare results from different labs. Other factors complicating the interpretation of existing literature include the difference in experimental design between studies and the use of only a single dose of LPS and/or a few time points. Consequently, assessing time- and dose-dependent changes in neuroinflammation and behavior following systemic LPS administration is not straightforward.

The present study was therefore designed to evaluate central effects of systemic LPS administration at several time points by combining multiple techniques to quantify neuroinflammation and behavioral changes. To our knowledge, such an extended and multidisciplinary approach has not yet been reported in this field. First, the kinetics of neuroinflammation following peripheral immune activation were assessed using a transgenic mouse line that carries the luciferase gene under the transcriptional control of the mouse GFAP promoter. GFAP is an intermediate filament protein that is predominantly expressed by astrocytes,

and is upregulated when astrocytes are activated [182]. This makes the bioluminescent Gfap-luc mouse model an ideal tool to quantify astrocyte activation, as marker of neuroinflammation, in living mice. Systemic LPS administration to these Gfap-luc mice led to a time- and dose-dependent increase in brain bioluminescence that peaked at 6 h after LPS administration and then gradually faded over time. The upregulation of GFAP at 6 h post systemic LPS injection demonstrates that astrocytes respond rapidly to a peripheral immune challenge. This early response of brain cells to peripheral immune activation has also been shown in another bioluminescent mouse model where systemic LPS administration induced a time- and dose-dependent increase in the expression of CCAAT enhancer binding protein (C/EBP), a regulator of inflammation [233]. However, C/EBP upregulation peaked at 24 h post-LPS, a time point at which GFAP expression has decreased substantially, suggesting that astrocyte activation might be an early and short-lasting response to peripheral immune stimulation, while other inflammatory processes in the brain persist. Gfap-luc mice treated with the highest dose of LPS (2.5 mg/kg) died during scanning at 6 h after LPS. This was unexpected as the same dose was not lethal in NMRI mice tested throughout the rest of the study. One possible explanation for this discrepancy may be a strain-related difference in LPS sensitivity as previously described in models for acute lung injury [234] and inflammation-induced depression [235]. Other potential causes for the unexpected mortality in Gfap-luc mice treated with a high dose of LPS might be found in the experimental procedure to measure bioluminescence. It is possible, for example, that the toxic effects of isoflurane and/or potassium bound to luciferin become lethal in combination with a high dose of LPS.

To confirm glial activation using a different technique and another glial cell type, Iba1 expression was quantified in the hippocampal dentate gyrus of LPS-treated FVB wild-type mice. This brain structure is associated with stress and depression [228-230], and commonly studied in models of LPS-induced neuroinflammation [231, 232]. Iba1 is expressed in microglia and its expression is elevated under pathological conditions [232, 236-238]. Consistent with astrocyte activation found in the Gfap-luc mouse, LPS-treated FVB wild-type mice showed a robust increase in Iba1 reactivity in the dentate gyrus. These results indicate that microglia, in addition to astrocytes, also show signs of activation in response to

systemic LPS administration and are in line with previous reports of increased Iba1 immunoreactivity in the hippocampus of LPS-treated mice [232, 239].

Acute systemic LPS administration is known to induce a transient release of cytokines in the periphery and CNS [161, 186, 240]. In agreement with the literature, the present study showed that serum levels of cytokines that are involved in the acute phase response of inflammation (IL-1β, IL-6, TNF-α) were upregulated 2 h after peripheral LPS administration. Serum levels of IFN-γ, however, were only increased 6 h post-treatment, suggesting that the release of this cytokine was probably not triggered by LPS directly, but by downstream effects of earlier released cytokines. Serum levels of most pro-inflammatory cytokines had returned to baseline values at 24 h. However, at this time point the serum levels of IL-6 and the chemokine MCP-1 were still slightly elevated in all LPS-treated mice, demonstrating that the immune system was still mildly activated in the periphery. IL-10, an anti-inflammatory cytokine that plays a role in regulating the intensity and duration of the inflammatory response, remained elevated in the serum of mice treated with a high dose of LPS. The fact that IL-10 levels were no longer elevated at 24 h in the serum of mice treated with a low LPS dose indicates that anti-inflammatory pathways return to baseline quicker after a less pronounced immune activation.

Cytokines from the periphery can pass the BBB and reach the brain through humoral, neural and cellular pathways [26, 28, 241]. LPS has been shown to affect BBB permeability in several ways. Apart from early findings that LPS disrupts the BBB, LPS is now also known to exert direct effects on tight junction regulation [242] and cytokine release from endothelial cells in the brain [243]. However, the present study did not measure the integrity of the BBB and did not account for the fact that cytokines from the periphery can enter the brain through a leaky BBB. Despite this limitation it was found that the time-dependent brain profiles of IL-6, TNF-α and MCP-1 matched the serum profile, suggesting that these cytokines are expressed at a similar rate in the brain and/or that they can easily cross the BBB. Although IL-1β is known to pass the BBB [244], its brain levels were only significantly elevated at 6 h post-LPS in mice receiving 2.5 mg/kg, but not in mice receiving 0.63 mg/kg LPS. These findings are in line with results described by Puentener and colleagues [186], who did not find an increase in IL-1β brain levels at 3 hours after acute i.p. administration. Erickson and coworkers, in contrast, described an elevation of

IL-1β brain levels in mice at 24 h after a single dose of LPS [161]. The present study was unable to reliably detect brain levels of IFN-γ and IL-10. Based on the large number of samples below detection limit in all treatment groups, this was likely due to a sensitivity issue and not to lack of cytokine levels in the brain. However, the strong increase in brain levels of IL-6, TNF-α and MCP-1 confirmed that systemic LPS administration leads to a pro-inflammatory status in the brain. The brain levels of most cytokines returned to baseline at 24 h, while levels of the chemoattractant MCP-1 remained elevated. This indicates that there is still mild neuroinflammation present at this time point and coincides with the time course of astrocyte activation in the Gfap-luc mouse model and Iba1 immunoreactivity in the dentate gyrus of LPS-treated mice. This study did not account for regional differences of cytokine profiles in the brain. However, results from several other studies have pointed out that there might be a spatio-temporal component to LPS-induced cytokine production in the brain [240, 245-247]. Future research focusing on the identification of local changes in neuroinflammation may help to identify brain areas that are involved in inflammation-associated depression.

In addition to evaluating the LPS-induced peripheral and central immune responses as described above, the second aim of this study was to investigate the main and side effects of peripheral LPS administration on behavior. Some indications already exist that at 24 h after acute peripheral LPS injection depressive-like behavior can be observed in rodents. However, the nature and characteristics of LPS-induced sickness behavior can substantially confound measurements of depressive-like behavior in commonly used paradigms. For example, sick animals show reduced motor activity which can confound measures of immobility, used to estimate despair in inescapable conditions (e.g. tail suspension and forced swim test) [99]. Therefore, studies focusing on depressive-like behavior should also include measures of sickness. Several groups have already used a combination of behavioral tests for that purpose. In some of these studies a time window was identified in which sickness had dissipated while depressive-like behavior was still present. However, findings from different labs often vary. Some groups showed that LPS-treated mice display increased immobility in the tail suspension and forced swim test at 24 h, a time point at which motor activity in the open field test had returned to baseline [97, 98]. In contrast, other groups still observed reduced locomotor

activity as an indication of sickness at this time after LPS administration [104, 105, 248]. Studies measuring sickness by evaluating social behavior are also not clear on the duration of LPS-induced sickness. Some groups, for example, have shown that social behavior returned to normal at 24 h post-LPS [96, 104, 249], while others still report deficits in social behavior at this time point [102]. Hence, we evaluated the dose-dependency and time course of LPS-induced changes in behavior across a panel of assays that are commonly used to study sickness, anxiety and depressive-like behavior in rodents. Sickness, as measured by decreased locomotion in the OFT, occurred at 2 h post-LPS treatment and had dissipated at 24 h in mice treated with a low dose of LPS. Animals treated with higher doses of LPS, however, still showed reduced locomotor activity at this point, indicating that sickness remained present in these mice. This timing coincided with signs of sickness seen in the SIH test where the baseline temperature (Temp1) of LPS-treated mice remained elevated at 24 h post-LPS, thereby confounding measures of anxiety (dT). Depressive-like effects as evaluated by immobility time in the TST and FST were very low at all measured time points, and can be considered biologically irrelevant here due to the co-occurrence of sickness. Furthermore, it is worth to note that we used naive mice at each time point in our behavioral tests to avoid differences in confounding habituation effects (due to repeated testing) between sick and control animals.

From the sucrose preference experiment it becomes clear that the concentration of sucrose is a key factor for sucrose preference in mice. As seen on the last day of the familiarization phase (D4), the sucrose preference increased with sucrose concentration, with a ceiling effect being reached at 5% sucrose. Mice exposed to 5-10% sucrose also clearly drank much more than their normal daily intake, i.e. on a day where they were exposed to water only. However, this was not the case in mice receiving 1-2% sucrose showing that the total volume intake also depends on the sucrose concentration. Moreover, our data reveal that LPS reduced sucrose preference in a time-dependent manner. These findings are in line with previous results showing that LPS administration to mice decreased their sucrose consumption [97] and sucrose preference [96] for up to 2 days after administration, while leaving their water and food intake unaltered [97]. Despite the fact that in our study there was also no difference in water intake between treatment groups during the first 24 h after LPS administration (data

not shown), it is important to mention that at this time the total volume intake in LPS-treated mice was reduced to approximately half of the normal daily intake. This suppressed drinking suggests that sickness still seems to be a confounding factor when measuring sucrose preference during the first 24 h after LPS administration and points out that caution is needed when interpreting LPS-induced reduction in sucrose preference as a measure of anhedonia.

Our data clearly show that acute systemic administration of LPS leads to a strong but ephemeral activation of the peripheral immune system with accompanying neuroinflammation and behavioral effects. Inflammation-associated depression in humans, however, is linked to chronic, persistent inflammation [70, 221]. This makes acute LPS administration to mice a less attractive translational model for inflammation-associated depression in humans. Interestingly, Kubera and coworkers recently described a mouse model in which repeated LPS injections given at one month intervals induced a chronic state of anhedonia, indicating that chronic LPS administration might be a more relevant approach to induce depressive-like behavior [250]. In that study, the prolonged anhedonia in response to repeated LPS administration was only observed in female, but not in male mice. In another study, a less elaborate model of repeated LPS administration was shown to induce depressive-like behavior in absence of sickness in male rats [245]. It is possible that as hypothesized for the human situation, a chronic inflammatory tone is needed to elicit depressive-like behavior in rodents. However, future work is needed to evaluate whether repeated LPS administration in rodents is a more valid model of inflammation-associated depression.

In conclusion, the present set of experiments using various assays and readouts confirmed that there is a strong crosstalk between the immune system and the brain, both on a neuroimmune and neurobehavioral level. Acute systemic LPS administration in mice caused a marked but transient increase in pro- and anti-inflammatory cytokines in the periphery. The time course of the systemic inflammation coincided with neuroinflammation as seen by astrocyte activation in Gfap-luc mouse, increased Iba1 immunoreactivity in the hippocampus, and elevated cytokine levels in the brain. Moreover, thorough investigation of several primary parameters across a panel of behavioral assays showed that systemic LPS administration induced sickness lasting for up to 48 hours. This time-dependent profile coincided with mild depressive-like behavior. However, due to

overlapping time windows and rather mild effects on depressive-like behavior per se, it is not possible to separate sickness from depressive-like behavior in the present rodent model.

4

Characterization of the cytokine and anhedonia response to LPS in rats

Based on:

Systematic analysis of the cytokine and anhedonia response to peripheral lipopolysaccharide administration in rats.

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ABSTRACT

Several lines of evidence indicate that inflammatory processes may play a role in the pathogenesis of depression, at least in subsets of vulnerable individuals. Inflammation-associated behavioral changes are commonly studied in rodents by administration of bacterial LPS. It has been proposed that systemic injection of LPS induces a biphasic response, in which sickness is followed by an episode of depressive-like behavior. However, the time window in which potential depressive-like behavior occurs is not very clear and findings often vary across labs. In this study, we characterized the time course of LPS-induced immune activation in rats by measuring serum levels of analytes relevant to inflammation at several time points. Then we used the sucrose preference test to evaluate anhedonia, as a measure of depressive-like behavior, in response to a single LPS injection. Finally, we tested whether repeated LPS exposure or sub-chronic restraint stress altered the anhedonia response to a subsequent acute LPS challenge.

We report that systemic administration of LPS robustly increased circulating levels of corticosterone, leptin, pro- and anti-inflammatory cytokines, and chemokines. Serum concentrations of most analytes peaked within 6 h after LPS injection and had returned to baseline values by 24 h. Chemokine levels, however, remained elevated for up to 96 h thereby indicating that the immunological response to LPS lasted for several days. The time course of sickness corresponded with the kinetics of corticosterone and cytokine release as it was clearly present from 2 to 24 h. LPS-induced anhedonia, as measured by decreased sucrose preference, lasted up to 96 h. Repeated LPS administration reduced sickness but did not influence the anhedonic response to acute LPS. Sub-chronic restraint stress did not alter the effects of a subsequent LPS challenge or cause anhedonia by itself.

In this study, we systemically characterized the kinetics of immune mediator release and behavioral changes following systemic LPS administration in rats. We presented a sucrose preference protocol that provides a way of separating LPS-induced anhedonia from sickness. Using this protocol, more elaborate rodent models can be developed to study the mechanisms underlying inflammation-associated depression in humans.

4.1 Introduction

Major depressive disorder, or depression, is a serious medical illness with a life time prevalence of around 16% [60]. It is predicted that by 2030 depression will be the second leading cause of disability worldwide [61]. Clinical manifestations of depression include a range of symptoms, such as depressed mood, anhedonia (inability to experience pleasure from naturally rewarding activities), feelings of worthlessness or excessive guilt, decreased appetite and weight, fatigue and recurrent suicidal ideations [62]. For many years, pharmacological research in depression has been focused on the monoamine theory, which proposes that depression is caused by decreased monoamine function in the brain and that drugs which correct this deficit, such as selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) can treat the disorder [251, 252]. Though the monoamine systems are clearly involved in the aetiology of depression, it is now generally accepted that a more complex interplay between genetics and environmental factors underlies its pathophysiology. Findings from clinical studies indicate that inflammatory processes might also be involved in the pathogenesis of depression, at least in a subset of susceptible individuals (for reviews see [10, 30, 89, 99, 221]).

Based on these observations, several rodent models of inflammation-associated depression have been developed. One of the most used approaches in these models involves administration of bacterial LPS, which is a potent activator of the immune system. Behavioral studies have shown that systemic LPS injection in rodents induces a sickness response, which is characterized by hypolocomotion, social withdrawal, fatigue, anorexia and alterations in sleep patterns and cognition [99]. There are some indications that LPS-induced sickness is followed by a depressive-like phenotype, in which rodents display behavior similar to clinically relevant symptoms of depression in humans [96-98]. However, the time window in which potential depressive-like behavior occurs is not very clear and findings often vary across labs. For example, some studies indicate that depressive-like behavior can be observed in the absence of sickness 24 h after systemic LPS administration [96-98], while others report that at this time sickness is still present and hence confounds measurements of depressive-like behavior [102, 104, 105, 160]. Indeed, sick animals display reduced locomotor activity, which can confound measurements of immobility

used to estimate behavioral despair in paradigms such as the forced swim test [99]. Moreover, sick animals show reduced eating and drinking behavior, thereby potentially confounding measures of sweetened fluid intake in paradigms designed to evaluate anhedonia. Therefore it is of crucial importance to include measures of sickness when assessing depressive-like behavior.

In a previous study we characterized behavioral changes induced by systemic LPS injection in mice [160]. Results from this work indicate that the time course of sickness and anhedonia can be evaluated by measuring total volume intake and sucrose preference in the SPT. In order to extend this study, we chose to characterize sickness and the anhedonic response to systemic LPS injection using a SPT in rats. First, the dose dependency of LPS-induced changes in behavior during the first 24 h after LPS administration was evaluated across a panel of behavioral assays. After identifying a dose that induced potential anhedonia, the immunological response to systemic LPS was measured by quantifying serum levels of corticosterone, leptin, and a selection of cytokines and chemokines over time. Then, the time course of sickness and the anhedonic response to systemic LPS was assessed using an optimized SPT. Finally, this optimized SPT was used to test whether pre-exposure to repeated LPS administration or sub-chronic restraint stress influences the anhedonic response to a subsequent LPS challenge.

4.2 Materials and methods

4.2.1 Animals and LPS

All studies were conducted in male Sprague-Dawley rats (Harlan, The Netherlands) weighing 200-220 g on arrival. Animals were housed in groups of 4 in plexiglass individually ventilated cages (L x W x H: 43 x 32 x 18 cm; Tecniplast, Italy) for one week prior to an experiment to acclimate. Procedure rooms were maintained at a temperature of 22 ± 2 °C and a humidity of 54 ± 2%, with a 12:12 h light-dark cycle (lights on at 06:00 AM. with a 30 minute dim and rise phase). Unless mentioned otherwise, food and water were available ad libitum. All experimental protocols were approved by the Institutional Ethical Committee on Animal Experimentation, in compliance with Belgian law (Royal Decree on the protection of laboratory animals dd. April 6, 2010) and conducted in facilities accredited by the AAALAC.
LPS from Escherichia coli (serotype 055:B5) was purchased from Sigma-Aldrich and freshly dissolved in sterile saline prior to injection.

4.2.2 Serum collection

To measure the effect of peripheral LPS administration on serum levels of a selection of analytes, rats were injected i.p. with either vehicle or 0.63 mg/kg LPS (n = 12 per group). Just before, and at 1 h, 2 h, 6 h, 24 h, 48 h, 72 h and 96 h after the immune challenge, rats were briefly anesthetized by inhalation of 2% isoflurane, 0.5 mL of whole blood was collected from the tail artery and the rats were returned to their home cage. Serum was obtained by keeping the blood samples in Vacutainer SST II Advance blood tubes (BD Biosciences, product ID 367955) for 30 minutes at room temperature. Then the samples were centrifuged (1300 g, 10 min, room temperature), aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C until further use.

4.2.3 Cytokine measurements

Serum levels of chemokine (C-X-C motif) ligand 1 (CXCL1), IFN-γ, IL-1β, IL-6, IL-10, leptin, MCP-1, macrophage inflammatory protein-1 α (MIP-1α) and TNF-α were simultaneously determined using a rat cytokine/chemokine magnetic bead panel kit from Merck Millipore. This assay is based on Luminex™ technology in which capture antibodies specific for individual cytokines are coated with magnetic beads that have a distinct emitting fluorescence pattern. All steps in the assay were conducted according to the manufacturer's instructions. A Bio-Plex 200 System (Bio-Rad) was used to measure the fluorescent signal and the data was analyzed using Bio-Plex Manager 5.0 software (Bio-Rad) with five-parameter logistic regression curve fitting. Cytokine levels below detection limit were assigned a value equal to the lowest detectable value of that cytokine. Cytokine values outside of the average + 3 times standard deviation range were considered outliers and were excluded from all calculations. This was the case for less than 2% of all measured cytokines.

4.2.4 Corticosterone measurements

Serum concentrations of corticosterone were determined using a commercial ELISA kit supplied by Enzo Life Sciences (product ID 900-097). All procedures were carried out as per manufacturer's instructions.

4.2.5 Behavioral tests

The OFT and FST setups were custom-made. In these paradigms, groups of naive rats (n = 12 per group) were injected i.p. with 0, 0.31, 0.63, or 1.25 mg/kg LPS and tested at 2 h, 6 h, or 24 h after LPS administration. This dose range of LPS was selected based on results from our previous experiments in mice [160].

Open field test

The OFT setup consisted of a circular arena with a diameter of 1.2 m. A video camera with an infrared filter was fixed 1 m above the arena and infrared illumination was provided from the bottom for optimal detection and tracking of the rats. This setup allowed the testing of one rat at a time. Exactly 2 sec after

detection of a rat, tracking was started and performed for 10 min using EthoVision 6.1 software (Noldus, The Netherlands), set up to detect immobility time and distance moved. In this test, exploratory behavior by the animal was used to measure locomotor activity.

Forced swim test

The FST setup consisted of four independent plexiglass cylinders (diameter 19 cm) which were flushed and filled with water (30 cm deep, 24-25 °C). The four cylinders allowed testing of four rats at once. A video camera with an infrared filter was fixed onto a frame in front of each cylinder and infrared illumination was provided to allow optimal detection and tracking of the rats. Exactly 2 sec after detection of each individual rat, tracking was started and performed using EthoVision 6.1 software (Noldus, The Netherlands). Each FST test consisted of two sessions: a 15 min training session on the day before LPS administration, and a 6 min test session at the relevant time after LPS. Immobility time and distance moved (based on center point of gravity of the detected surface) were measured during each session and the duration of immobility was evaluated as a measure of behavioral despair.

Sucrose preference test in fluid deprived rats

Animals were single-housed in IVCs (L × W × H: 35 × 31 × 16 cm; Tecniplast, Italy) fitted with two 250 mL drinking bottles and ad libitum access to food. Each of the drinking bottles contained either filtered tap water or a 1% sucrose solution. The location of each bottle on the cage was randomized every day, with half the animals receiving sucrose on the left, and the other half on the right. Prior to LPS administration, rats were familiarized to the sucrose solution by presenting them with W/W or W/S for 24 h each on 2 consecutive days. Then, the rats were fluid deprived overnight and injected i.p. with 0, 0.31, 0.63 or 1.25 mg/kg LPS (n = 12 per group). At 2 h, 6 h and 24 h after LPS administration all rats were exposed to pre-weighed drinking bottles containing W/S. After 1 h the bottles were removed and weighed using Software Wedge for Windows 1.2 (TAL Technologies).

Sucrose preference test in undeprived rats

All of the remaining SPT experiments started with a 4 day familiarization phase, during which the rats were presented for 24 h with two water-filled bottles (W/W) on familiarization day 1 (FAM1) and FAM3, or one water- and one 1% sucrose-filled bottle (W/S) on FAM2 and FAM4. The bottles were removed between 08:00 and 09:00 AM each day and weighed using Software Wedge for Windows 1.2. Then, the animals were weighed and freshly prepared bottles were put onto the cages.

To assess the effect of a single peripheral bolus of LPS on anhedonia over time, the test phase started by weighing and injecting rats with either vehicle or 0.63 mg/kg i.p. LPS (n = 12 per group). Immediately after LPS administration, the rats were put into their home cage and given access to W/S for 4 consecutive days. This study was performed three times using naive animals for each experiment and the data was pooled prior to analysis so that the final n = 36 per treatment group.

The effect of repeated systemic LPS injection on anhedonia was evaluated by randomizing rats across 4 experimental groups, i.e. 5 days vehicle + vehicle on test day (5 Veh + Veh), 5 days vehicle + LPS on test day (5 Veh + LPS), 5 days of LPS + vehicle on test day (5 LPS + Veh) and 5 days of LPS + LPS on test day (5 LPS + LPS) (n = 12 per group). After the familiarization phase, a pre-exposure phase was introduced in which rats from the 5 days of vehicle groups were injected i.p. with vehicle while rats from the 5 days of LPS groups received a daily i.p. injection of 0.63 mg/kg LPS for 5 consecutive days. All rats had ad libitum access to food and water during the pre-exposure phase. Three days after the last LPS administration, rats were injected with an acute bolus of either vehicle or 0.63 mg/kg i.p. LPS and presented with W/S for 24 h for 4 consecutive days.

To assess the effect of stress on LPS-induced anhedonia, rats were randomized across 4 experimental groups, i.e. no stress + vehicle (NS + Veh), no stress + LPS (NS + LPS), stress + vehicle (S + Veh) and stress + LPS (S + LPS) (n = 12 per group) and a manipulation phase was introduced in between the familiarization phase and the test phase. During this manipulation phase, animals in the stress groups were weighed and subjected to 1 h of physical restraint per day using a transparent rat restrainer (D x H: 5 x 23 cm; length adjusted to tightly enclose the rat) for 5 consecutive days. To control for

possible effects of handling stress, rats from the no stress groups were weighed, handled and put back in their home cage. All rats had ad libitum access to food and water during the manipulation phase. The test phase started 3 days after the manipulation phase by injecting the rats i.p. with either vehicle or 0.63 mg/kg LPS. Immediately after LPS administration, all animals were presented with W/S for 4 consecutive days.

Evaluation of parameters in sucrose preference test

In all SPT experiments, the amount drunk by a rat was determined by subtracting the weight of a bottle at the start of the observation period and at the end (taking fluid density as 1 g/mL). Total fluid intake was taken as the total change in volume from both bottles combined, while sucrose preference was calculated as a percentage of consumed sucrose solution of the total fluid intake. A total fluid intake greater than the mean +2x standard deviation was considered to be an invalid measure that probably resulted from leaking bottles. Invalid measures were replaced by the mean of all the bottles either on the relevant side (for W/W) or for either water or sucrose (for W/S). This happened for less than 2% of bottle measurements in any given experiment. Total volume intake was evaluated as a primary measure for sickness behavior (reduction versus normal daily intake), while sucrose preference was used as a measure of anhedonia. Change in body weight was calculated by subtracting the weight at a given time point from the weight at the start of the experiment. These time points are specified for each experiment in the results section.

4.2.6 Statistical analysis

Data were analyzed using SPSS Statistics software (Version 20 for Windows, IBM Inc). ANOVA or rmANOVA was performed to assess the statistical significance of differences between treatment groups. A Greenhouse-Geisser correction epsilon (ε) was used in case of repeated measures analysis to correct for potential violation of the sphericity assumption [184]. This correction multiplies both the numerator and the denominator degrees of freedom by epsilon and the significance of the F-ratio is evaluated with the new degrees of freedom, resulting in a more conservative statistical test. To account for the skewness of the data distribution, concentrations of serum analytes were log-

transformed prior to analysis. Significance was accepted for the ANOVAs and rmANOVAs when $p < 0.05$. When appropriate, post-hoc comparisons were made by using an independent samples t-test with a Bonferroni-corrected p-value. For consistency between the analysis and the visualization of serum analyte concentrations, the group means and its SEM were back-transformed and visually presented on a logarithmic scale. All other data are expressed as mean ± SEM on a linear scale.

4.3 Results

4.3.1 LPS causes sickness and anhedonia in a dose- and time-dependent manner

The total distance travelled in the OFT is a general measure for exploration and can be used as a marker of sickness behavior. Factorial ANOVA revealed a main effect of LPS dose ($F(3,110) = 11.1$, $p < 0.001$) and time point ($F(2,110) = 15.6$, $p < 0.001$) for total distance travelled. Post-hoc analysis indicated that systemic LPS administration reduced locomotor activity in a dose-dependent manner at 2 h (figure 4.1, OFT). This LPS-induced reduction in exploration was more pronounced at 6 h after LPS, but disappeared at 24 h.

The effect of systemic LPS administration on behavioral despair was evaluated in the FST by placing the rats in a water-filled cylinder from which they cannot escape and measuring the time they remained immobile. Factorial ANOVA demonstrated that there was no main effect of LPS dose or time point tested. Explorative post-hoc analysis indicated that rats injected with 0.63 mg/kg LPS showed a potential increase in immobility time at 6 h after administration (figure 4.1, FST). Such an immobility response was not observed at any of the other time points or LPS doses used.

In the sucrose preference paradigm, sickness is evaluated by measuring the total volume of fluid an animal consumes during a pre-defined observation period, while sucrose preference is used as marker for anhedonia. rmANOVA revealed a significant time x LPS dose interaction for total volume intake ($F(6,80) = 12.3$, $p < 0.001$, $\varepsilon = 0.98$). Post-hoc analysis indicated that LPS reduced total volume intake at 6 h and 24 h to a similar extend at all doses (figure 4.1, SPT), suggesting suppression of drinking as a consequence of sickness. No main effect of time or LPS dose was found for sucrose preference. However, explorative post-hoc analysis demonstrated that at 24 h, sucrose preference was significantly reduced in rats that were administered with 0.63 or 1.25 mg/kg LPS (figure 4.1, SPT). Interestingly, rats injected with 0.31 mg/kg LPS did not show reduced sucrose preference, while at this time they drank much less than vehicle-treated rats. This suggests that LPS-induced anhedonia is potentially detectable at a dose of 0.63 mg/kg and higher.

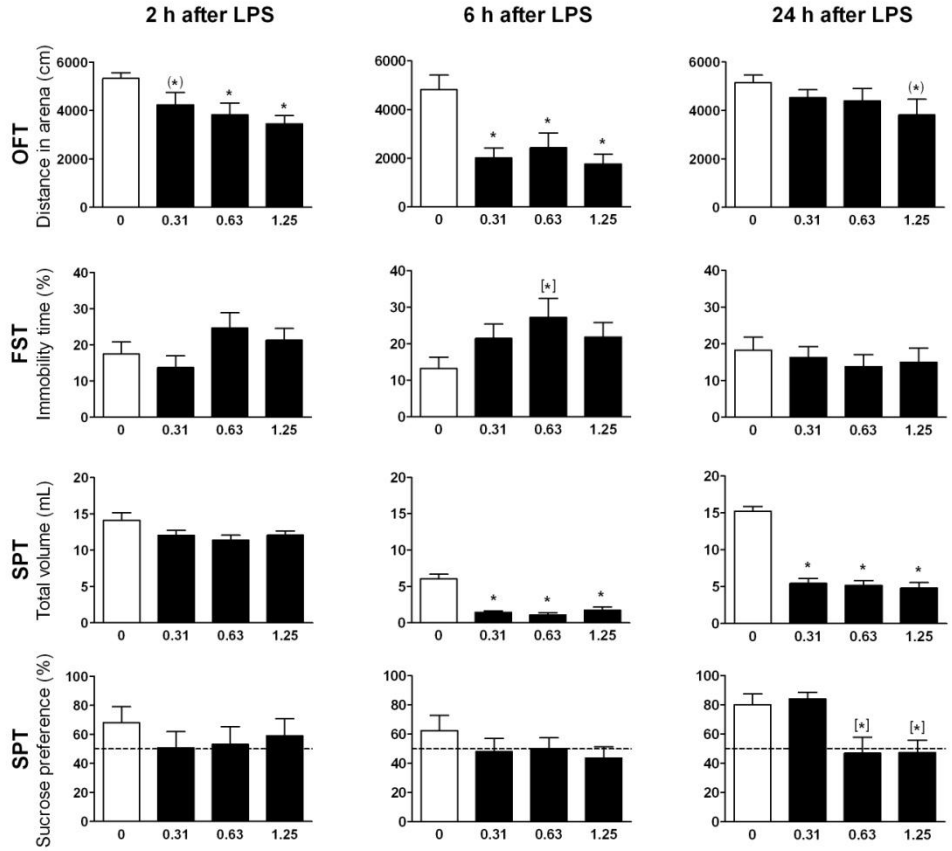

Figure 4.1 LPS causes sickness and anhedonia in rats. Intraperitoneal injection of LPS induced sickness behavior, as seen by reduced locomotor activity in the open field test (OFT) and decreased total volume intake in the sucrose preference test (SPT). At 24 h after administration, a dose of 0.63 and 1.25 mg/kg LPS reduced sucrose preference, thereby potentially indicating development of anhedonia. However, a single i.p. injection of LPS did not induce clear depressive-like behavior in the forced swim test (FST). Please note that in the OFT and FST naive animals were used at all time points, whereas in the SPT rats were tested repeatedly. Dashed lines indicate chance level for sucrose preference. Graphs are plotted as mean + SEM (n = 12 per group). OFT and FST data were analyzed by multivariate ANOVA, SPT data by rmANOVA, and followed by independent samples t-test. (*) $0.1 < p < 0.05$; * $p < 0.05$ compared to 0 mg/kg LPS group; [*] $p < 0.05$ compared to 0 mg/kg LPS group in absence of rmANOVA effects.

4.3.2 LPS increases serum levels of corticosterone, cytokines and chemokines in a time-dependent manner

Based on the strong behavioral effects of 0.63 mg/kg LPS, it was decided to analyze the effect of this particular LPS dose on the release of a selection of hormones and cytokines in serum over time. Factorial rmANOVA revealed a significant time x LPS interaction for the analytes corticosterone ($F(7,140) = 11.2$, $p < 0.001$, $\varepsilon = 0.47$), CXCL1 ($F(7,133) = 56.7$, $p < 0.001$, $\varepsilon = 0.30$), IFN-γ ($F(7,140) = 39.8$, $p < 0.001$, $\varepsilon = 0.29$), IL-1β ($F(7,140) = 14.9$, $p < 0.001$, $\varepsilon = 0.28$), IL-6 ($F(7,140) = 76.6$, $p < 0.001$, $\varepsilon = 0.19$), IL-10 ($F(7,140) = 35.1$, $p < 0.001$, $\varepsilon = 0.22$), Leptin ($F(7,140) = 6.5$, $p < 0.001$, $\varepsilon = 0.53$), MCP-1 ($F(7,140) = 288.4$, $p < 0.001$, $\varepsilon = 0.38$), MIP-1α ($F(7,140) = 51.1$, $p < 0.001$, $\varepsilon = 0.33$) and TNF-α ($F(7,140) = 68.2$, $p < 0.001$, $\varepsilon = 0.23$). Post-hoc analysis showed that serum levels of corticosterone were elevated at 2 h, 6 h and 24 h, and fell below control values at 48 h after LPS administration (figure 4.2). Furthermore, LPS caused a strong but short-lasting increase in the serum concentrations of most cytokines. Interestingly, the peak of this release did not occur at the same time for all cytokines. IL-10 and TNF-α peaked at 1 h, while CXCL1, IL-1β, IL-6, MCP-1 and MIP-1α reached their peak release at 2 h after LPS administration. IFN-γ and leptin were the only analytes that peaked at 6 h post-LPS. Apart from the chemokines CXCL1, MCP-1 and MIP-1α all cytokines had returned to control levels by 24 h.

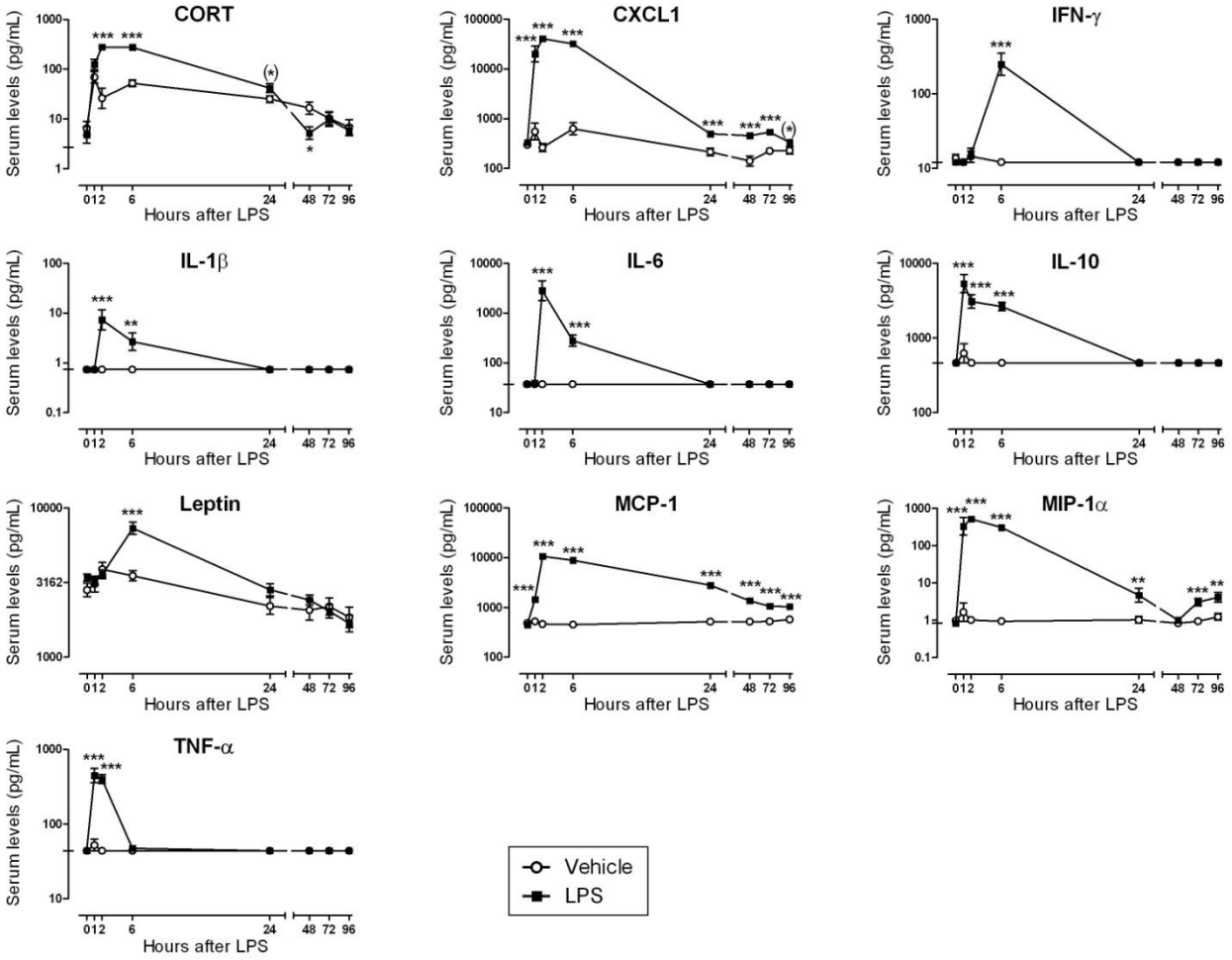
CORT
CXCL1
IFN-γ
IL-1β
IL-6
IL-10
Leptin
MCP-1
MIP-1α
TNF-α
Serum levels (pg/mL)
Hours after LPS
0 1 2 6 24 48 72 96
Vehicle
LPS

Figure 4.2 Serum profile of a selection of analytes at multiple time points after LPS administration. Time curves of a selection of cytokines, chemokines, leptin and corticosterone quantified in serum at 0 h, 1 h, 2 h, 6 h, 24 h, 48 h, 72 h and 96 h after LPS injection (0.63 mg/kg, i.p.). The detection limit of each analyte is indicated by a tick on the y-axis of its individual graph. Detection limits that fall below the lowest value on the y-axis are not presented. Graphs are plotted as mean ± SEM (n = 12 per group). Data were analyzed by rmANOVA followed by independent samples t-test. (*) $0.1 < p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle.

4.3.3 LPS-induced anhedonia can be measured in the sucrose preference test

Based on the results from the first SPT study (figure 4.1) and the time course of LPS-induced cytokine and chemokine release (figure 4.2), it was decided to perform an extended SPT study to analyze the effects of peripheral LPS administration over a longer period of time. In this optimized experimental design, undeprived rats were subjected to a familiarization and a test phase. The purpose of the familiarization phase was to assess normal daily intake volume, familiarize the animals with exposure to sucrose and ensure a stable sucrose preference baseline. To evaluate the growth rate of rats during each day of the familiarization and test phase, the body weight change was calculated against their weight at the beginning of the first day of the familiarization phase. Factorial rmANOVA showed that there was a main effect of time ($F(3,210) = 563,2$, $p < 0.001$, $\varepsilon = 0.69$) and LPS assignment ($F(1,70) = 5.1$, $p < 0.05$, $\varepsilon = 0.69$) for change in body weight during the familiarization phase. Post-hoc analysis indicated that rats in the LPS group had a statistically significant lower change in body weight at familiarization day 1 (FAM1) as compared to animals in the vehicle group (figure 4.3, top left panel). However, this difference was very small and can be considered as not biologically relevant. Rats from both groups continuously grew about 5 g per day throughout the familiarization phase, regardless of exposure type (W/W versus W/S). This indicates that the caloric value of sucrose did not influence the change in body weight.

For total volume intake during the familiarization phase, there was a flavor x repeat interaction ($F(1,90) = 8.9$, $p < 0.01$), but no effect of LPS assignment. Post-hoc analysis indicated that the total volume intake increased substantially on days that rats were exposed to W/S when compared to W/W days. This increase was slightly reduced upon retesting (i.e. FAM4 versus FAM2) (figure 4.3, middle left panel).

There were no time or group effects on sucrose preference during the familiarization phase and the rats showed a stable sucrose preference of around 80% on both W/S days (figure 4.3, bottom left panel).

In the test phase, the effect of systemic LPS on change in body weight, total daily intake volume and sucrose preference was assessed over time. Factorial rmANOVA revealed a strong time x LPS interaction ($F(3,210) = 83.3$, $p < 0.001$, $\varepsilon = 0.83$) for change in body weight during the test phase. Post-hoc analysis

showed that systemic LPS injection reduced weight during the first 2 days after injection (D1 and D2) and that this weight decrease remained statistically significant throughout the test phase (figure 4.3, top right panel).

For total volume intake during the test phase, there was a time x LPS interaction ($F(3,210) = 50.0$, $p < 0.001$, $\varepsilon = 0.87$). In the first 24 h after administration (D1), LPS reduced total volume intake to less than one third of the normal daily water intake, suggesting suppression of drinking as a consequence of sickness (figure 4.3, middle right panel). On D2, LPS-injected rats still drank significantly less than rats that received vehicle, but their total volume intake was no longer lower than the normal daily water intake, thereby indicating that sickness had dissipated. No differences in total volume intake were found on D3 and D4 after LPS treatment.

A time x LPS interaction ($F(3,210) = 2.8$, $p < 0.05$, $\varepsilon = 0.91$) was also found for sucrose preference during the test phase. Post-hoc analysis revealed that systemic LPS administration reduced sucrose preference to close to chance level (i.e. 50%) on D1 (figure 4.3, bottom right panel). Interestingly, the LPS-induced decrease of sucrose preference lasted until D3, a time point at which total volume intake had returned to control levels suggesting anhedonia in the absence of sickness on D2 and D3.

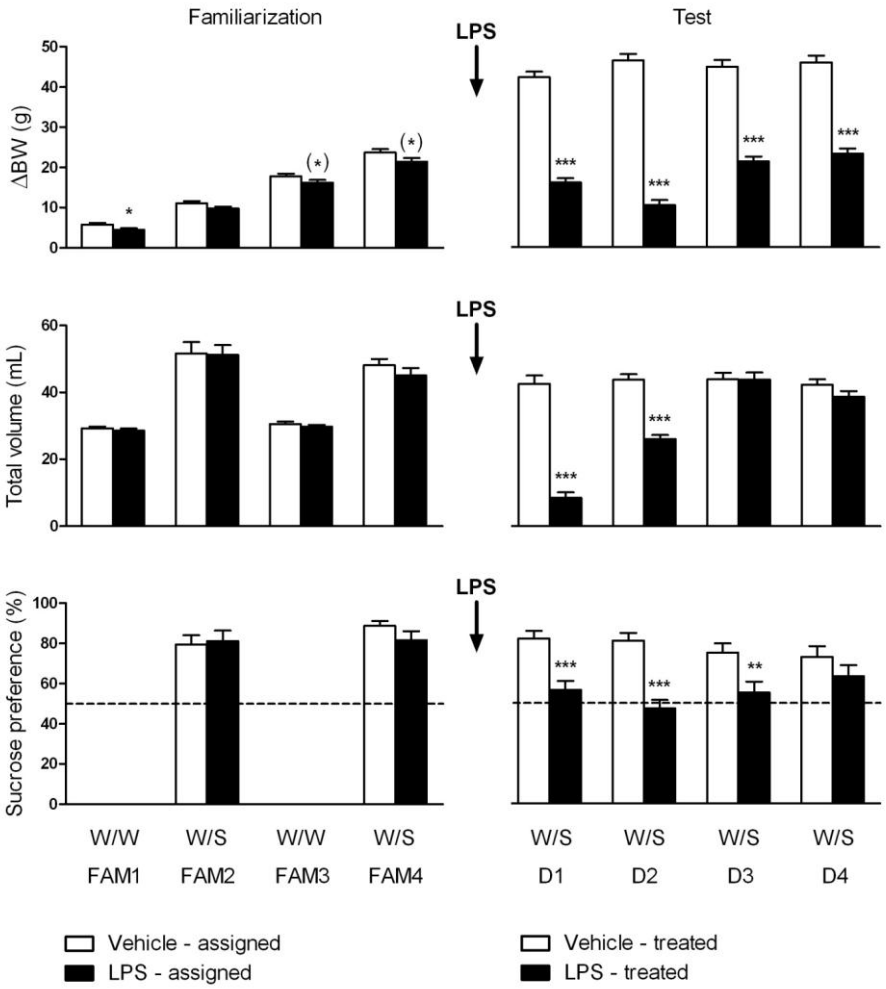

Figure 4.3 LPS reduces body weight, fluid intake and sucrose preference in rats.
An optimized sucrose preference test was used to evaluate the anhedonic response to LPS. During the familiarization phase of the sucrose preference test (left), rats were exposed to 2 bottles of water (W/W) on familiarization day 1 (FAM1) and FAM3, while on FAM2 and FAM4 one bottle was filled with water and the other bottle contained a 1% sucrose solution (W/S). Three days after the familiarization phase, rats were injected i.p. with 0.63 mg/kg LPS or vehicle and voluntary consumption of water and sucrose was measured during a period of 24 h for 4 days (D1-D4) in the test phase (right). Dashed lines indicate chance level for sucrose preference. Graphs are plotted as mean + SEM and represent pooled data from 3 separate, but identical, studies using 12 naive animals per treatment group in each experiment (total n = 36 per treatment group). Data were analyzed by rmANOVA followed by independent samples t-test. (*) $0.1 < p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle.

4.3.4 Repeated LPS exposure protects against acute LPS-induced sickness but not anhedonia

The duration of inflammatory processes associated with depression is thought to be chronic rather than acute. In this experiment, the anhedonic response to a more prolonged immune challenge was investigated by first injecting the rats with LPS on 5 consecutive days (pre-exposure phase) and measuring sucrose preference after an acute LPS injection 3 days later (test phase).

The effect of repeated LPS administration on the growth rate of rats was determined by calculating the change in body weight versus their weight just before the first LPS injection in the pre-exposure phase. Factorial rmANOVA demonstrated a time x pre-exposure interaction for change in body weight during this pre-exposure phase. Post-hoc analysis showed that rats receiving vehicle injections continuously grew, while rats pre-exposed to LPS showed reduced weight change at all days of the pre-exposure phase (figure 4.4 A).

At the beginning of the test phase, rats that were pre-exposed to LPS weighed significantly less than animals that received vehicle pre-exposure (i.e. 288.9 ± 2.6 g versus 314.5 ± 3.6 g, $p < 0.001$; data not shown). To evaluate the weight change after a subsequent acute LPS injection, weight measures during the test phase were subtracted from the weight at the start of the test phase. rmANOVA revealed that there was a time x pre-exposure x LPS interaction ($F(3,132) = 13.9$, $p < 0.001$, $\varepsilon = 0.76$) for change in body weight in the test phase. Post-hoc analysis indicated that all rats lost weight after receiving the acute LPS challenge (figure 4.4 B). Animals that received LPS during the pre-exposure phase, however, lost far less weight after the acute LPS injection than rats that were pretreated with vehicle. Moreover, rats pre-exposed to LPS recovered faster from the acute LPS challenge than rats that received acute LPS after vehicle pretreatment.

There was a time x pre-exposure x LPS interaction for total volume intake during the test phase. All groups that received LPS in the test phase drank less than vehicle-injected animals on the first day after acute LPS administration (figure 4.4 C). However, LPS-pretreated rats drank much more upon a subsequent acute LPS challenge than animals that were pre-exposed to vehicle. On the second day after the final LPS injection, the total volume drank by rats pre-exposed to LPS had returned to control levels, while this took until day 3 for vehicle-pretreated rats.

Finally, factorial rmANOVA revealed that for sucrose preference during the test phase, there was a main effect of LPS ($F(1,44) = 24.4$, $p < 0.001$, $\varepsilon = 0.93$) but not of time or pre-exposure. Rats that were administered with LPS during the test phase had a reduced sucrose preference when compared to vehicle-injected animals (figure 4.4 D). However, due to the absence of other main effects no further post-hoc analyses could be made.

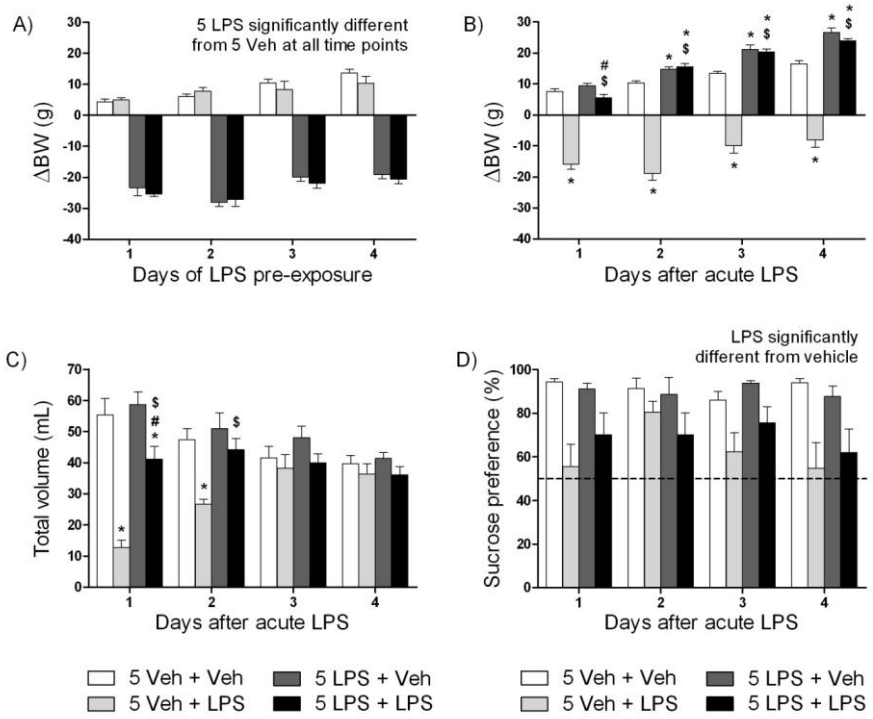

Figure 4.4 Repeated LPS pre-exposure protects against LPS-induced sickness but not anhedonia. After the familiarization phase (data not shown), rats received daily i.p. injections of either 0.63 mg/kg LPS (5 LPS) or vehicle (5 Veh) for 5 consecutive days. Three days after this pre-exposure phase, rats were administered an acute systemic injection of either 0.63 mg/kg LPS or vehicle (Veh) and voluntary consumption of water and sucrose was measured during a period of 24 h for 4 days. Repeated peripheral LPS administration reduced body weight during the pre-exposure phase (A). At the beginning of the test phase, rats pre-exposed to LPS had a significant lower weight than animals that received vehicle pre-exposure (288.9 ± 2.6 g versus 314.5 ± 3.6 g, $p < 0.001$; data not shown). Weight only decreased mildly upon rechallenge with LPS, while weight reduction in LPS naive rats was more pronounced (B). On the first day of the test phase, LPS-challenged rats drank less than their vehicle-injected controls but this effect was less pronounced in rats that were pre-exposed to LPS (C). Sucrose preference was reduced in LPS-treated rats but no effect of pre-exposure was found (D). Dashed lines indicate chance level for sucrose preference. Graphs are plotted as mean + SEM (n = 12 per group). Data were analyzed by rmANOVA followed by independent samples t-test. * $p < 0.05$ compared to 5 veh + Veh, # $p < 0.05$ compared to 5 LPS + Veh, $ $p < 0.05$ compared to 5 Veh + LPS. 5 Veh + Veh: 5 days of vehicle followed by acute vehicle, 5 Veh + LPS: 5 days of vehicle followed by acute LPS, 5 LPS + Veh: 5 days of LPS followed by acute vehicle, 5 LPS + LPS: 5 days of LPS followed by acute LPS.

4.3.5 Stress does not influence the anhedonic response to a subsequent LPS challenge

Stress, which is a risk factor for depression, is known to influence immunological responses. To test whether stress influences the anhedonic response to a subsequent immune challenge, rats were first exposed to 1 h of restraint stress per day for 5 consecutive days (manipulation phase) and then systemically injected with LPS three days later (test phase).

The effect of sub-chronic restraint stress on the growth rate of rats was determined by calculating the change in body weight during the manipulation phase versus the weight just before the first stress session. Factorial rmANOVA revealed a time x stress interaction ($F(3,138) = 145,6$, $p < 0.001$, $\varepsilon = 0.69$) for change in body weight during the manipulation phase. Post-hoc analysis indicated that stressed rats continuously lost weight from the first stress session until the last, while non-stressed rats grew steadily during the manipulation phase (figure 4.5 A).

Rats that were stressed weighed significantly less than non-stressed animals at the beginning of the test phase (i.e. 299.7 ± 3.1 g versus 324.9 ± 2.4 g, $p < 0.001$; data not shown). Weight changes induced by a subsequent acute LPS challenge were determined by subtracting weight measures from the rats' weight at the beginning of the test phase. There was a time x LPS ($F(3,132) = 23.9$, $p < 0.001$, $\varepsilon = 0.63$) and a stress x LPS ($F(1,44) = 16.0$, $p < 0.001$, $\varepsilon = 0.63$) interaction for change in body weight during the test phase. Post-hoc analysis showed that LPS decreased weight in stressed and non-stressed rats (figure 4.5 B). This LPS-induced weight loss was most pronounced in the first 2 days after administration and then started to recover over time. For total volume intake during the test phase, there was a time x stress x LPS interaction ($F(3,132) = 4.5$, $p < 0.01$, $\varepsilon = 0.75$). On the first 2 days after administration, stressed and non-stressed rats that were injected with LPS drank significantly less than animals that received vehicle (figure 4.5 C). On the third day after LPS administration, stressed rats that received LPS drank less than their vehicle-injected controls, while the total volume intake of LPS-treated non-stressed rats had returned to control values. Finally, factorial rmANOVA indicated that there was a significant LPS effect ($F(1,44) = 59,0$, $p < 0.001$, $\varepsilon = 0.85$), but no main effect of time or stress for sucrose preference during the test phase. LPS-treated rats had a lower sucrose preference than vehicle-injected animals, but the lack

of main effects of time and stress did not allow further post-hoc analysis (figure 4.5 D).

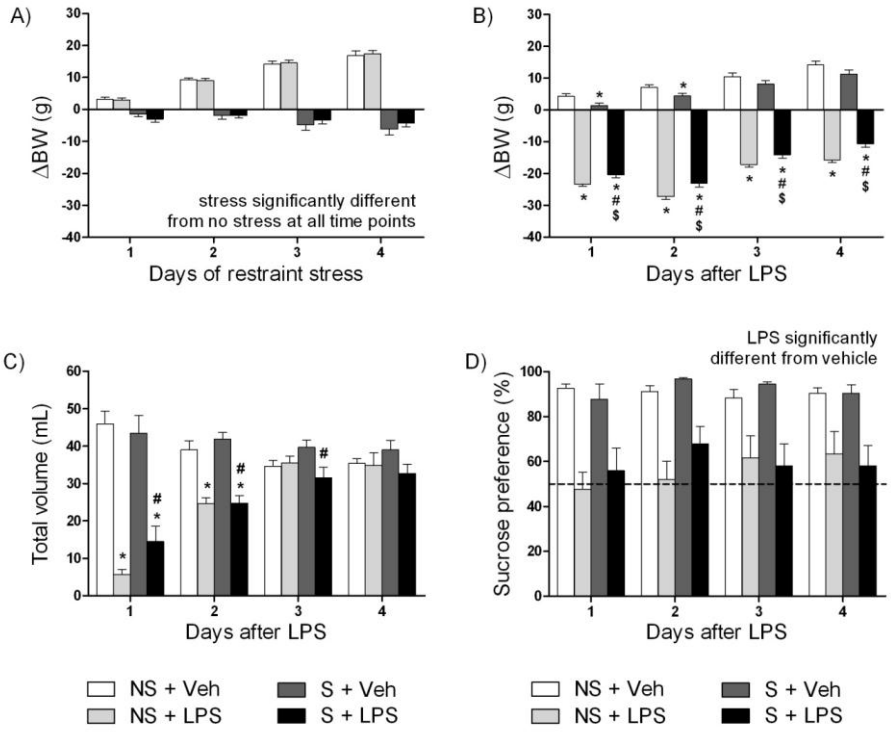

Figure 4.5 Stress does not influence the anhedonic response to a subsequent LPS challenge. After the familiarization phase (data not shown), rats were exposed to 1 h of restraint stress daily for 5 consecutive days. Three days after the last stress session animals received an i.p. injection of either vehicle or 0.63 mg/kg LPS. Daily restraint stress reduced body weight (A). At the beginning of the test phase, rats that were stressed during the manipulation phase had a significant lower body weight than animals that were non-stressed (299.7 ± 3.1 g versus 324.9 ± 2.4 g, $p < 0.001$; data not shown). A subsequent acute LPS challenge reduced weight in non-stressed rats, and to a slightly lesser extent in stressed animals (B). Systemic LPS administration also reduced total volume intake (C) and sucrose preference (D), but no differences could be found between stressed and non-stressed rats. Dashed lines indicate chance level for sucrose preference. Graphs are plotted as mean + SEM (n = 12 per group). Data were analyzed by rmANOVA followed by independent samples t-test. * $p < 0.05$ compared to NS + Veh, # $p < 0.05$ compared to S + Veh, $ $p < 0.05$ compared to S + LPS. NS: non-stressed, S: stressed, Veh: vehicle.

4.4 Discussion

Anhedonia, or the inability to experience pleasure from naturally rewarding activities, is a hallmark symptom of clinical depression. Where other key features such as depressed mood are difficult to measure in laboratory animals, anhedonia can be estimated fairly easy by measuring the preference an animal develops for a sweetened solution relative to water. It is suggested that a decrease in this preference reflects a state of anhedonia [253]. Earlier studies showed that peripheral administration of a single bolus of LPS may induce an episode of anhedonia in rodents. However, contrasting findings are reported and the use of different experimental procedures and study designs makes it difficult to compare results between labs.

In a previous study, we characterized LPS-induced behavioral changes in mice and demonstrated that the time course of sickness and anhedonia can be evaluated by measuring total volume intake and sucrose preference in the SPT [160]. To extend this work, we assessed the anhedonic response to LPS in rats. First, the dose dependency of LPS-induced behavioral changes was evaluated in assays commonly used to measure sickness and depressive-like behavior in rodents. Sickness, as measured by reduced locomotor activity in the OFT, was present as soon as 2 h after LPS administration and started to dissipate at 24 h. However, total volume intake in the SPT was still decreased at this time, thereby indicating that sickness had not disappeared completely. Interestingly, sucrose preference in rats injected with the low dose of LPS (0.31 mg/kg) had returned to control levels at 24 h, despite the fact that these animals still drank much less than vehicle-treated rats. Rats injected with higher doses of LPS, in contrast, showed reduced total volume intake and decreased sucrose preference at this time. This may indicate that at a dose of 0.63 mg/kg (and higher), LPS causes more pronounced anhedonia and therefore this particular dose was selected for further experimentation.

The immunological response to LPS in rats is not as well documented as is the case for mice. Currently available literature on the effect of systemic LPS administration on circulating levels of inflammatory mediators in the rat is limited by the number of analytes measured and/or time points used. As previously shown in the mouse [160], we found that LPS administration in rats

led to a rapid release of proinflammatory cytokines such as TNF-α, IL-1β and IL-6. Serum levels of these cytokines peaked between 1 and 2 h after LPS and had returned to baseline levels at 24 h, thereby indicating their importance in the acute phase of inflammation. These findings are in line with Goble et al., who previously reported rapid, but short lasting, increases in circulating IL-1β and IL-6 in LPS-challenged rats [254]. In our study, the release of the anti-inflammatory cytokine IL-10 peaked at 1 h and faded in the first 24 h after LPS. This confirms that the strong inflammatory response to a peripheral immune challenge is tightly regulated and rapidly attenuated by anti-inflammatory mediators. The appetite suppressing hormone leptin, whose primary function is to regulate energy balance [255], is also known to be an important mediator of sickness during systemic inflammation [256]. In line with other studies [257, 258], we found that peripheral LPS administration increased circulating levels of leptin. Although this effect was short lasting, it is not unlikely that it plays a role in the reduction of fluid intake and body weight that follows after LPS injection. The LPS-induced changes in serum levels of most analytes had dissipated by 24 h. However, circulating levels of CXCL1, MCP-1 and MIP-1α remained elevated up to 96 h posttreatment. These chemokines play an important role in leukocyte migration and activation, and their serum profile suggests that the immunological response to systemically administered LPS lasted for several days. Activation of the immune system is known to deregulate the HPA axis, a physiological finding which is frequently observed in depression [28]. In agreement with previous findings [254, 259], we found that systemic LPS injection increased serum levels of corticosterone. This release of corticosterone into the circulation occurred promptly after LPS administration and had decreased substantially by 24 h. Corticosterone levels also increased in vehicle-injected rats. However, this happened to a lesser extent than following LPS administration and probably occurred as a consequence of stress related to the experimental procedure.

From our extended SPT study it becomes clear that a single bolus of LPS reduces fluid intake in the first 2 days after administration. More specifically, on the first day after administration, LPS decreased total volume intake to approximately one third of the normal daily intake (i.e. unchallenged on a W/W day). This suppressed drinking is a clear indication of sickness and precludes interpreting

reduced sucrose preference as a sign of anhedonia. On the second day, however, LPS-injected rats also drank less than vehicle-treated controls but their total volume intake had reached normal daily intake levels. Therefore, the reduced sucrose preference seen on this day can be interpreted as an anhedonic response to the LPS challenge. This became even clearer on the third day where there was no difference in total volume intake between treatment groups, but still a significant reduction in sucrose preference in LPS-injected rats. The fact that rats treated with LPS started to gain weight at this day further indicates that sickness had dissipated at this point. In line with previously reported rat data [100, 260], LPS administration did not alter water intake at any of the time points (data not shown). This highlights the importance of including measures of total volume intake to estimate sickness in paradigms such as the SPT.

In our model of acute systemic LPS administration, anhedonia was present in the absence of apparent sickness from 2 to 3 days post-injection. Depressive episodes in humans, however, can last up to several months [261]. Moreover, inflammation-associated depression in humans develops on a background of persistent inflammation. In order to mimic the human situation more closely, we decided to evaluate the anhedonic response to a longer lasting immune challenge. It was found that pre-exposure to LPS reduced the sickness response to a subsequent acute LPS challenge, thereby suggesting the induction of tolerance to LPS. This phenomenon involves downregulation of proinflammatory responses to repeated LPS exposure and is thought to protect an organism from excessive tissue damage and the development of pathological states during prolonged or uncontrolled inflammation [262, 263]. Moreover, in our study, the secondary LPS challenge reduced sucrose preference in all rats, regardless of their pre-exposure. This indicates that in our model to study anhedonia, repeated LPS administration does not offer an advantage over a single peripheral injection. However, Kubera and coworkers recently described a model in which repeated LPS injections given at one-month intervals induced a chronic state of anhedonia in female, but not in male mice [250]. Although our study was performed in male rats, it may be possible that a more specific or elaborate LPS dosing scheme is required to induce more pronounced and/or longer lasting anhedonia.

Stress is a major risk factor for the development of depression [264]. In a second, more indirect approach to create an anhedonia model that relates to the human situation, we tested whether exposure to stress alters anhedonic responses to a subsequent immune challenge. It was observed that repeated daily exposure to restraint stress decreased body weight, thereby indicating that our stress protocol was stressful indeed. Previous work by other labs has shown that repeated restraint stress can induce depressive-like behavior, including anhedonia [265-269]. Moreover, in studies using another model of psychological stress, social disruption was shown to aggravate the sickness response to a secondary LPS challenge [270, 271]. However, the repeated restraint stress protocol in our study did not alter measures of sickness and anhedonia in response to a subsequent LPS challenge. These findings are in line with results from a study by Wohleb et al., where repeated social defeat stress in mice did not exacerbate anxiety behavior following a secondary LPS challenge [172]. Still, in that study the sickness response to LPS was higher in stressed versus non-stressed mice. It is possible that the rather mild restraint stress protocol used in our study was not sufficient to alter the sickness and/or anhedonic response to a subsequent immune challenge. Indeed, in most studies where anhedonia was reported following repeated restraint stress, the animals were restrained for several hours per day (versus 1 hour in our study), during several weeks (versus 5 days in our study) [266, 268, 269], and when shorter lasting restraint stress protocols were used, anhedonia was evaluated immediately after the last restraint session [265].

In the experiments where we tested the effect of LPS pre-exposure or stress on the anhedonia response to a subsequent LPS administration, LPS-injected rats showed reduced sucrose preference across the test phase. These findings are not in line with the acute LPS experiment where sucrose preference was only reduced at day 2 and 3 but had recovered at day 4. This discrepancy can potentially be explained by the fact that in our more elaborate experimental protocols a third phase was introduced between the familiarization and test phase. It may be possible that this extra week of individual housing and handling confounded measures of sucrose preference after a subsequent systemic LPS injection.

In conclusion, this study provides a systematic analysis of the time course of cytokine release and behavioral changes following peripheral LPS administration in rats. We report a SPT protocol that includes measurements of total volume intake, sucrose preference and body weight and demonstrated that by assessing these measurements and their interaction, this SPT protocol provides a way of separating LPS-induced anhedonia from sickness. Our results indicate that a single systemic injection of LPS in rats increases serum levels of corticosterone, leptin and pro- and anti-inflammatory cytokines in the first 24 h following administration. The time window in which these analytes are elevated corresponds to the period of sickness. However, serum concentrations of some chemokines remain elevated up to 4 days post-LPS, indicating that the immunological response to LPS has not dissipated completely. Measures of anhedonia in the SPT confirm these findings as during day 2 and 3 anhedonia seems to be present in the apparent absence of sickness. Although this anhedonic response is robust, it only lasts for 2 days and therefore caution is needed when studying the mechanisms underlying inflammation-associated depression using a single LPS injection in rats. In an attempt to mimic the chronic nature of depression in humans more carefully, our SPT protocol was used to test whether pre-exposure to repeated LPS administration or sub-chronic stress influenced the anhedonic response to a subsequent LPS challenge. We found that repeated administration of LPS reduces the sickness response but does not influence the anhedonic response to LPS. Moreover, we observed that pre-exposure to sub-chronic restraint stress does not alter the effects of a subsequent LPS injection, or cause long-lasting anhedonia by itself. Taken together, these findings provide insights into the behavioral consequences of peripheral immune activation using LPS and may contribute to the development of better rodent models of inflammation-associated depression.

5

Characterization of the central nervous system response to peripheral TNF-α administration in mice

Based on:

Peripheral administration of tumor necrosis factor-alpha induces neuroinflammation and sickness but not depressive-like behavior in mice.

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ABSTRACT

Clinical observations indicate that activation of the TNF-α system may contribute to the development of inflammation-associated depression. In this study, we tested the hypothesis that systemic upregulation of TNF-α is sufficient to induce central neuroinflammation and behavioral changes relevant to depression. We report that a single intraperitoneal injection of TNF-α in mice increased serum and brain levels of the proinflammatory mediators TNF-α, IL-6 and MCP-1, in a dose- and time-dependent manner, but not IL-1β. Protein levels of the anti-inflammatory cytokine IL-10 increased in serum but not in the brain. The transient release of immune molecules was followed by glial cell activation as indicated by increased astrocyte activation in bioluminescent Gfap-luc mice and elevated immunoreactivity against the microglial marker Iba1 in the dentate gyrus of TNF-α-challenged mice. In addition, we evaluated TNF-α-injected mice in a panel of behavioral tests commonly used to study sickness and depressive-like behavior in rodents. Our behavioral data indicate that systemic administration of TNF-α leads to a strong sickness response characterized by reduced locomotor activity, decreased fluid intake and body weight loss. Depressive-like behavior could not be separated from sickness at any of the time points studied. Taken together, these results demonstrate that peripheral TNF-α affects the central nervous system at a neuroimmune and behavioral level.

5.1 Introduction

Clinical depression is a chronic, disabling psychiatric condition that affects over 120 million people worldwide [272]. It is predicted that by 2030, depression will be the second leading cause of disability in the world [61]. Although its etiology remains poorly understood, it is generally accepted that depression is a multifactorial disorder with numerous interacting systems underlying its pathogenesis. A number of clinical observations suggest that dysregulation of the immune system might also play a role in the development of depression, at least in a subset of susceptible individuals. For example, depression frequently occurs as a comorbidity of medical conditions characterized by a chronic inflammatory component including rheumatoid arthritis [73], cancer [74], type 2 diabetes [75] stroke [76], obesity [77] and coronary artery disease [78]. Even in absence of other medical illness, depressed patients often show elevated circulating levels of inflammatory mediators such as proinflammatory cytokines and the acute-phase CRP [79, 80]. Moreover, up to half of cancer and hepatitis C patients that receive therapeutic administration of proinflammatory cytokines eventually develop depressive symptoms [137, 219, 220].

There are several indications that TNF-α is one of the key cytokines involved in the pathogenesis of inflammation-associated depression. For example, recent meta-analyses confirmed that circulating levels of TNF-α are significantly higher in depressed patients compared with healthy control subjects [80, 145]. Moreover, elevated plasma levels of TNF-α are associated with treatment resistance to conventional antidepressants [154]. In hepatitis C patients that are chronically treated with interferon-α, increased blood levels of TNF-α correlate with the development of depressive symptoms [144]. Furthermore, peripheral administration of anti-TNF-α antibodies improves depressed mood in patients suffering from psoriasis [155], Crohn's disease [156] and rheumatoid arthritis [157]. TNF-α antagonism has also been shown to improve treatment resistant depression in a subgroup of patients with high baseline inflammatory biomarkers [158].

Inflammation-associated depression is often studied in rodents by peripheral administration of an immunostimulant such as bacterial LPS. It is known that systemic injection of LPS elicits a widespread immune response, characterized by the release of numerous immune mediators and the occurrence of sickness, a

behavioral state comprised of symptoms such as malaise, lethargy, decreased motor activity and appetite, sleep disturbances, and increased sensitivity to pain [25, 108]. There are some indications that this sickness response is followed by a phase of depressive-like behavior [96-98]. However, the characteristics of sickness can substantially confound the evaluation of depressive-like behavior in behavioral tests. For example, sick animals display reduced exploration, which can potentially interfere with measurements of immobility used to estimate behavioral despair in paradigms such as the forced swim and tail suspension test [99]. Moreover, sick animals eat and drink less, which can bias measures of sweetened fluid intake in assays designed to evaluate anhedonia (the inability to experience pleasure from naturally rewarding activities). Using a panel of behavioral paradigms in mice, we recently demonstrated that it is difficult to separate depressive-like behavior from sickness following acute peripheral LPS administration [160].

Based on the fact that systemic LPS administration induces a broad immune response, and the clinical data linking TNF-α to human inflammation-associated depression, we hypothesized that peripheral administration of TNF-α itself may provide a more specific approach to study depressive-like behavior in mice. Indeed, systemic administration of TNF-α has already been shown to have central effects as indicated by increased proinflammatory gene expression in the brain and the development of sickness [2, 273]. Moreover, intracerebroventricular (i.c.v.) injection of TNF-α was shown to lead to depressive-like behavior in mice [274, 275]. However, to our knowledge, no study has systematically assessed the effect of peripheral TNF-α administration on neuroinflammation and depressive-like behavior over time. Therefore, the present series of experiments aimed at characterizing the central effects of systemic TNF-α injection by combining multiple techniques to quantify neuroinflammation and behavioral changes. First, serum and brain levels of immune mediators were quantified at several time points after systemic TNF-α administration. Next, transgenic bioluminescent Gfap-luc mice were used to evaluate the time course of TNF-α-induced astrocyte activation, as a marker of glial cell activation in vivo. Then, the occurrence of glial cell activation was confirmed by immunohistochemistry using the microglial marker Iba1. Finally, TNF-α-injected mice were tested in a panel of behavioral paradigms to assess whether depressive-like behavior could be separated over time from sickness.

5.2 Materials and methods

5.2.1 Animals and TNF-α

All animal care and use was performed in accordance with the Guide for the Care and Use of Laboratory Animals (NRC) and experimental protocols were approved by the Institutional Ethical Committee on Animal Experimentation, according to applicable regional law. Male NMRI mice were purchased from Charles River Laboratories (France), male wild-type FVB mice from Janvier (France), and male transgenic Gfap-luc mice (FVB/N-Tg(Gfap-luc)-Xen) were obtained from Taconic Laboratories (USA). The latter animals express luciferase under the transcriptional control of the GFAP promoter [181] and are commonly used as a model system for noninvasive quantification of astrocyte activation in living animals over time [160, 225, 226]. Unless mentioned otherwise, animals were housed in groups of 4 per cage under a normal 12:12 h light-dark cycle (lights on at 06:00 AM with a 30 min dim and rise phase). Food and water were available ad libitum.
Recombinant mouse TNF-α was purchased from Biolegend (product ID 575208) and dissolved in sterile PBS prior to injection.

5.2.2 Cytokine measurements

10-week-old male NMRI mice were injected i.p. with 0, 63, 125 or 250 µg/kg TNF-α (n = 6-7 per group) and sacrificed by decapitation at 2 h, 6 h or 24 h. This dose range was based on results from the open field test (see section 2.5). Serum and whole brain samples were collected and processed as previously described [160]. Concentrations of IFN-γ, IL-1β, IL-6, IL-10, MCP-1, and TNF-α were determined in each sample using a mouse cytokine/chemokine magnetic bead panel kit from Merck Millipore. All steps in the assay were conducted according to the manufacturer's instructions. Cytokines levels below detection limit were assigned a value equal to the lowest detectable value of that cytokine.

5.2.3 In vivo bioluminescence imaging

Astrocyte activation in 10-week-old male Gfap-luc mice was quantified before (baseline) and at 2 h, 6 h, 24 h, 48 h, 72 h and 96 h after i.p. administration of either 0, 63 or 250 µg/kg TNF-α (n = 7 per group). Brain bioluminescence was detected as described previously [160]. Briefly, Gfap-luc mice were anesthetized by inhalation of 2% isoflurane in 1 l/min oxygen, shaved on the head and injected with 126 mg/kg D-luciferin (Promega, product ID E1601) in the tail vein. Three minutes later the animals were scanned with a CCD camera (IVIS Imaging System® 200 Series, PerkinElmer) mounted on a dark box. Photon emission from the whole brain was measured using Living Image® 3.2 software (PerkinElmer) in a ROI that was kept constant across mice. Bioluminescence coming from the ears was considered to be basal GFAP activity and was excluded from the ROI. Imaging signals were measured in physical units of surface radiance (photons/s/cm^2/steradian [sr]).

5.2.4 Immunohistochemistry

10-week-old male FVB mice were injected i.p. with vehicle or 250 µg/kg TNF-α (n = 8 per group) and tissue was collected 24 h later. Immunohistochemical staining of Iba1 protein in the dentate gyrus of the hippocampus was performed using a rabbit polyclonal anti-Iba1 primary antibody (1 : 500, Wako Chemicals) and a fluorescent Alexa 555 goat anti-rabbit secondary antibody (1 : 500, Invitrogen), as previously described [160].

5.2.5 Behavioral tests

All behavioral tests were performed on 10-week-old male NMRI mice. The OFT and FST setups were custom-made and were described in detail previously [160].

Two independent OFTs were performed in this study. In the first OFT, mice were injected with 0, 63, 250 or 1000 µg/kg TNF-α (n = 10 per group) and repeatedly tested at 2 h, 6 h, 24 h and 48 h after administration. However, 2 of the 10 mice that received 1000 µg/kg TNF-α died during the first 24 h after injection. Therefore, it was decided to take 250 µg/kg TNF-α as the highest test dose for

all experiments and to repeat the OFT with a vehicle and 125 µg/kg TNF-α group.

In the FST, mice (n = 10 per group) were injected with 0, 63, 125 or 250 µg/kg TNF-α and tested at 2 h, 6 h, 24 h and 48 h after administration.

The SPT started by single-housing the animals in IVCs (L × W × H: 35 × 31 × 16 cm; Tecniplast, Italy) fitted with two 250 mL drinking bottles and ad libitum access to food. Each bottle contained either filtered tap water or a 5 % sucrose solution. The location of the bottles on the cage was randomized during every exposure session with half of the animals receiving sucrose on the left, and half on the right. The SPT protocol lasted for 5 days and consisted of a familiarization and a test phase. The familiarization phase started on day 1 by exposing all mice to W/S for 24 h. On day 2, the animals had free access to W/W until 4:00 PM, after which they were fluid-deprived overnight. The test phase started on day 3 by injecting mice i.p. with 0, 63, 125 or 250 µg/kg TNF-α (n = 10 per group). To test the effects of TNF-α at 2 h, 6 h, 24 h and 48 h, the animals were presented with W/S during a 1 h exposure period at these time points. Mice were fluid-deprived in between exposure periods. In order to avoid a protracted deprivation period between the 24 h and 48 h time point, mice were given access to W/W from 4:00 to 5:00 PM on day 4.

In the SPT study using a within-subject design it became clear that exposing the thirsty animals to W/S at 2 h affected the total volume intake at 6 h (less thirsty). To exclude that the effects of TNF-α were confounded by retesting the same animals over time, the SPT study was repeated in an independent between-subject design study using separate groups of TNF-α challenged mice that were either tested at 6 h or 24 h. These mice underwent the same familiarization phase as described above. At the beginning of the test phase, the animals were injected with 0, 63, 125 or 250 µg/kg i.p. TNF-α (n = 10 per group). At 6h post-TNF-α, mice from the 6 h time point group were exposed to W/S for a 1 h period. Animals from the 24 h time point were allowed to drink W/W for 1 h at 6 h post-TNF-α in order to avoid a protracted deprivation period between TNF-α administration and the 24 h time point. At 24 h, mice from the 24 h group were presented with W/S during a 1 h exposure period.

In both SPT studies, the amount drunk by a mouse was determined by subtracting the weight of a bottle at the start of an exposure period and at the end (taking fluid density as 1 g/mL). Total fluid intake was calculated as the

total change in volume from both bottles combined. A fluid intake that was greater than the mean +2x standard deviation was considered to be an invalid measure that probably resulted from leaking bottles. Invalid measures were replaced by the group mean of the relevant solution (water or sucrose). This occurred for less than 4 % of all bottle measurements. Sucrose preference was calculated as the percentage of consumed sucrose solution of the total fluid intake.

5.2.6 Statistical analysis

SPSS Statistics software version 20 (IBM Inc) was used for data analysis. ANOVA or rmANOVA was performed to determine the statistical significance of differences between treatment groups. To correct for potential violation of the sphericity assumption, a Greenhouse-Geisser correction epsilon (ε) was used for repeated measures analysis [184]. This correction multiplies both the numerator and the denominator degrees of freedom by ε and the significance of the F-ratio is evaluated with the new degrees of freedom, resulting in a more conservative statistical test. To account for the skewness of the data distribution, bioluminescence measurements and cytokine concentrations were log-transformed prior to analysis. ANOVAs and rmANOVAs were considered statistically significant if $p < 0.05$, while $0.1 < p < 0.05$ was considered to indicate a trend effect. When appropriate, post-hoc comparisons were made by using an independent samples t-test with a Bonferroni-corrected p-value. For consistency between the analysis and the visualization of bioluminescence measurements and cytokine concentrations, the group means and its SEM were back-transformed and visually presented on a logarithmic scale. All other data are expressed as mean ± SEM on a linear scale.

5.3 Results

5.3.1 TNF-α increases immune mediator levels in serum and brain

To characterize the immunological response to peripheral TNF-α injection, serum and brain levels of several immune factors were quantified at 2 h, 6 h and 24 h after administration. Factorial ANOVA showed a significant time x dose interaction on serum levels of IL-6, TNF-α and MCP-1 (IL-6: $F(6,68) = 13.4$, $p < 0.001$; TNF-α: $F(6,66) = 15.7$, $p < 0.001$; MCP-1: $F(6,68) = 7.2$, $p < 0.001$), a main effect of time and dose on serum levels of IL-10 (time: $F(2,68) = 5.3$, $p < 0.01$; dose: $F(3,68) = 4.8$, $p < 0.01$) and a main effect of dose on serum levels of IL-1β ($F(3,68) = 3.2$, $p < 0.05$). No effect of time or dose was found on serum levels of IFN-γ. Post-hoc analysis demonstrated that serum levels of IL-6, TNF-α and MCP-1 peaked at 2 h after systemic injection of TNF-α and then gradually waned over time (figure 5.1, left). The TNF-α-induced release of IL-10 followed a different time course as serum levels of this cytokine were only elevated at 6 h post-TNF-α. At 24 h, the serum concentrations of IL-6, TNF-α and IL-10 had returned to baseline values, while MCP-1 levels remained significantly elevated in mice that were injected with 250 µg/kg TNF-α.

For brain tissue, a significant time x dose interaction was found on protein levels of IL-6, TNF-α and MCP-1 (IL-6: $F(6,67) = 6.4$, $p < 0.001$; TNF-α: $F(6,67) = 70.2$, $p < 0.001$; MCP-1: $F(6,67) = 15.4$, $p < 0.001$) and a main effect of dose on IFN-γ levels ($F(3,67) = 4.0$, $p < 0.05$). No significant effect of time or dose could be detected on brain levels of IL-1β or IL-10. Post-hoc analysis revealed that brain levels of IL-6 and TNF-α peaked at 2 h and had dissipated by 6 h (figure 5.1, right). However, at 6 h there was still a trend for elevated IL-6 levels h in mice that had received 250 µg/kg TNF-α. Comparable to the time course of its release in serum, brain levels of MCP-1 remained strongly elevated from 2 h until 6 h posttreatment. At 24 h, there was still a trend for increased MCP-1 levels in mice injected with 250 µg/kg TNF-α.

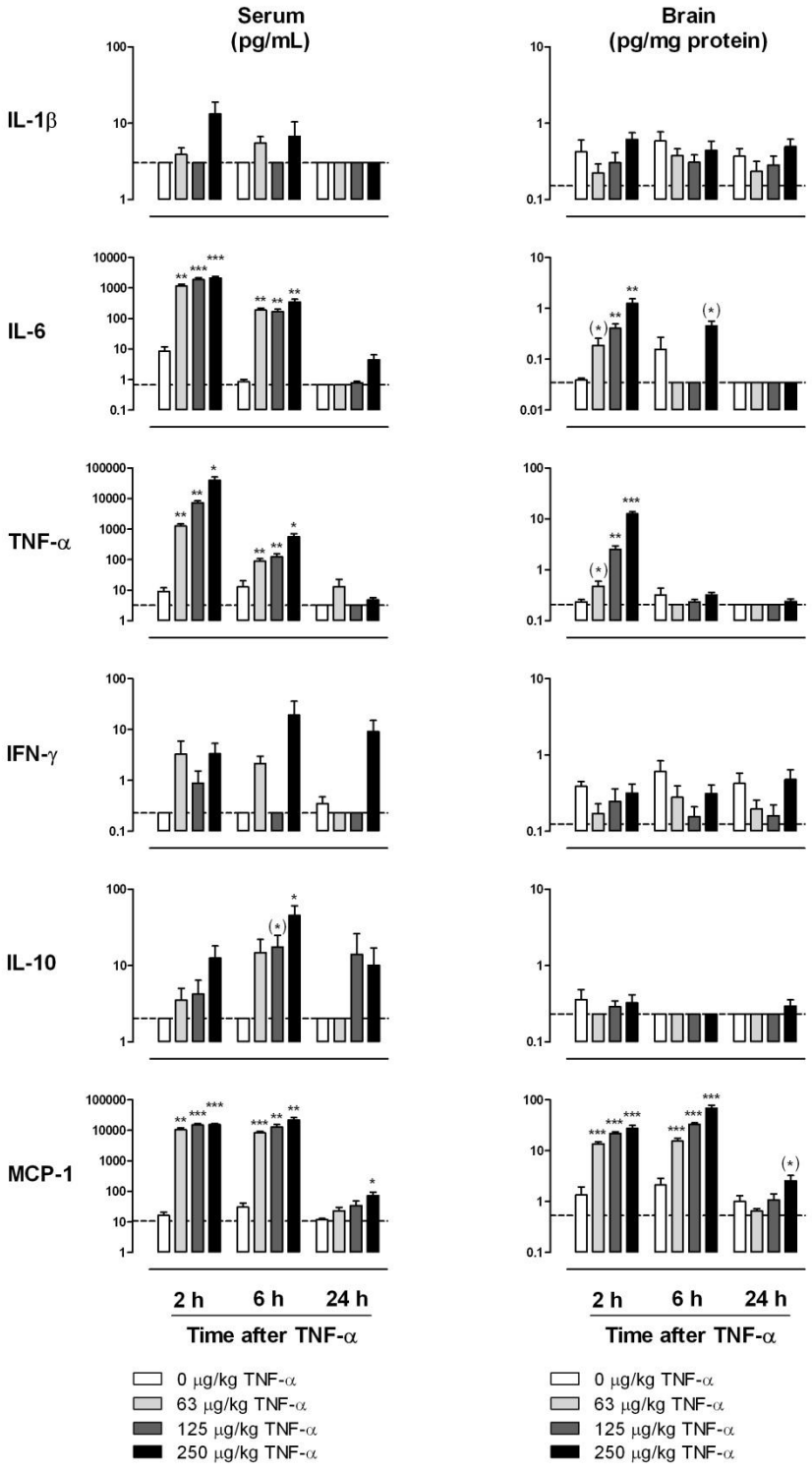
Serum
(pg/mL)
Brain
(pg/mg protein)
IL-1β
IL-6
TNF-α
IFN-γ
IL-10
MCP-1
2 h
6 h
24 h
Time after TNF-α
0 μg/kg TNF-α
63 μg/kg TNF-α
125 μg/kg TNF-α
250 μg/kg TNF-α

Figure 5.1 Peripheral TNF-α administration induces the release of immune mediators in serum and brain. Comparison of a selection of cytokines and the chemokine MCP-1 measured in serum (left) and brain (right) at 2 h, 6 h and 24 h after i.p. TNF-α injection. Note that serum concentrations are shown as pg/mL while brain levels are expressed in pg/mg protein. Dashed lines indicate the detection limit of the measured analyte. Graphs are plotted as mean + SEM (n = 6-7 per group). Data were analyzed by ANOVA followed by independent samples t-test. (*) $0.1 < p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0 µg/kg TNF-α.

5.3.2 TNF-α induces glial cell activation

To quantify the effects of systemic TNF-α administration on astrocyte activation over time, Gfap-luc mice were injected i.p. with different doses of TNF-α and bioluminescence was measured at specific time points. Factorial rmANOVA revealed a significant time x dose interaction ($F(12,84) = 5.8$, $p < 0.001$, $\varepsilon = 0.53$) for photons emitted per second in the brain ROI. Post-hoc analysis demonstrated that at 6 h post-administration, a strong bioluminescent signal was present in the brain of TNF-α-injected mice (figure 5.2). This signal was higher in mice injected with 250 µg/kg TNF-α as compared to mice that received 63 µg/kg TNF-α. Brain bioluminescence in mice treated with 63 µg/kg TNF-α reached control levels at 24 h, while it took up to 72 h to normalize for animals injected with 250 µg/kg TNF-α.

In order to confirm TNF-α-induced activation of glial cells by using a different technique and focusing on another cell type, it was decided to perform immunohistochemistry using a microglial activation marker. The expression of Iba1 was quantified in the hippocampal dentate gyrus at 24 h after systemic injection of vehicle or 250 µg/kg TNF-α. This brain structure was chosen based on its association with stress and depression [228-230]. Pairwise comparison demonstrated that immunoreactivity against Iba1 in the dentate gyrus at 24 h was significantly higher in TNF-α-injected mice when compared to mice that received vehicle ($F(1,13) = 7,3$, $p < 0.05$) (figure 5.3).

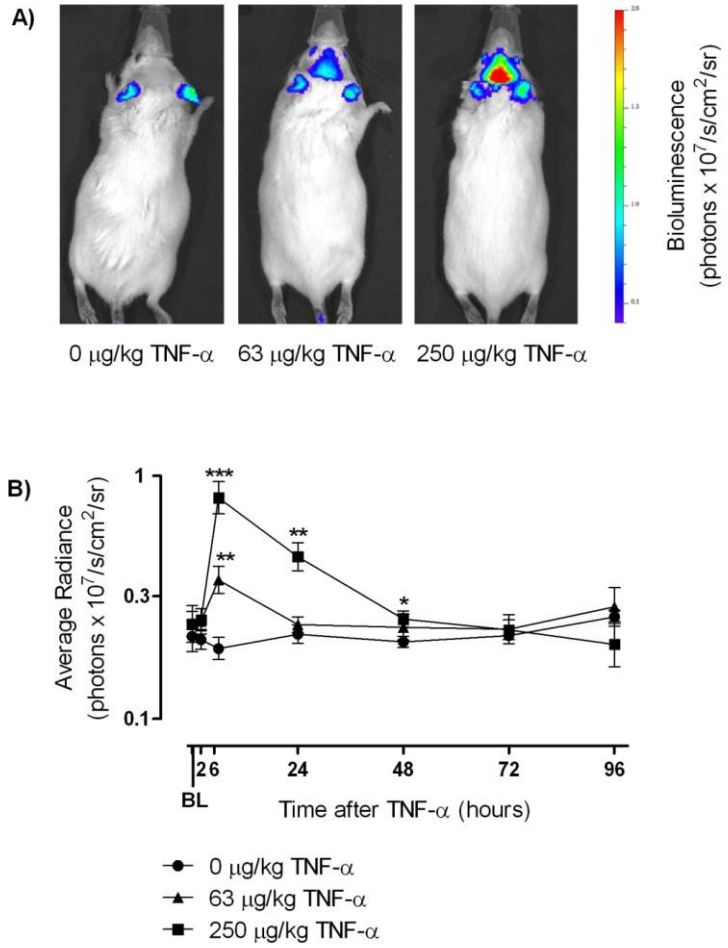

Figure 5.2 TNF-α causes astrocyte activation in vivo. Intraperitoneal injection of TNF-α caused a clear bioluminescent signal in the brain of Gfap-luc mice, as shown on representative images taken at 6 h after injection (A). This signal peaked at 6 h and then gradually waned over time (B). The color scale indicates the number of photons emitted from the animal per second. The graph is plotted as mean ± SEM (n = 7 per group). Data were analyzed by rmANOVA followed by independent samples t-test. BL = baseline. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to 0 µg/kg TNF-α.

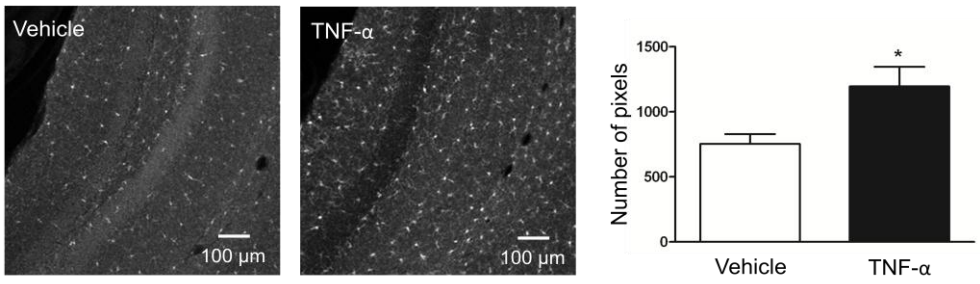

Figure 5.3 TNF-α increases Iba1 immunoreactivity in the dentate gyrus. TNF-α (250 µg/kg, i.p.) caused a strong upregulation of the microglial activation marker Iba1 in the hippocampal dentate gyrus at 24 h post-administration.The panel on the left shows representative images (10x). Image quantifications of n = 8 per group are depicted in the bar chart (right). Graph is plotted as mean + SEM. Data were analyzed by ANOVA followed by independent samples t-test. * $p < 0.05$ compared to vehicle.

5.3.3 TNF-α causes sickness but no depressive-like behavior

Sickness behavior in rodents is commonly evaluated by measuring changes in body weight and by assessing their locomotor activity in the OFT. Unfortunately, 2 out of 10 mice that were injected with 1000 µg/kg TNF-α died within 24 h after administration. These subjects were removed from the analyses, resulting in a group size of n = 8 for this dose. rmANOVA showed a time x dose interaction for change in body weight ($F(6,68) = 24.9$, $p < 0.001$, $\varepsilon = 0.95$). Post-hoc analysis demonstrated that there was a dose-dependent weight reduction at 24 h and 48 h after TNF-α administration (figure 5.4 A, ΔBW, left). Mice that were injected with 63 µg/kg and 250 µg/kg TNF-α started to gain weight at 48 h, while mice in the 1000 µg/kg TNF-α group continued to lose weight.

A significant time x dose interaction was found for total distance travelled in the OFT ($F(9,102) = 10.2$, $p < 0.001$, $\varepsilon = 0.70$). At 2 h after systemic application, TNF-α reduced locomotor activity in a dose-dependent manner (figure 5.4 A, OFT, left). By 6 h, the total distance travelled by mice administered with 63 µg/kg TNF-α had normalized to control levels, while it further declined in animals from the 250 µg/kg and 1000 µg/kg group. At 24 h, animals from the 250 µg/kg group had recovered whereas this took up to 48 h for mice injected with 1000 µg/kg TNF-α.

Based on the mortality rate of 20 % in mice that received 1000 µg/kg TNF-α, it was decided to take 250 µg/kg TNF-α as the highest dose and to introduce a 125 µg/kg TNF-α group in all of the behavioral experiments that followed. To test the effect of this additional dose on body weight and locomotor activity, a second, independent OFT was performed. rmANOVA showed a time x dose interaction for change in body weight ($F(2,36) = 6.0$, $p < 0.05$, $\varepsilon = 0.74$) and a main effect of time ($F(3,54) = 14.2$, $p < 0.001$, $\varepsilon = 0.66$) and dose ($F(1,18) = 7.0$, $p < 0.05$, $\varepsilon = 0.66$) for distance travelled in the OFT. Post-hoc analysis revealed that the weight of mice injected with 125 µg/kg TNF-α was reduced at 24 h, but not anymore at 48 h (figure 5.4 A, ΔBW, right). Moreover, systemic administration of 125 µg/kg TNF-α decreased the distance travelled at 6 h, but not at any of the other time points measured (figure 5.4 A, OFT, right).

In the FST an animal is placed in a water-filled cylinder from which it cannot escape. Behavioral despair can be evaluated in this paradigm by quantifying the time an animal remains immobile, which can be confirmed by measuring the

total distance it swims. rmANOVA revealed a significant effect of time, but not of dose, for total distance ($F(3,108) = 20.4$, $p < 0.001$, $\varepsilon = 0.77$) and immobility time ($F(3,108) = 38.0$, $p < 0.001$, $\varepsilon = 0.75$). Post-hoc analysis showed that compared to the 2 h time point all animals swam less and remained immobile longer at 6 h, 24 h and 48 h post-TNF-α (figure 5.4 B). This happened independently of the TNF-α dose given and indicates habituation to the experimental procedure during retesting.

In the SPT an animal's preference for a sweetened solution versus water is measured. This paradigm allows evaluating sickness by assessing total volume intake while reductions in sucrose preference can be used as a measure for anhedonia, which is a key symptom of depression. rmANOVA demonstrated a significant effect of time ($F(3,108) = 26.0$, $p < 0.001$, $\varepsilon = 0.98$) and dose ($F(3,36) = 4.0$, $p < 0.05$, $\varepsilon = 0.98$) for total volume intake. Moreover, there was an effect of time ($F(3,108) = 23.5$, $p < 0.001$, $\varepsilon = 0.96$) and a trend for dose ($F(3,36) = 2.6$, $p = 0.068$, $\varepsilon = 0.96$) for sucrose preference. At 2 h and 6 h after administration, animals that were injected with 250 µg/kg TNF-α drank significantly less than vehicle-treated controls, while mice that received 125 µg/kg TNF-α only showed reduced fluid intake at 6 h (figure 5.4 C, left). Sucrose preference was lower at 2 h and 6 h in animals injected with 250 µg/kg TNF-α but not at lower doses (figure 5.4 C, right).

All animals, including the vehicle-injected controls showed reduced total volume intake at 6 h when compared to the other time points. This probably resulted from the fact that the fluid-deprived mice were allowed to drink at 2 h and hence were less thirsty at 6 h. To exclude that the effects of TNF-α were confounded by retesting the same animals over time, the SPT study was repeated for the 6 h and 24 h time points using separate groups of TNF-α challenged mice. In this second SPT study there was a main effect of time point ($F(1,72) = 5.7$, $p < 0.05$) and dose ($F(3,72) = 9.0$, $p < 0.001$) for total volume intake, and a main effect of dose ($F(3,72) = 3.3$, $p < 0.05$), but not time point, for sucrose preference. Post-hoc analysis revealed that using naive animals for each time point stabilized total volume intake in mice injected with vehicle (figure 5.5 A). At 6 h post-administration, all TNF-α-treated mice drank less than their vehicle-injected controls. Volume intake at 24 h was only significantly reduced in mice administered with 250 µg/kg TNF-α. Sucrose preference across

both time points was reduced in mice that received 125 µg/kg TNF-α, but not at any of the other doses (figure 5.5 B).

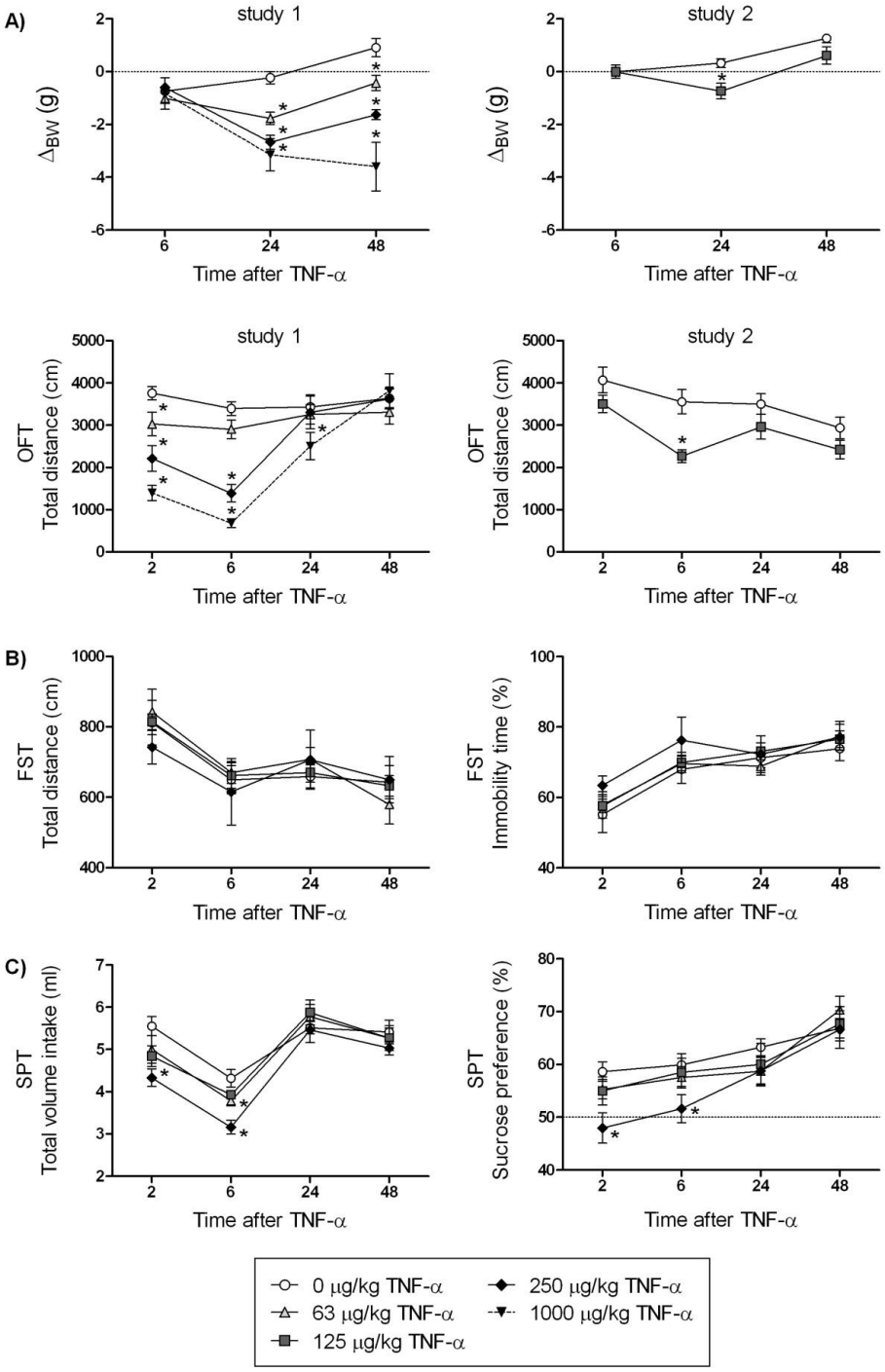
A)
study 1
study 2
ΔBW (g)
Time after TNF-α
OFT
Total distance (cm)
B)
FST
Total distance (cm)
Immobility time (%)
C)
SPT
Total volume intake (ml)
Sucrose preference (%)
0 μg/kg TNF-α
63 μg/kg TNF-α
125 μg/kg TNF-α
250 μg/kg TNF-α
1000 μg/kg TNF-α

Figure 5.4 TNF-α causes sickness, but no clear depressive-like behavior. Systemic injection of TNF-α caused body weight loss (A, ΔBW), reduced locomotor activity in the open field test (A, OFT) and decreased total fluid intake in the sucrose preference test (C). Measures of behavioral despair in the forced swim test were not affected by administration of TNF-α (B). A high dose of TNF-α did decrease sucrose preference in the SPT but this can be considered biologically irrelevant due to the overlapping time course of sickness. The dashed line in the SPT indicates the chance level (50%) for sucrose preference. Please note that the y-axis does not start at 0 for the FST and SPT data. Graphs are plotted as mean ± SEM (n = 10 per group, except n=8 for 1000 µg/kg TNF-α). Data were analyzed by rmANOVA followed by independent samples t-test. * $p < 0.05$ compared to 0 µg/kg TNF-α.

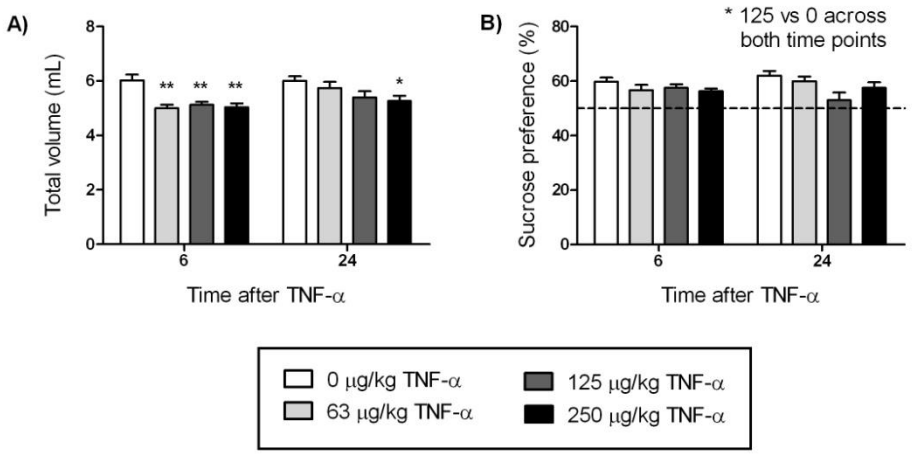

Figure 5.5 TNF-α decreases fluid intake and sucrose preference. Separate groups of naive animals were injected i.p. with TNF-α and tested in the sucrose preference test at either 6 h or 24 h. Dashed line indicates chance level for sucrose preference. Graphs are plotted as mean + SEM (n = 10 per group). Data were analyzed by ANOVA followed by independent samples t-test. * $p < 0.05$, ** $p < 0.01$ compared to 0 μg/kg TNF-α at the same time point.

5.4 Discussion

A substantial set of literature data indicates a link between activation of the immune system and depression, at least in subpopulations of patients. Several clinical observations suggest that TNF-α is one of the key cytokines contributing to the development of inflammation-associated depression. In this series of experiments, we tested whether peripheral administration of TNF-α in mice is able to induce neuroinflammation as well as behavioral changes relevant to human depression.

TNF-α is a pleiotrophic cytokine that plays an important role in the early stages of inflammatory responses and in triggering the release of downstream immune molecules [141-143]. To assess the effect of peripheral TNF-α administration on immune activation in mice, we measured serum and brain levels of a selection of immune mediators. As expected, systemic injection of TNF-α caused a robust dose-dependent increase in circulating levels of TNF-α. Due to the fact that recombinant mouse TNF-α was administered, it was not possible to discriminate injected from endogenous TNF-α. However, previous studies have shown that systemic injection of TNF-α upregulates cytokine gene expression in the liver [2, 239], thereby indicating that TNF-α is capable of eliciting a broad immunological response. In line with these findings, we found that peripheral TNF-α administration increased circulating levels of the proinflammatory immune mediators IL-6 and MCP-1. Moreover, the peak release of these factors was followed by an increase in the serum concentration of IL-10. This cytokine is a potent anti-inflammatory mediator that plays a role in attenuating inflammatory responses and suppressing the expression of proinflammatory cytokines [7]. Apart from MCP-1 levels in mice treated with the highest dose of TNF-α, the concentration of all cytokines had returned to baseline values at 24 h. This indicates that the inflammatory response to a single injection of TNF-α is short-lasting. Our cytokine data corroborates with findings from a recent study where systemic TNF-α was also reported to increase circulating levels of pro- and anti-inflammatory mediators [2]. However, not all of our findings are in line with those described by Skelly et al. In our study, for example, TNF-α-induced increases in IL-6 were of a higher magnitude than the ones previously described [2]. Moreover, in contrast to Skelly's data, we were not able to detect statistically significant increases in IL-1β levels. This was unexpected as TNF-α is

known to induce the expression and release of IL-1β [2, 239]. These discrepancies may in part result from differences in experimental protocols, including the use of dissimilar recombinant TNF-α, mouse strains and gender, blood sampling methods and potentially the sensitivity of the techniques used to quantify cytokine levels.

Cytokines from the periphery can pass the BBB through various mechanisms and access the brain [28]. TNF-α influences these processes in several ways. For example, TNF-α increases the permeability of the BBB [276], thereby facilitating the passage of relatively large molecules such as cytokines from the blood into the brain. Moreover, TNF-α stimulates the release of the chemokine MCP-1, which increases BBB permeability even further and subsequently drives the infiltration of leukocytes into the brain [3]. In line with these findings, we found that brain levels of TNF-α, IL-6 and MCP-1 transiently increased in response to peripheral TNF-α administration. As we did not assess the integrity of the BBB, it is not clear whether these immune mediators entered the brain through a leaky BBB and/or if they were actively produced and released locally in the brain. However, based on the fact that systemic administration of TNF-α was previously shown to increase cytokine transcript expression in the brain [2, 239], it is likely that cytokine brain levels in our study not only resulted from passive diffusion from the periphery.

The neuroinflammatory response to peripheral TNF-α was further characterized using a transgenic mouse line that expresses luciferase under the transcriptional control of the GFAP promoter. GFAP is an intermediate filament protein that is predominantly expressed by astrocytes, and its expression is upregulated when astrocytes are activated [182]. These Gfap-luc mice thus allow noninvasive quantification of GFAP mRNA expression, as a marker of astrocyte activation, in living mice over time. We found that systemic administration of TNF-α caused a strong dose- and time-dependent activation of astrocytes. This TNF-α-induced astrocyte activation occurred after the peak release of proinflammatory cytokines and lasted for 2 days, thereby suggesting that the brain sequelae to a peripheral immune challenge may propagate in absence of the initial stimulus.

Although the quantification of glial cell activation using bioluminescence imaging offers numerous advantages, this technique does not allow for spatial discrimination of specific brain regions. However, previous work has shown that neuroinflammatory responses to a peripheral immune challenge are brain region

specific [240, 277, 278]. To confirm glial activation at a cellular level, focusing on another cell type and a specific brain area, we quantified the expression of Iba1 in the hippocampus. This brain structure is associated with depression and has previously been shown to display immune cell activation following a peripheral immune challenge [160, 163, 279]. In the brain, Iba1 is primarily expressed by microglia and its expression is upregulation upon microglial activation [236]. We observed increased Iba1 immunoreactivity in the hippocampus of FVB wild-type mice at 24 h after peripheral administration of TNF-α. Consistent with measures of astrocyte activation in Gfap-luc mice, TNF-α injection increased Iba1 immunoreactivity in the hippocampus of FVB wild-type mice. This indicates that, in addition to astrocytes, microglia also show signs of activation following peripheral TNF-α administration.

Activated microglia are known to release proinflammatory cytokines, particularly TNF-α and IL-1β. However, brain levels of these cytokines were not elevated at the time point at which we observed microglial activation. This may partly be explained by the fact that we quantified cytokine levels in the whole brain, and not in specific brain regions. Moreover, assessing protein levels of cytokines in the brain is hampered by the limited sensitivity of available quantification techniques. This problem could be overcome by quantifying transcript expression using quantitative PCR, which is more sensitive approach than measuring protein levels of immune mediators in brain tissue. However, the activity of cytokines and chemokines is not only limited by their gene expression, but also regulated at the posttranscriptional and posttranslational level [7, 280, 281]. Therefore, assessing protein levels of cytokines is suggested to be a more accurate indicator of cytokine activity [161]. Irrespective of differences in assay sensitivity, our data align with results from previous studies showing that cytokine expression in the hippocampus and hypothalamus was no longer elevated at 24 h after peripheral TNF-α administration [2].

To our knowledge, no study has systematically assessed the time course of sickness and depressive-like behavior following systemic TNF-α administration. After confirming that peripheral administration of TNF-α induces a central inflammatory response, we evaluated the time course of TNF-α-induced behavioral changes across a panel of assays commonly used to study sickness and depressive-like behavior in rodents. Our behavioral data demonstrate that TNF-α dose-dependently induces sickness during the first 24 h after systemic

administration. This could be seen as a decrease in body weight, reduced exploration in the OFT and suppressed drinking in the SPT. In contrast to i.c.v. administration [274, 275], peripherally injected TNF-α did not affect measures of behavioral despair in the FST. Moreover, mild signs of anhedonia observed in the SPT overlapped with the time course of sickness and can therefore be considered biologically irrelevant. One limitation in our study is the within-subject design for the behavioral paradigms. This approach allowed reduction of animal numbers but also led to habituation of the animals to some of the experimental paradigms. Such habituation effects were observed in vehicle-injected control animals upon retesting in the FST (i.e. less swimming and longer immobility time) and in the SPT (i.e. less drinking at 6 h than at 2 h). To rule out that effects of TNF-α were missed because of habituation during retesting, the SPT study was repeated using separate groups of naive animals for the 6 h and 24 h time points. From this experiment it also became clear that peripheral TNF-α administration induced sickness, but not anhedonia. Taken together, the behavioral data indicate that acute systemic injection of TNF-α is not a reliable model to induce depressive-like behavior in mice. However, because of the strong but short-lasting effects of TNF-α on neuroinflammation and behavior, it may be possible that prolonged or intermittent administration of TNF-α, leading to chronic upregulation of cytokines, offers a more valid approach to study depressive-like behavior in rodents. Such chronic TNF-α administration would mimic the human situation where inflammation-associated depression is believed to develop on a background of sustained, low-grade inflammation.

In conclusion, the present set of experiments using a variety of techniques and readouts demonstrated that systemically administered TNF-α induced a strong, temporal release of immune mediators in the circulation and the brain. This release of inflammatory factors was followed by glial cell activation, as measured by astrocyte activation in the Gfap-luc mouse and increased Iba1 immunoreactivity in the hippocampus of FVB wild-type mice. Moreover, systemic administration of TNF-α led to a strong sickness response and mild signs of anhedonia. However, due to the overlapping time course of these behavioral states it was not possible to unambiguously distinguish depressive-like behavior from sickness.

6

Summary, General discussion & Future perspectives

Summary and general discussion

Clinical depression is a heterogeneous disorder with unknown etiology. Although the exact pathogenesis is still unclear, most experts in the field agree that depression is caused by a complex interaction between genetic and environmental factors. Several lines of evidence indicate that inflammatory processes may also be involved in the development of depression, at least in subsets of vulnerable individuals. For instance, depression frequently occurs as a comorbidity of medical conditions characterized by chronic inflammatory processes. Even in absence of other medical illness, many depressed patients display marked alterations in inflammatory cytokine levels and immune cell activity. Moreover, therapeutic administration of proinflammatory cytokines is able to elicit depression in psychiatric healthy subjects. The fact that these cytokines are typically administered intravenously indicates that activation of the immune system in the periphery has profound effects on the brain.

Validated animal models are needed to elucidate the biological underpinnings of inflammation-associated depression and to screen for novel drugs. Despite extensive scientific research during the past decades, no such animal models are currently available. Several groups have proposed that peripheral immune activation using stimuli such as LPS or proinflammatory cytokines can be used to model inflammation-associated depression in rodents. It is suggested that these immune stimuli elicit a biphasic response in which an initial episode of sickness is followed by a depressive-like phenotype. However, the use of different experimental approaches and reports of contrasting results makes it difficult to interpret currently available literature. In this study, a combination of techniques and readouts was used to investigate the time course of central effects induced by peripheral immune stimuli. The main immune stimulus used throughout the study is LPS, an immunostimulant that mimics the early phases of a bacterial infection. Although fairly high doses of LPS have been used, it is shown that humans are about 100,000-fold more sensitive to LPS than rodents [282]. Moreover, the fact that even a low dose of LPS was found to have mood altering effects in humans [283, 284] supports the idea that LPS injection in mice is a suitable model to study the molecular mechanisms of inflammation-associated behavioral changes.

Figure 6.1 summarizes the major biological findings of this study. We demonstrated that injection of LPS into the abdominal cavity triggers a rapid and robust release of immune molecules into the circulation, thereby causing systemic inflammation. This "cytokine storm" is able to access the CNS and elevate brain levels of inflammatory immune mediators. Once in the brain, these immune molecules activate astrocytes and microglia as demonstrated by increased expression of activation markers by these cell types. In addition, the inflammatory response to peripheral LPS administration is accompanied by behavioral changes. These behavioral changes are characterized by a strong sickness component and mild depressive-like behavior. However, due to overlapping time windows it is not possible to separate these behavioral states.
LPS elicits a broad immune response, as demonstrated by the release of multiple inflammatory molecules and the occurrence of sickness. Based on clinical data linking TNF-α to inflammation-associated depression, we investigated whether administration of this particular proinflammatory cytokine induces a depressive-like state that is less confounded by sickness. Using a similar experimental design as for the LPS study we demonstrated that intraperitoneal injection of TNF-α also induces the systemic release of multiple inflammatory molecules. Although the extent and nature of TNF-α-mediated cytokine release differs slightly from the one evoked by LPS, it became clear that both immune stimuli cause comparable central responses. Accordingly, peripheral administration of TNF-α triggers a time-dependent release of inflammatory molecules in the brain and activates glial cells. These inflammatory responses are accompanied by sickness and mild depressive-like behavior. Unfortunately, as was the case for LPS, the time course of these behavioral states overlapped and therefore it is not possible to identify a clear depressive-like phenotype. However, the fact that peripheral injection of TNF-α is able to induce neuroinflammation and behavioral alterations confirms that the TNF-α system is an important player in immune-to-brain communication. Future work should determine whether prolonged or deregulated activation of TNF-α signaling offers a more suitable model to induce depression-related symptoms.

The consistent and systematic use of immune challenges, time points and experimental readouts in this thesis allows answering fundamental questions relevant to inflammation-associated depression.

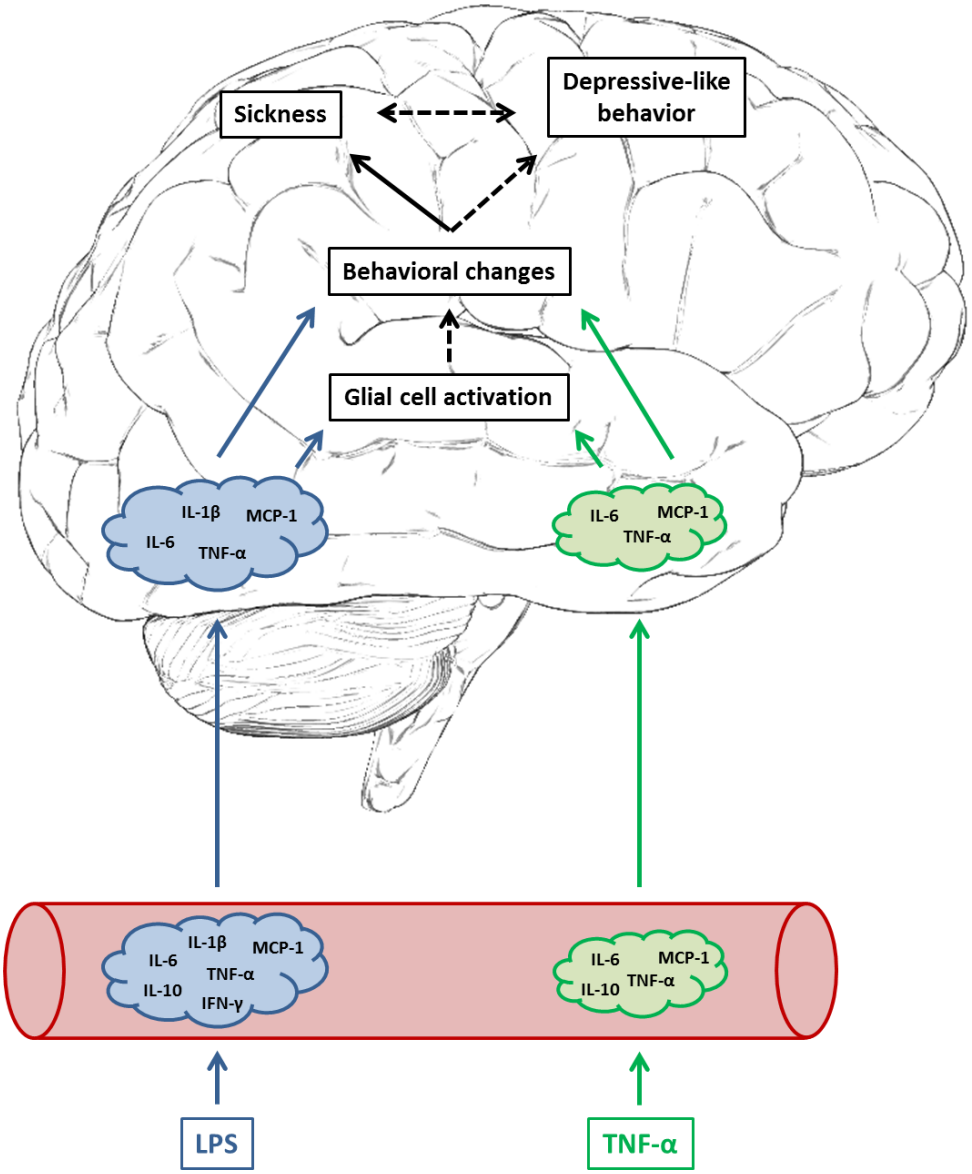

Figure 6.1 Summary of the major findings in this thesis. Using a combination of techniques and readouts, it was found that peripheral administration of the immunostimulant LPS induces the systemic release of several immune molecules (left). Some of these mediators are able to access the brain and trigger the activation of glial cells such as microglia and astrocytes. These inflammatory responses are accompanied by behavioral changes including sickness and mild depressive-like behavior. However, due to overlapping time windows it is difficult to separate these behavioral states. Peripheral administration of the proinflammatory cytokine TNF-α leads to a less pronounced release of inflammatory mediators than LPS, but has comparable central consequences (right).

Does peripheral immune activation induce neuroinflammation?

Inflammation involves a complex and coordinated response of the immune system to infections and/or tissue damage. In the absence of tools to routinely evaluate inflammation in living tissue, quantification of humoral factors such as cytokines and other immune molecules can be used to assess the activation state of the immune system at a given time point. For many years, the gold standard for assessing cytokine concentrations in biological samples involved the use of enzyme-linked immunosorbent assays (ELISAs). This technique is restricted by the fact that only a single protein of interest can be measured per assay run. Multiplex immunoassays which allow detection of large numbers of proteins in limited sample volumes have recently become commercially available [285]. In **chapters 3** and **4**, we used such immunoassays to characterize the nature and kinetics of the inflammatory response to peripheral LPS administration by quantifying serum levels of a panel of immune molecules at multiple time points. It was found that LPS induces a rapid and robust release of pro- and anti-inflammatory cytokines that peaks and wanes within the first day after injection. These findings are in accordance with results from Grigoleit et al., who reported a comparable time course of cytokine release following peripheral LPS administration in humans [286, 287]. Taken together, these data suggest that the inflammatory response to LPS fades within a couple of hours. However, by showing that serum levels of chemokines remained elevated for up to 4 days, we demonstrated that a single bolus of LPS affects the release of immune mediators for several days.

The sensitivity of multiplex immunoassays typically lies within the picogram range. Cytokine concentrations in brain homogenate usually fall below this detection limit, thereby making it difficult to assess cytokine protein levels in the brain. Hence, most studies that characterize cytokine profiles in the brain do so by quantifying gene transcripts of these immune molecules. However, the activity of cytokines and chemokines is not only limited by their gene expression, but also regulated at the posttranscriptional and posttranslational level [7, 280, 281]. Therefore, it is suggested that assessing protein levels of cytokines is a more accurate indicator of cytokine activity [161]. In **chapter 3**, we applied a multiplex immunoassay on brain homogenate and demonstrated that systemic administration of LPS induces a strong upregulation of immune molecule levels in the brain. In contrast to Qin et al., who reported that brain

concentrations of TNF-α remained elevated for up to 10 months after LPS administration [239], we found that brain levels of proinflammatory cytokines rapidly returned to control values. Corroborating our results, Erickson et al. also reported that brain levels of inflammatory cytokines were no longer significantly elevated beyond 24 h after peripheral LPS administration. This suggests that the neuroinflammatory response to peripherally administered LPS has dissipated by 24 h. However, we and others demonstrated that brain levels of the chemokine MCP-1 are still elevated at this point, thereby indicating that inflammatory processes are still taking place in the brain.

Future studies should be conducted to identify the cellular source of brain cytokines. LPS is previously shown to make the BBB leaky, which would stimulate passive leakage of cytokines into the brain. Therefore, it is possible that the increased brain levels of immune mediators in this study resulted uniquely from passive diffusion of circulating cytokines. However, differences in the time course of serum and brain profiles indicate that not all brain molecules originated in the periphery. For instance, brain levels of IL-1β are detectable at 6 h post-LPS, a time point at which IL-1β serum concentrations had returned to baseline values. Moreover, serum levels of IL-6 in LPS-treated mice are 10-fold higher at 2 h than at 6 h, while IL-6 brain levels are of the same magnitude at these time points. The idea that at least a portion of the immune mediators was produced in the brain is further confirmed by previous studies showing increased gene transcripts of inflammatory molecules following peripheral immune activation [96, 104, 245, 288].

Quantification of glial cell activation markers offers another way of assessing neuroinflammation. In **chapter 2** we combined in vivo imaging techniques to simultaneously quantify the activation state of astrocytes and microglia in living animals. In order to achieve this, a radioligand that binds specifically to TSPO was administered to transgenic Gfap-luc mice. Brain bioluminescence was measured to evaluate astrocyte activation while positron emission tomography allowed quantification of activated microglia. This experimental approach offers several advantages over conventional mRNA transcript and protein detection techniques. For example, it provides a quick way to monitor the activation state of glial cells in real-time. Moreover, these imaging techniques are non-invasive and can be performed in the same animal over time, thereby drastically reducing animal numbers when studying glial cell activation in longitudinal

studies. Using this in vivo imaging approach we demonstrated that peripheral administration of a single bolus of LPS increases the expression of glial cell activation markers in a time-dependent manner. These findings provide additional support to the idea that peripheral immune activation induces inflammatory responses in the brain and that these processes are locally mediated. In this regard, the use of bioluminescent mouse models is particularly interesting as they allow quantification of gene transcription in real-time. Generating transgenic mice with a reporter gene coupled to the promoter region of specific proinflammatory cytokines could be used to monitor de novo synthesis of these immune mediators in the brain.

Based on the comparison of time profiles, it appears that glial cell activation occurs after the peak release of proinflammatory cytokines in the brain. This is not surprising as astrocytes and microglia are known to express cytokine receptors and undergo rapid molecular and functional changes upon activation of these receptors. There are some indications that glial cell activation promotes neuronal survival during neuroinflammation [289]. Consequently, glial cell abnormalities have been demonstrated in several neurodegenerative conditions, including depression [290, 291]. Future studies are needed to establish the molecular mechanisms that underlie glial cell activation and to investigate how glial cell activation influences neuronal function. Conditional knockout mice that lack cytokine receptors in astrocytes or microglia could be used to elucidate the role of specific immune mediators in these processes.

In conclusion, the results described in **chapters 2** and **3** confirm that peripheral immune activation using LPS induces neuroinflammation in a timely fashion. These neuroinflammatory responses are robust, but fairly short-lasting and therefore probably not sufficient to induce the morphological and neurological changes observed in clinical depression. It needs to be noted, though, that we did not focus on regional effects and hence cannot exclude that cytokine release and/or glial activation persisted in specific brain areas. Previous work has indicated that the neuroinflammatory response to systemic inflammation is brain region specific [240, 277, 278] and focusing on spatio-temporal immune processes could help identifying those brain structures that are important in the development of inflammation-associated depression.

Does systemic LPS administration induce depressive-like behavior?

The behavioral response to LPS in rodents has been extensively described. Overall, systemic LPS administration induces a state of sickness that lasts for 1 to 2 days [99]. Some studies propose that this initial sickness response is followed by a depressive-like phenotype in which rodents display behavior similar to clinically relevant symptoms of depression in humans. However, the time window in which this potentially depressive-like behavior occurs is not very clear and results from different labs often vary. Moreover, studies focusing on LPS-induced behavioral changes frequently show variations in experimental design, which makes it difficult to compare results between research groups.

In **chapter 3**, LPS-injected mice were evaluated in a panel of automated behavioral assays optimized to measure sickness, anxiety and depressive-like behavior in an observer-independent manner. In line with previous reports, we found that systemic administration of a single bolus of LPS elicits a strong sickness response. Measures of anxiety and depressive-like behavior, however, are less convincing and difficult to interpret due to the confounding effects of sickness. These results are in contrast with findings from O'Connor et al., who used a comparable behavioral analysis and reported depressive-like behavior in absence of sickness at 24 h after peripheral LPS administration. However, in that study, mice were repeatedly tested and therefore results are potentially confounded by habituation effects. Indications of such habituation effects are apparent in mice from the vehicle-injected group, which were much less active upon retesting in the open field test. The LPS-treated mice, in contrast, may not have experienced habituation effects during retesting because they displayed sickness and therefore did not explore the open field arena during the first time they were tested. Similar habituation effects can also be seen in another study where repeated testing of mice in the open field test decreased line crossing in control animals [104]. However, in contrast to the O'Connor study [98], LPS still caused a significant reduction in locomotor activity at 24 h post-administration here. In our study, we avoided confounding habituation effects by using naive mice at all time points and demonstrated that it is not possible to convincingly separate depressive-like behavior from sickness following a single systemic LPS injection in mice.

Characterizing the kinetics of neuroinflammation and behavioral changes allowed us to compare the time course of central responses to peripheral immune

activation. We found that brain levels of proinflammatory cytokines peaked rapidly after an immune stimulus, while glial cell activation occurred with a delay of a couple of hours. Glial cells express a variety of cytokine receptors and it is likely that the upregulation of glial activation markers occurred as a direct result of stimulation of these receptors [292]. Unfortunately, our experimental design did not allow the identification of the factors causing the behavioral changes observed in immune-challenged mice. Based on the overlap between the peak of proinflammatory cytokine release and the onset of sickness behavior, it is likely that these immune mediators play a direct role in the development of sickness. Indeed, TNF-α, IL-1β and IL-6 have well described actions on the hypothalamus, including suppression of appetite, induction of fever and stimulation of the HPA axis by increasing CRH release [293, 294]. Apart from influencing brain targets to induce sickness, there are several mechanisms through which excessive and/or sustained cytokine release in the brain can contribute to the development of depressive symptoms. As described in section 1.2.3, proinflammatory cytokines impair neurotransmission by reducing the synthesis of neurotransmitters and increasing their reuptake [9]. Cytokines also increase glutamate release from astrocytes, which can alter synaptic plasticity, mediate glutamate-induced excitotoxicity and lead to reduced production of neurotrophic factors [110-112]. Moreover, inflammatory cytokines increase the activity of IDO and K3MO, two enzymes in the kynurenine pathway of tryptophan breakdown (figure 1.4) [135]. Sustained activation of these enzymes impairs serotonergic neurotransmission in the brain and eventually causes neurodegeneration through generation of neurotoxic tryptophan metabolites and reactive oxygen species [131].

There are some suggestions that the origin of proinflammatory cytokines determines their behavioral outcome. For instance, Bay-Richter et al. proposed that inflammation in the periphery causes sickness, while depressive-like behavior results from centrally produced cytokines [245]. This idea is supported by studies showing that proinflammatory cytokines induce depressive-like behavior in absence of sickness when injected intracerebroventricularly [274, 275, 295]. However, the evidence of depressive-like behavior in these studies is not very convincing and an equal amount of publications indicate that i.c.v. administration of proinflammatory cytokines or LPS does induce sickness [273, 296, 297]. Therefore it is unlikely that the distinction between sickness and

depressive symptoms merely results from cytokines released in different compartments. We believe that it is not the origin of cytokines that causes depressive-like symptoms, but rather the duration of exposure to proinflammatory mediators. Indeed, this would correspond to the clinical situation where systemic administration of IFN-α during immunotherapy first results in neurovegetative symptoms and later in mood and cognitive symptoms. Keeping this in mind, it is likely that the neurobiological consequences of inflammation are particularly important for the psychomotor and neurovegetative aspects of depression [295].

Can peripheral LPS administration be used to model behavioral endophenotypes of depression?

There is no validated animal model that mimics the entire complex syndrome of depression. Many depressive symptoms are only verbally expressed and can therefore not be modeled or assessed in animals. To date, only distinct symptoms of the human disease can be induced and analyzed in rodents. There is a high degree of similarity between inflammation-induced sickness and depression. For example, during both sickness and depression, there is a withdrawal from the physical and social environment that is accompanied by motor and cognitive impairments [298]. Moreover, anhedonia, which is part of the sickness response to inflammation, is also a key symptom of depression. However, the similarity between sickness and depression is only partial. Sickness is an adaptive response to infection by pathogens and fully reversible when the pathogen is cleared. Depression, on the other hand, can persist for months and does not always have a clear inflammatory trigger. It is possible that inflammation-associated depression is a maladaptive version of cytokine-induced sickness, which may occur in vulnerable individuals with exacerbated and/or sustained immune activation [99]. If this is the case, inflammation might account for certain depressive subtypes and rodent models using an immune stimulus could induce certain endophenotypes of inflammation-associated depression.

In **chapters 3** and **4** we showed that peripheral administration of LPS induces mild behavioral despair in the forced swim and tail suspension test. However, this did not occur in a dose-dependent manner and should be considered

biologically irrelevant due to the overlapping sickness-related reduction in locomotor activity.

In **chapter 4**, we optimized a sucrose preference protocol in rats and demonstrated that by including measures of total volume intake and sucrose preference, LPS-induced anhedonia can be separated over time from sickness. These findings seem to contradict with the lack of behavioral despair in our previous experiments. However, it is possible that the inflammatory response to LPS has a stronger and/or longer lasting effect on specific brain circuits, such as the reward system, and therefore induces behavioral changes like anhedonia, but not despair. This would correlate to the human situation where depressed patients also not necessarily display all possible symptoms of depression.

Anhedonia is one of the key symptoms of clinical depression and induction of anhedonia in rodents would be an important step in modeling behavioral endophenotypes of human depression. Although our sucrose preference protocol showed a clear anhedonic response to systemic LPS administration there are some limitations that should be taken into account. First of all, anhedonia was only assessed using a sucrose preference test. Confirming LPS-induced anhedonia using other anhedonia-assessing paradigms such as intracranial self-stimulation [299] or the recently described urine sniffing test [300] would help in supporting our findings. Secondly, functional readouts in the sucrose preference test strongly depend on the experimental setup. For example, we demonstrated that the preference for sucrose augments with increasing sucrose concentrations and found that rats showed a higher innate hedonic preference for sucrose than mice. Moreover, in **chapter 4** it became clear that changes in our standardized SPT protocol affected measures of sucrose preference. For instance, expanding the protocol by introducing a phase in which animals were exposed to repeated LPS injections or sub-chronic restraint stress changed sucrose preference in the control animals and seemingly altered the time course of anhedonia following a subsequent LPS challenge. However, due to the absence of statistically significant main effects these differences could not be analyzed in detail and it cannot be excluded that increasing the number of animals per group would omit these discrepancies. Nonetheless, these findings indicate that changing the experimental protocol affects functional readouts and can lead to potential misinterpretation of results in the sucrose preference test. Thirdly, our rat data (**chapter 4**) seemingly contradict the sucrose preference

results in mice (**chapter 3**). Indeed, there were no evident signs of LPS-induced anhedonia in mice. However, the use of different protocols makes it difficult to compare results from these experiments. Fourthly, LPS-induced anhedonia only lasted for 3 days. Anhedonic episodes related to depression in humans, in contrast, have a more chronic nature and can last up to several months. This indicates that bolus administration of LPS in rodents may be useful to study the acute mechanisms causing anhedonia, but is not sufficient to induce molecular changes that alter the reward circuit on the long term.

From a translational point of view, Eisenberger et al. demonstrated that peripheral administration of a low dose of LPS induces a depressed mood in humans [283]. Using neuroimaging, this group also demonstrated that inflammation alters reward-related neural responding and that these anhedonia-associated responses mediate the effects of inflammation on depressed mood. Such neuroimaging studies in humans are particularly useful to identify the neuronal circuits and brain areas that are involved in the development of inflammation-associated depressive symptoms. For example, imaging studies in people that underwent typhoid vaccination indicated that the psychomotor consequences of peripheral inflammation are mediated by midbrain dopaminergic nuclei [301], while inflammation-associated mood changes occur through alterations in subgenual anterior cingulate activity and mesolimbic connectivity [86]. Imaging studies in IFN-α treated hepatitis C patients also showed that peripheral immune activation alters neuronal activity in depression-related brain areas, including the prefrontal cortex, nucleus accumbens, putamen and anterior cingulate cortex [302, 303]. Rodent models could be used to study these alterations at the molecular level and hence evaluate which cell types and processes are involved in mediating these changes.

Is peripheral administration of TNF-α sufficient to induce neuroinflammation and depressive-like behavior in mice?

In **chapters 2, 3** and **4** we characterized the inflammatory and central effects of peripheral LPS administration and demonstrated that LPS causes a widespread immune response that is characterized by the release of multiple immune mediators. In addition, we found that LPS-induced behavioral changes are relatively short-lived and dominated by sickness. In an attempt to elicit more

specific depression-related responses, we characterized the central effects of peripheral TNF-α administration. This particular cytokine was chosen because clinical data suggests that TNF-α is one of the key cytokines involved in inflammation-associated depression. Moreover, it is reported that central administration of TNF-α induces depressive-like behavior in mice [274, 275].

In **chapter 5**, we showed that intraperitoneal administration of TNF-α elicits the systemic release of numerous inflammatory molecules. Some of these mediators, including TNF-α, IL-6 and MCP-1 are able to access the brain and induce a profound neuroinflammatory response as indicated by the activation of astrocytes and microglia. These findings are in line with results from a study by Skelly et al. in which peripheral administration of TNF-α was found to induce proinflammatory gene expression in the brain [2]. Using a panel of behavioral assays, we demonstrated that peripheral TNF-α administration also elicits behavioral changes. Unfortunately, these behavioral changes are dominated by sickness and no depressive-like phenotype could be observed. Nonetheless, given the increasing attention in literature on correlating specific systemic inflammatory mediators with neuropsychiatric conditions, our data provide useful information on the likely CNS inflammatory profiles resulting from systemic elevations of TNF-α.

Although the central effects of an acute bolus injection of TNF-α were strong, they were only short-lasting. Based on the hypothesis that inflammation-associated depression results from sustained, low-grade inflammation, it is expected that chronic or intermittent administration of TNF-α may provide a more suitable approach to induce depressive-like behavior in rodents. However, future studies are needed to confirm this theory as recent in vitro work has demonstrated that preincubating human monocytes with TNF-α reduces their inflammatory response to a subsequent TNF-α challenge [304]. If this phenomenon also occurs in vivo, it is likely that the magnitude and/or duration of TNF-α-induced immune cell activation decreases upon repeated TNF-α administration. This does not necessarily imply that chronic TNF-α administration is unable to induce a depressive phenotype in rodents. It is possible, for example, that injection of exogenous TNF-α induces behavioral alterations through direct effects on the molecular pathways that mediate inflammation-associated depression.

The fact that TNF-α was able to trigger the release of downstream immune molecules suggests that TNF-α is a central mediator of inflammation. This raises the idea that blocking the TNF-α-mediated signaling cascade is sufficient to dampen inflammatory responses. Indeed, TNF-α antagonism is already a widely used therapeutical strategy for treating disease with an inflammatory component [305]. Interestingly, anti-TNF-α therapy was previously shown to reduce depressive symptoms that frequently occur as a comorbidity of these inflammatory conditions [155-157]. Raison et al. demonstrated that anti-TNF-α therapy improved treatment resistant depression in a subgroup of patients with high baseline inflammatory biomarkers [158]. Future studies are needed, though, to determine whether inhibition of TNF-α is sufficient to block the inflammatory and behavioral consequences of systemic inflammation.

Translational implications

A tremendous amount of literature suggests a link between inflammation and depression. It is still not clear whether inflammation is a cause or consequence of depression. Several clinical studies have indicated that administration of cytokines, or cytokine inducers such as LPS or vaccination can cause behavioral symptoms that overlap with those found in depression [30]. This favors the idea that inflammation contributes to the development of depression, at least in a subset of vulnerable individuals. Although the exact pathophysiological mechanisms of inflammation-associated depression have not been elucidated, there is a common believe that it results from a sustained, low-grade proinflammatory tone in the body. In this regard, it is proposed that subsequent activation of inflammatory pathways leads to increased neurodegeneration and reduced neurogenesis, which ultimately causes the behavioral phenotype of depression. This view has been strengthened by a wide variety of preclinical studies demonstrating that immune activation, and the accompanying release of proinflammatory mediators, is able to induce profound behavioral changes. Although future work is needed to improve the translational capacity of rodent models of inflammation-associated depression, the current study has provided indications that peripheral immune activation has central effects that may be relevant in developing depressive symptoms. Moreover, the research techniques and rodent models that have been validated and optimized in this study could be used to screen for novel drug targets and therapeutic approaches to treat inflammation-associated depression. Using a comparable LPS model in mice, O'Connor et al. already identified IDO as a critical molecular mediator of inflammation-triggered depressive-like behavior [98]. Although it is debatable whether LPS induces a clear depressive-like episode, it can still be used to investigate the downstream effects of immune activation and hence contribute to the identification of mechanisms that lead to depressive symptoms.

In light of clinical observations suggesting immune activation in subpopulations of depressed patients, our findings have several diagnostic and therapeutical implications. A continuing challenge in depression is the lack of biomarkers for patient stratification, i.e. a clear way of classifying what is a highly heterogeneous disorder [221]. Identification of peripheral biomarkers that aid in the diagnosis or prediction of treatment response would be of particular interest. Altered blood levels of specific cytokines and other molecules related to immune

activation are systematically reported in depressed patients [306]. A number of studies have indicated that some of these immune molecules hold promise in predicting the onset of depression [307-309] and treatment response [154, 310, 311]. However, it is worth noting that biomarker research in inflammation-associated depression is hampered in several ways. For example, blood levels of inflammatory markers are heavily influenced by genetic and environmental factors. Moreover, the lack of standardized methods for sample collection, sample measurement and data analysis limit the comparison of results between labs [285]. Inconsistencies in patient populations and the fact that depression is generally not considered as a heterozygous disorder makes it even more difficult to draw solid conclusions from currently available studies. Keeping these limitations in mind, there is a need for hypothesis-driven investigation of biomarkers in well-defined patient subpopulations. Based on the highly heterozygous nature of depression these investigations should not be designed to identifying single biomarkers, but focus on panels of analytes. Such an approach may lead to the discovery of specific and sensitive biomarkers for depression subtypes, categories or even specific symptoms.

If inflammation is able to induce depressive symptoms, it is to be expected that anti-inflammatory treatment could be used to alleviate them. Indeed, an immense body of preclinical research indicates that anti-inflammatory agents and cytokine antagonists can block the development of behavioral changes following immune activation [312]. In addition, studies using transgenic animals have indicated that knocking out components of proinflammatory cytokine signaling pathways induces an antidepressant phenotype [313-315], while deleting components of anti-inflammatory cytokine machinery increases depressive-like behavior [316]. The idea that anti-inflammatory treatment has antidepressant properties is also supported by clinical observations. Adding minocycline, a compound with general anti-inflammatory properties, to antidepressant medication was reported to have antidepressant and antipsychotic effects in patients with psychotic depression [317]. Adjunctive treatment with cyclooxygenase inhibitors, which inhibit the production of proinflammatory cytokines, greatly enhances the therapeutical efficacy of conventional antidepressants [318, 319]. Blocking the production of the inflammatory mediator prostaglandin E2 using acetylsalicylic acid shortens the onset of action of antidepressants in depressed patients [320]. Several meta-

analyses have indicated that food supplementation of anti-inflammatory polyunsaturated fatty acids such as omega 3 is beneficial in adults suffering from depression [321, 322]. More targeted approaches such as antagonizing specific proinflammatory cytokines are also shown to be successful in alleviating depressive symptoms. Peripheral administration of anti-TNF-α antibodies, for example, improved depressed mood in patients suffering from psoriasis [155], Crohn's disease [156] and rheumatoid arthritis [157]. TNF-α antagonism has also been shown to improve treatment-resistant depression [158]. In that study, however, depressive symptoms only improved in a subgroup of patients with high baseline inflammatory biomarkers. This stresses the importance of patient stratification and personalized medicine in highly heterozygous disorders such as depression. Taken together, these findings confirm that depressed patients could benefit from anti-inflammatory agents. However, several issues should be considered before adding anti-inflammatory drugs to standard antidepressant treatment. First of all, there is an urgent need to identify patients that will potentially respond to anti-inflammatory agents. Raison et al. successfully separated responders from non-responders by quantifying pre-treatment levels of the inflammatory marker CRP [158]. Secondly, it needs to be decided whether general anti-inflammatory compounds or more specific approaches will be used. Based on the highly pleiotrophic role of the immune system in health and disease, targeting specific pathways related to inflammation-associated depression would be preferred over general immune suppression. However, such pathways have not yet been identified and future research should elucidate whether they can be found amongst factors that are responsible for immune cell activation, cytokine release and/or action, production of second messenger and effector molecules. Thirdly, it is reported that reducing inflammation in the periphery is able to improve depression scores [158, 318, 319]. Strictly speaking, depressive symptoms are mediated in the brain. This raises the question whether dampening immune activation in the periphery is sufficient to attenuate neuroinflammation and inflammation-associated depressive symptoms or if brain penetrating anti-inflammatory agents are needed. The imaging techniques that were optimized and used in this thesis provide a valuable tool to potentially answer this question in rodents. Finally, the benefit/risk ratio of add-on anti-inflammatory drugs should always be considered. For example, treating depression by suppressing inflammatory processes would impair the prognosis

of patients suffering from infections or malignancies. Moreover, anti-inflammatory agents cannot be used to treat comorbid depression in patients receiving proinflammatory cytokine treatment as immunosuppression would reduce the therapeutic efficacy of such therapies.

Conclusion and future directions

Several lines of evidence suggest that inflammatory processes contribute to the development of depression in vulnerable individuals. Despite major scientific efforts, the pathophysiological mechanisms through which inflammation causes depressive symptoms remain elusive. One of the factors impeding current research and the development of novel therapeutic strategies is the lack of validated animal models to study the molecular processes leading to inflammation-associated depression. Using a combination of multiplex immunoassays and non-invasive imaging in rodents we found that peripheral immune activation increased cytokine levels in the brain and subsequently causes glial cell activation. These neuroinflammatory responses are robust, but short-lasting. Inflammation-associated depression, in contrast, is thought to develop on a background of sustained, low-grade inflammation. This may explain why the behavioral changes observed in our studies were transient and dominated by sickness. Based on clinical observations in patients receiving proinflammatory cytokine treatment, it is believed that sustained immune activation leads to a biphasic response in which sickness is followed by depressive symptoms. The fact that these depressive symptoms only develop after several weeks of treatment suggests that the molecular mechanisms underlying inflammation-associated depression need to be activated chronically in order to develop a depressive phenotype. Therefore, future rodent studies should aim at inducing longer lasting inflammatory responses. This could be achieved by repeated or chronic administration of an immune challenge such as LPS. However, repeated administration of LPS induces a state of tolerance in which innate immune response to subsequent LPS challenges are severely blunted [323]. Regardless of LPS tolerance, Kubera et al. recently described a mouse model in which repeated, intermittent LPS challenges induced a chronic depressive phenotype in mice [250]. The authors of that study proposed that the depressogenic effect of their treatment scheme was not directly caused by the release of proinflammatory cytokines but resulted from LPS-induced oxidative stress. It needs to be noted, though, that depressive symptoms occurred in female, but not in male, mice and that depressive-like behavior was only assessed by evaluating anhedonia using a sucrose preference test. Infection with life pathogens offers another way of inducing longer lasting inflammatory

responses in rodents. It needs to be kept in mind, though, that inflammatory processes induced by life infections are more elaborate than the ones evoked by immunostimulants such as LPS [186]. Moreover, life infections can cause tissue damage, which makes it difficult to attribute molecular changes and/or altered behavior to the action of specific immune molecules. To avoid tissue damage, O'Connor et al. injected mice with Bacillus Calmette-Guérin (BCG), which is an attenuated form of Mycobacterium bovis [324]. Using this model of persistent immune activation, the authors demonstrated development of depressive-like behavior in absence of sickness. Interestingly, they also showed that this depressive phenotype was mediated by chronic cytokine-induced activation of the tryptophan-degrading enzyme IDO.

Patients suffering from autoimmune disorders are at increased risk of developing depression [325-327]. In line with these findings, depressive symptoms are also reported in rodent models of autoimmunity, including the experimental autoimmune encephalomyelitis mouse model for multiple sclerosis [328, 329] and a lupus-prone mouse strain [330, 331]. However, these models are characterized by broad and elaborate (neuro)immunological processes, which makes it again difficult to link specific processes to behavioral outcomes. In a more direct approach, Hayley et al. repeatedly infused the proinflammatory cytokine IFN-γ into the brain of mice and found anhedonic behavior after several administrations [295]. These results indicate that chronic administration of specific cytokines might also provide a way of inducing prolonged inflammation and depressive-like behavior.

Taken together, the studies described above suggest that sustained immune activation can elicit depressive-like behavior in rodents. It needs to be noted, though, that in most of these studies the inflammation-associated behavioral changes were not assessed systematically. Therefore, more work is needed to confirm that these protocols induce clear depressive-like behavior before they can be used to study the molecular mechanisms of inflammation-associated depression. Automated and standardized behavioral assays such as the ones described in this thesis should be used to facilitate validation of depressive symptoms.

In our studies we have used young adult rodents to evaluate the central effects of peripheral immune activation. Timing the immune challenge at other stages in life may have different and/or more pronounced functional and behavioral

consequences. It is known that the immune system plays an important role in shaping the brain during development [332]. Consequently, several studies have indicated that prenatal or early post-natal immune activation has detrimental effects on brain development and leads to behavioral alterations relevant to mood disorders later in life [333, 334]. Other studies showed that aging is associated with a proinflammatory tone in the body. Moreover, the aged immune system is considered to be in a "triggered" state, thereby producing exaggerated inflammatory responses to systemic immune challenges [13, 335, 336]. Consequently, aged rodents are reported to be more susceptible to the neuroinflammatory and behavioral effects of peripheral immune activation [104, 105]. These findings seem to align with the human situation where inflammatory responses to an immune challenge last longer in aged adults at risk for depression [337]. Future research should clarify whether changing the nature or timing of immune stimuli offers more valid approaches to study the molecular mechanisms of inflammation-associated depression in rodents. Based on the behavioral work presented in this thesis, it is advised to include proficient measures of sickness in such future studies.

Preclinical and clinical research on the pathological processes underlying depression has historically focused on neurons. Based on reports of immune activation and glial cell abnormalities in depressed patients, it is likely that these cell types also play an important role in the development of depression. However, the neurobiological mechanisms underlying glial cell activation and the behavioral consequences of inflammatory mediator release are still largely unknown. More work is needed to relate given behavioral effects of immune molecules to specific actions in a well-defined area in the brain [99]. Microarray technology can be used on specific brain areas to simultaneously screen a vast number of genes that are potentially up- or down-regulated in rodent models of inflammation-associated depression and even in postmortem brain tissue of depressed patients. In addition, optogenetics could be utilized to control and monitor the activity of individual cells in living animals and evaluate the effects of those manipulations on behavior in real-time. Generating conditional knockout animals that lack cytokines, or their receptor, in specific cell types provides another way of investigating the role of individual cytokines. Using this approach Quintana et al. demonstrated that IL-6 production by astrocytes plays an important role in exploration and anxiety behavior [338]. The fact that mice with

deficiencies in the astrocytic IL-6 system developed a phenotype that differs from total IL-6 knockout mice [339] stresses the cell-specific function of immune molecules.

In addition to characterizing the triggers and mechanisms of neuroinflammation, additional research is needed to identify the downstream effects of immune activation. Consequently, it is important to elucidate the mechanisms through which immune cell activation induces neuronal dysfunction and how this may lead to the behavioral aspects of inflammation-associated depression. Studying abnormalities in the kynurenine pathway of tryptophan breakdown would be particularly interesting. Many reports have already linked disturbances in this pathway to neurodegenerative and neuropsychological conditions. However, large scale clinical research on these disturbances has been hampered by difficulties in measuring tryptophan metabolites. This has historically been done by labor intensive and time consuming techniques such as high-performance liquid chromatography. Recent technological advances, however, could be used to investigate alterations in tryptophan metabolism in depression. For example, PET imaging of radioligand labeled synthetic analogs of tryptophan could be utilized to non-invasively detect abnormalities of tryptophan metabolism in the brain. Such tracers are already clinically used in the field of oncology, where IDO activation is measured to evaluate immuno-resistance of brain tumors [340, 341]. Moreover, immunoassays can be developed using recently generated antibodies against tryptophan metabolites. This would allow quick and high-throughput quantification of these metabolites in patient samples. In addition, antibodies against tryptophan metabolites could be used to restore imbalances in the kynurenine pathway of tryptophan breakdown and hence hold promise for therapeutical interventions in certain types of depression.

To summarize, the results of this thesis confirm that peripheral immune activation elicits a robust neuroinflammatory response that is accompanied by behavioral alterations. It is becoming clear that these processes are a double-edged sword. Where at first inflammation activates pathways that are neuroprotective and needed to restore homeostasis, overstimulation of these pathways can induce a depressive phenotype. Further research on the molecular mechanisms of inflammation-associated depression can therefore contribute to

the identification of disease-specific biomarkers and lead to the discovery of novel therapeutical strategies to treat certain subtypes of depression.

7

Samenvatting

Samenvatting

Depressie is een ernstige neuropsychiatrische aandoening die wordt gekenmerkt door een verminderde levenslust of zware neerslachtigheid. Ongeveer 1 op de 6 personen heeft ooit in zijn leven met depressie te kampen. Naast een sterk verminderde levenskwaliteit hebben depressieve patiënten een verhoogd mortaliteitsrisico. Zo is de kans op zelfdoding 20 keer hoger bij personen met een depressie. Bovendien verlaagt depressie de prognose en overlevingskansen van patiënten die lijden aan een andere aandoening. Depressie kan op verschillende manieren behandeld worden en er zijn verschillende klassen van antidepressieve medicatie beschikbaar. Spijtig genoeg schieten deze antidepressiva op vele vlakken tekort. Zo duurt het dikwijls weken voordat ze werkzaam zijn en veroorzaken ze vaak ongewenste bijwerkingen. Zelfs wanneer een depressie succesvol behandeld wordt zullen 50-80% van de patiënten opnieuw hervallen. Uiteindelijk ontwikkelt een op de drie patiënten een vorm van depressie die onbehandelbaar is.

Ondanks de hoge prevalentie en aanzienlijke socioeconomische impact, is er zeer weinig geweten over de oorzaak van depressie. De meeste experts zijn het eens dat een depressie veroorzaakt wordt door een complex samenspel van genetische en omgevingsfactoren. Er zijn verschillende aanwijzingen dat onstekingsprocessen ook kunnen bijdragen tot de ontwikkeling van depressie. Zo komen depressieve symptonen vaak voor bij patiënten die leiden aan een inflammatoire aandoening. Bovendien worden er vaak ontstekingsmerkers aangetroffen in het bloed van depressieve patiënten. Een directere aanwijzing dat inflammatie mogelijks bijdraagt aan het totstandkomen van depressie komt van klinische observaties bij hepatitis C en kanker patiënten die behandeld worden met immuunstimulerende therapiën. Ongeveer de helft van deze patiënten ontwikkelt namelijk symptomen die ook voorkomen bij ideopathische depressie.

Om de mechanismen van complexe aandoeningen zoals depressie te bestuderen zijn goed gevalideerde diermodellen noodzakelijk. Verschillende knaagdierstudies hebben aangetoond dat het toedienen van immuunstimulerende agentia gedragsveranderingen veroorzaakt die sterk lijken op bepaalde symptomen van depressie bij de mens. Het doel van deze thesis was het opzetten en karakteriseren van zulke knaagdiermodellen om zo de

onderliggende mechanismen van inflammatie-geassocieerde depressie te bestuderen. Volgende hypothese werd gesteld: immuunactivatie in de periferie leidt tot ontstekingsprocessen in de hersenen en veroorzaakt depressieve symptomen. Om deze stelling te testen werden verschillende onderzoekstechnieken en gedragstesten ontwikkeld en op punt gesteld. Zo werden multiplex immunoassays gebruikt voor het karakteriseren van immuunresponsen in de periferie en in het brein. Bovendien werd het tijdsverloop van immuuncelactivatie in de hersenen van muizen geanalyseerd met behulp van niet-invasieve beeldvormingstechnieken zoals bioluminescentie en positron emissie tomografie. Naast het bestuderen van immunologische processen werden ook de gedragsveranderingen die volgen op perifere immuunactivatie in kaart gebracht. Hierbij werd er vooral aandacht besteed aan het scheiden van algemeen ziektegedrag en symptomen van depressief gedrag bij knaagdieren.

De resultaten in deze thesis tonen aan dat het toedienen van een immuunstimulus in de buikholte van knaagdieren leidt tot een sterke vrijzetting van immuunmoleculen in het bloed. Bovendien werden deze immuunmediatoren aangetoond in de hersenen, hetgeen erop wijst dat er een immuunrespons plaatsvond in het brein. Door gebruik te maken van niet-invasieve beeldvorming kon vervolgens worden aangetoond dat immuuncellen in de hersenen tekenen van activatie vertonen. Dit bevestigt dat immuunactivatie in de periferie leidt tot een inflammatoire respons in de hersenen. Onze gedragsdata wijzen erop dat deze immunologische processen gepaard gaan met uitgesproken gedragsveranderingen. Deze gedragsveranderingen zijn kortstondig en worden vooral gekenmerkt door algemene ziektesymptomen. Dit maakt het moeilijk om een duidelijk depressief fenotype te onderscheiden. Toekomstig onderzoek moet uitwijzen of chronische immuunstimulatie leidt tot meer uitgesproken depressief gedrag.

De bevindingen van deze thesis duiden erop dat er een sterke wisselwerking is tussen het immuunsysteem en de hersenen. Verder onderzoek naar de pathologische relevantie van ontstekingsprocessen in de ontwikkeling van depressie kan verschillende implicaties hebben. Zo kan het bijvoorbeeld leiden tot de ontdekking van specifieke biomerkers die gebruikt kunnen worden voor diagnose en patiëntstratificatie bij depressie. Bovendien kan het bestuderen van inflammatie-geassocieerde ziektemechanismen leiden tot de ontdekking van

nieuwe therapeutische strategiën om bepaalde types van depressie te behandelen.

Reference List

[1] Lyman M, Lloyd DG, Ji X, Vizcaychipi MP, Ma D. Neuroinflammation: The role and consequences. Neurosci Res 2013.

[2] Skelly DT, Hennessy E, Dansereau MA, Cunningham C. A Systematic Analysis of the Peripheral and CNS Effects of Systemic LPS, IL-1Beta, TNF-alpha and IL-6 Challenges in C57BL/6 Mice. PLoS One 2013;8:e69123.

[3] D'Mello C, Le T, Swain MG. Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation. J Neurosci 2009;29:2089-102.

[4] Saunders BM, Frank AA, Orme IM, Cooper AM. Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection. Infect Immun 2000;68:3322-6.

[5] Dantzer R. Cytokine-induced sickness behaviour: a neuroimmune response to activation of innate immunity. Eur J Pharmacol 2004;500:399-411.

[6] Opal SM, DePalo VA. Anti-inflammatory cytokines. Chest 2000;117:1162-72.

[7] Iyer SS, Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol 2012;32:23-63.

[8] Medzhitov R. Origin and physiological roles of inflammation. Nature 2008;454:428-35.

[9] Miller AH, Haroon E, Raison CL, Felger JC. Cytokine targets in the brain: impact on neurotransmitters and neurocircuits. Depress Anxiety 2013;30:297-306.

[10] Blume J, Douglas SD, Evans DL. Immune suppression and immune activation in depression. Brain Behav Immun 2011;25:221-9.

[11] Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev 2011;91:461-553.

[12] Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 2005;308:1314-8.

[13] Perry VH, Cunningham C, Holmes C. Systemic infections and inflammation affect chronic neurodegeneration. Nat Rev Immunol 2007;7:161-7.

[14] Boche D, Perry VH, Nicoll JA. Review: activation patterns of microglia and their identification in the human brain. Neuropathol Appl Neurobiol 2013;39:3-18.

[15] Hamby ME, Sofroniew MV. Reactive astrocytes as therapeutic targets for CNS disorders. Neurotherapeutics 2010;7:494-506.

[16] Volterra A, Meldolesi J. Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 2005;6:626-40.

[17] Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature 2010;468:232-43.

[18] Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X et al. Astrocyte-mediated control of cerebral blood flow. Nat Neurosci 2006;9:260-7.

[19] Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. Acta Neuropathol 2010;119:7-35.

[20] Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG et al. Glial cells in (patho)physiology. J Neurochem 2012;121:4-27.

[21] Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci 2009;32:638-47.

[22] Sofroniew MV. Reactive astrocytes in neural repair and protection. Neuroscientist 2005;11:400-7.

[23] Bilbo SD, Schwarz JM. The immune system and developmental programming of brain and behavior. Front Neuroendocrinol 2012;33:267-86.

[24] De KJ, Mostert JP, Koch MW. Dysfunctional astrocytes as key players in the pathogenesis of central nervous system disorders. J Neurol Sci 2008;267:3-16.

[25] Hart BL. Biological basis of the behavior of sick animals. Neurosci Biobehav Rev 1988;12:123-37.

[26] Banks WA. Blood-brain barrier transport of cytokines: a mechanism for neuropathology. Curr Pharm Des 2005;11:973-84.

[27] Holmes C. Review: systemic inflammation and Alzheimer's disease. Neuropathol Appl Neurobiol 2013;39:51-68.

[28] Capuron L, Miller AH. Immune system to brain signaling: Neuropsychopharmacological implications. Pharmacol Ther 2011;130:226-38.

[29] Morita S, Miyata S. Different vascular permeability between the sensory and secretory circumventricular organs of adult mouse brain. Cell Tissue Res 2012;349:589-603.

[30] Miller AH, Maletic V, Raison CL. Inflammation and its discontents: the role of cytokines in the pathophysiology of major depression. Biol Psychiatry 2009;65:732-41.

[31] Quan N, Banks WA. Brain-immune communication pathways. Brain Behav Immun 2007;21:727-35.

[32] Raison CL, Capuron L, Miller AH. Cytokines sing the blues: inflammation and the pathogenesis of depression. Trends Immunol 2006;27:24-31.

[33] Tracey KJ. Reflex control of immunity. Nat Rev Immunol 2009;9:418-28.

[34] Chatham WW, Kimberly RP. Treatment of lupus with corticosteroids. Lupus 2001;10:140-7.

[35] Almawi WY, Beyhum HN, Rahme AA, Rieder MJ. Regulation of cytokine and cytokine receptor expression by glucocorticoids. J Leukoc Biol 1996;60:563-72.

[36] Ciriaco M, Ventrice P, Russo G, Scicchitano M, Mazzitello G, Scicchitano F et al. Corticosteroid-related central nervous system side effects. J Pharmacol Pharmacother 2013;4:S94-S98.

[37] Seruga B, Zhang H, Bernstein LJ, Tannock IF. Cytokines and their relationship to the symptoms and outcome of cancer. Nat Rev Cancer 2008;8:887-99.

[38] Powell ND, Tarr AJ, Sheridan JF. Psychosocial stress and inflammation in cancer. Brain Behav Immun 2013;30 Suppl:S41-S47.

[39] Dugue B, Leppanen E, Grasbeck R. The driving license examination as a stress model: effects on blood picture, serum cortisol and the production of interleukins in man. Life Sci 2001;68:1641-7.

[40] Maes M, Song C, Lin A, De JR, Van GA, Kenis G et al. The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. Cytokine 1998;10:313-8.

[41] Bierhaus A, Wolf J, Andrassy M, Rohleder N, Humpert PM, Petrov D et al. A mechanism converting psychosocial stress into mononuclear cell activation. Proc Natl Acad Sci U S A 2003;100:1920-5.

[42] Carpenter LL, Gawuga CE, Tyrka AR, Lee JK, Anderson GM, Price LH. Association between plasma IL-6 response to acute stress and early-life adversity in healthy adults. Neuropsychopharmacology 2010;35:2617-23.

[43] Nakata A. Psychosocial job stress and immunity: a systematic review. Methods Mol Biol 2012;934:39-75.

[44] Steptoe A, Owen N, Kunz-Ebrecht S, Mohamed-Ali V. Inflammatory cytokines, socioeconomic status, and acute stress responsivity. Brain Behav Immun 2002;16:774-84.

[45] Jousilahti P, Salomaa V, Rasi V, Vahtera E, Palosuo T. Association of markers of systemic inflammation, C reactive protein, serum amyloid A, and fibrinogen, with socioeconomic status. J Epidemiol Community Health 2003;57:730-3.

[46] Koster A, Bosma H, Penninx BW, Newman AB, Harris TB, van Eijk JT et al. Association of inflammatory markers with socioeconomic status. J Gerontol A Biol Sci Med Sci 2006;61:284-90.

[47] Cole SW, Hawkley LC, Arevalo JM, Sung CY, Rose RM, Cacioppo JT. Social regulation of gene expression in human leukocytes. Genome Biol 2007;8:R189.

[48] Creswell JD, Irwin MR, Burklund LJ, Lieberman MD, Arevalo JM, Ma J et al. Mindfulness-Based Stress Reduction training reduces loneliness and pro-inflammatory gene expression in older adults: a small randomized controlled trial. Brain Behav Immun 2012;26:1095-101.

[49] von KR, Mills PJ, Mausbach BT, Dimsdale JE, Patterson TL, Ziegler MG et al. Effect of Alzheimer caregiving on circulating levels of C-reactive protein and other biomarkers relevant to cardiovascular disease risk: a longitudinal study. Gerontology 2012;58:354-65.

[50] Capoccia S, Berry A, Bellisario V, Vacirca D, Ortona E, Alleva E et al. Quality and timing of stressors differentially impact on brain plasticity

and neuroendocrine-immune function in mice. Neural Plast 2013;2013:971817.

[51] Grippo AJ, Francis J, Beltz TG, Felder RB, Johnson AK. Neuroendocrine and cytokine profile of chronic mild stress-induced anhedonia. Physiol Behav 2005;84:697-706.

[52] Nguyen KT, Deak T, Owens SM, Kohno T, Fleshner M, Watkins LR et al. Exposure to acute stress induces brain interleukin-1beta protein in the rat. J Neurosci 1998;18:2239-46.

[53] Nguyen KT, Deak T, Will MJ, Hansen MK, Hunsaker BN, Fleshner M et al. Timecourse and corticosterone sensitivity of the brain, pituitary, and serum interleukin-1beta protein response to acute stress. Brain Res 2000;859:193-201.

[54] Kwon MS, Seo YJ, Lee JK, Lee HK, Jung JS, Jang JE et al. The repeated immobilization stress increases IL-1beta immunoreactivities in only neuron, but not astrocyte or microglia in hippocampal CA1 region, striatum and paraventricular nucleus. Neurosci Lett 2008;430:258-63.

[55] O'Connor KA, Johnson JD, Hansen MK, Wieseler Frank JL, Maksimova E, Watkins LR et al. Peripheral and central proinflammatory cytokine response to a severe acute stressor. Brain Res 2003;991:123-32.

[56] Frank MG, Baratta MV, Sprunger DB, Watkins LR, Maier SF. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. Brain Behav Immun 2007;21:47-59.

[57] Sugama S, Fujita M, Hashimoto M, Conti B. Stress induced morphological microglial activation in the rodent brain: involvement of interleukin-18. Neuroscience 2007;146:1388-99.

[58] Nair A, Bonneau RH. Stress-induced elevation of glucocorticoids increases microglia proliferation through NMDA receptor activation. J Neuroimmunol 2006;171:72-85.

[59] Bowers SL, Bilbo SD, Dhabhar FS, Nelson RJ. Stressor-specific alterations in corticosterone and immune responses in mice. Brain Behav Immun 2008;22:105-13.

[60] Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry 2005;62:593-602.

[61] Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006;3:e442.

[62] American Psychiatric Association. Mood Disorders. In. *Diagnostic and Statistical Manual of Mental Disorders, Text Revision (DSM-IV-TR)*; 2000.

[63] Osby U, Brandt L, Correia N, Ekbom A, Sparen P. Excess mortality in bipolar and unipolar disorder in Sweden. Arch Gen Psychiatry 2001;58:844-50.

[64] Barth J, Schumacher M, Herrmann-Lingen C. Depression as a risk factor for mortality in patients with coronary heart disease: a meta-analysis. Psychosom Med 2004;66:802-13.

[65] Coleman SM, Katon W, Lin E, Von KM. Depression and death in diabetes; 10-year follow-up of all-cause and cause-specific mortality in a diabetic cohort. Psychosomatics 2013;54:428-36.

[66] Teng PR, Yeh CJ, Lee MC, Lin HS, Lai TJ. Depressive symptoms as an independent risk factor for mortality in elderly persons: results of a national longitudinal study. Aging Ment Health 2013;17:470-8.

[67] Kato M, Chang CM. Augmentation treatments with second-generation antipsychotics to antidepressants in treatment-resistant depression. CNS Drugs 2013;27 Suppl 1:S11-S19.

[68] Machado M, Iskedjian M, Ruiz I, Einarson TR. Remission, dropouts, and adverse drug reaction rates in major depressive disorder: a meta-analysis of head-to-head trials. Curr Med Res Opin 2006;22:1825-37.

[69] Williams JM, Russell IT, Crane C, Russell D, Whitaker CJ, Duggan DS et al. Staying well after depression: trial design and protocol. BMC Psychiatry 2010;10:23.

[70] Maes M, Yirmyia R, Noraberg J, Brene S, Hibbeln J, Perini G et al. The inflammatory & neurodegenerative (I&ND) hypothesis of depression: leads for future research and new drug developments in depression. Metab Brain Dis 2009;24:27-53.

[71] Chopra K, Kumar B, Kuhad A. Pathobiological targets of depression. Expert Opin Ther Targets 2011;15:379-400.

[72] Dantzer R, O'Connor JC, Lawson MA, Kelley KW. Inflammation-associated depression: From serotonin to kynurenine. Psychoneuroendocrinology 2010.

[73] Dickens C, McGowan L, Clark-Carter D, Creed F. Depression in rheumatoid arthritis: a systematic review of the literature with meta-analysis. Psychosom Med 2002;64:52-60.

[74] Evans DL, Charney DS, Lewis L, Golden RN, Gorman JM, Krishnan KR et al. Mood disorders in the medically ill: scientific review and recommendations. Biol Psychiatry 2005;58:175-89.

[75] Anderson RJ, Freedland KE, Clouse RE, Lustman PJ. The prevalence of comorbid depression in adults with diabetes: a meta-analysis. Diabetes Care 2001;24:1069-78.

[76] Schwartz JA, Speed NM, Brunberg JA, Brewer TL, Brown M, Greden JF. Depression in stroke rehabilitation. Biol Psychiatry 1993;33:694-9.

[77] Petry NM, Barry D, Pietrzak RH, Wagner JA. Overweight and obesity are associated with psychiatric disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Psychosom Med 2008;70:288-97.

[78] Khawaja IS, Westermeyer JJ, Gajwani P, Feinstein RE. Depression and coronary artery disease: the association, mechanisms, and therapeutic implications. Psychiatry (Edgmont) 2009;6:38-51.

[79] Howren MB, Lamkin DM, Suls J. Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. Psychosom Med 2009;71:171-86.

[80] Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK et al. A meta-analysis of cytokines in major depression. Biol Psychiatry 2010;67:446-57.

[81] Raison CL, Miller AH. Depression in cancer: new developments regarding diagnosis and treatment. Biol Psychiatry 2003;54:283-94.

[82] Musselman DL, Lawson DH, Gumnick JF, Manatunga AK, Penna S, Goodkin RS et al. Paroxetine for the prevention of depression induced by high-dose interferon alfa. N Engl J Med 2001;344:961-6.

[83] Strike PC, Wardle J, Steptoe A. Mild acute inflammatory stimulation induces transient negative mood. J Psychosom Res 2004;57:189-94.

[84] Wright CE, Strike PC, Brydon L, Steptoe A. Acute inflammation and negative mood: mediation by cytokine activation. Brain Behav Immun 2005;19:345-50.

[85] Reichenberg A, Yirmiya R, Schuld A, Kraus T, Haack M, Morag A et al. Cytokine-associated emotional and cognitive disturbances in humans. Arch Gen Psychiatry 2001;58:445-52.

[86] Harrison NA, Brydon L, Walker C, Gray MA, Steptoe A, Critchley HD. Inflammation causes mood changes through alterations in subgenual cingulate activity and mesolimbic connectivity. Biol Psychiatry 2009;66:407-14.

[87] Capuron L, Gumnick JF, Musselman DL, Lawson DH, Reemsnyder A, Nemeroff CB et al. Neurobehavioral effects of interferon-alpha in cancer patients: phenomenology and paroxetine responsiveness of symptom dimensions. Neuropsychopharmacology 2002;26:643-52.

[88] Capuron L, Miller AH. Cytokines and psychopathology: lessons from interferon-alpha. Biol Psychiatry 2004;56:819-24.

[89] Gibney SM, Drexhage HA. Evidence for a dysregulated immune system in the etiology of psychiatric disorders. J Neuroimmune Pharmacol 2013;8:900-20.

[90] Willner P. The validity of animal models of depression. Psychopharmacology (Berl) 1984;83:1-16.

[91] Belzung C, Lemoine M. Criteria of validity for animal models of psychiatric disorders: focus on anxiety disorders and depression. Biol Mood Anxiety Disord 2011;1:9.

[92] Wiborg O. Chronic mild stress for modeling anhedonia. Cell Tissue Res 2013.

[93] Berton O, Hahn CG, Thase ME. Are we getting closer to valid translational models for major depression? Science 2012;338:75-9.

[94] Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. Cytokine 2008;42:145-51.

[95] Kent S, Bluthe RM, Kelley KW, Dantzer R. Sickness behavior as a new target for drug development. Trends Pharmacol Sci 1992;13:24-8.

[96] Henry CJ, Huang Y, Wynne A, Hanke M, Himler J, Bailey MT et al. Minocycline attenuates lipopolysaccharide (LPS)-induced neuroinflammation, sickness behavior, and anhedonia. J Neuroinflammation 2008;5:15.

[97] Frenois F, Moreau M, O'Connor J, Lawson M, Micon C, Lestage J et al. Lipopolysaccharide induces delayed FosB/DeltaFosB immunostaining

within the mouse extended amygdala, hippocampus and hypothalamus, that parallel the expression of depressive-like behavior. Psychoneuroendocrinology 2007;32:516-31.

[98] O'Connor JC, Lawson MA, Andre C, Moreau M, Lestage J, Castanon N et al. Lipopolysaccharide-induced depressive-like behavior is mediated by indoleamine 2,3-dioxygenase activation in mice. Mol Psychiatry 2009;14:511-22.

[99] Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat Rev Neurosci 2008;9:46-56.

[100] Yirmiya R. Endotoxin produces a depressive-like episode in rats. Brain Res 1996;711:163-74.

[101] Zhu CB, Lindler KM, Owens AW, Daws LC, Blakely RD, Hewlett WA. Interleukin-1 receptor activation by systemic lipopolysaccharide induces behavioral despair linked to MAPK regulation of CNS serotonin transporters. Neuropsychopharmacology 2010;35:2510-20.

[102] Berg BM, Godbout JP, Kelley KW, Johnson RW. Alpha-tocopherol attenuates lipopolysaccharide-induced sickness behavior in mice. Brain Behav Immun 2004;18:149-57.

[103] Tonelli LH, Holmes A, Postolache TT. Intranasal immune challenge induces sex-dependent depressive-like behavior and cytokine expression in the brain. Neuropsychopharmacology 2008;33:1038-48.

[104] Godbout JP, Chen J, Abraham J, Richwine AF, Berg BM, Kelley KW et al. Exaggerated neuroinflammation and sickness behavior in aged mice following activation of the peripheral innate immune system. FASEB J 2005;19:1329-31.

[105] Godbout JP, Moreau M, Lestage J, Chen J, Sparkman NL, O' CJ et al. Aging exacerbates depressive-like behavior in mice in response to activation of the peripheral innate immune system. Neuropsychopharmacology 2008;33:2341-51.

[106] Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.

[107] Cunningham C, Campion S, Teeling J, Felton L, Perry VH. The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C). Brain Behav Immun 2007;21:490-502.

[108] Dantzer R. Cytokine-induced sickness behavior: where do we stand? Brain Behav Immun 2001;15:7-24.

[109] Felger JC, Lotrich FE. Inflammatory cytokines in depression: neurobiological mechanisms and therapeutic implications. Neuroscience 2013;246:199-229.

[110] Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De CE et al. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci 2001;4:702-10.

[111] Hardingham GE, Bading H. Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. Biochim Biophys Acta 2002;1600:148-53.

[112] Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev 2006;86:1009-31.

[113] Hashimoto K, Sawa A, Iyo M. Increased levels of glutamate in brains from patients with mood disorders. Biol Psychiatry 2007;62:1310-6.

[114] Nutt DJ. Relationship of neurotransmitters to the symptoms of major depressive disorder. J Clin Psychiatry 2008;69 Suppl E1:4-7.

[115] Pariante CM, Lightman SL. The HPA axis in major depression: classical theories and new developments. Trends Neurosci 2008;31:464-8.

[116] Pace TW, Miller AH. Cytokines and glucocorticoid receptor signaling. Relevance to major depression. Ann N Y Acad Sci 2009;1179:86-105.

[117] Capuron L, Raison CL, Musselman DL, Lawson DH, Nemeroff CB, Miller AH. Association of exaggerated HPA axis response to the initial injection of interferon-alpha with development of depression during interferon-alpha therapy. Am J Psychiatry 2003;160:1342-5.

[118] Silverman MN, Sternberg EM. Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. Ann N Y Acad Sci 2012;1261:55-63.

[119] Videbech P, Ravnkilde B. Hippocampal volume and depression: a meta-analysis of MRI studies. Am J Psychiatry 2004;161:1957-66.

[120] Dwivedi Y, Rizavi HS, Conley RR, Roberts RC, Tamminga CA, Pandey GN. Altered gene expression of brain-derived neurotrophic factor and

receptor tyrosine kinase B in postmortem brain of suicide subjects. Arch Gen Psychiatry 2003;60:804-15.

[121] Sen S, Duman R, Sanacora G. Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications. Biol Psychiatry 2008;64:527-32.

[122] Barrientos RM, Sprunger DB, Campeau S, Higgins EA, Watkins LR, Rudy JW et al. Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist. Neuroscience 2003;121:847-53.

[123] Koo JW, Duman RS. IL-1beta is an essential mediator of the antineurogenic and anhedonic effects of stress. Proc Natl Acad Sci U S A 2008;105:751-6.

[124] Ben Menachem-Zidon O, Goshen I, Kreisel T, Ben MY, Reinhartz E, Ben HT et al. Intrahippocampal transplantation of transgenic neural precursor cells overexpressing interleukin-1 receptor antagonist blocks chronic isolation-induced impairment in memory and neurogenesis. Neuropsychopharmacology 2008;33:2251-62.

[125] Widner B, Ledochowski M, Fuchs D. Interferon-gamma-induced tryptophan degradation: neuropsychiatric and immunological consequences. Curr Drug Metab 2000;1:193-204.

[126] Oxenkrug GF. Tryptophan kynurenine metabolism as a common mediator of genetic and environmental impacts in major depressive disorder: the serotonin hypothesis revisited 40 years later. Isr J Psychiatry Relat Sci 2010;47:56-63.

[127] Fujigaki H, Saito K, Fujigaki S, Takemura M, Sudo K, Ishiguro H et al. The signal transducer and activator of transcription 1alpha and interferon regulatory factor 1 are not essential for the induction of indoleamine 2,3-dioxygenase by lipopolysaccharide: involvement of p38 mitogen-activated protein kinase and nuclear factor-kappaB pathways, and synergistic effect of several proinflammatory cytokines. J Biochem 2006;139:655-62.

[128] Musso T, Gusella GL, Brooks A, Longo DL, Varesio L. Interleukin-4 inhibits indoleamine 2,3-dioxygenase expression in human monocytes. Blood 1994;83:1408-11.

[129] Yuan W, Collado-Hidalgo A, Yufit T, Taylor M, Varga J. Modulation of cellular tryptophan metabolism in human fibroblasts by transforming growth factor-beta: selective inhibition of indoleamine 2,3-

dioxygenase and tryptophanyl-tRNA synthetase gene expression. J Cell Physiol 1998;177:174-86.

[130] Wichers MC, Maes M. The role of indoleamine 2,3-dioxygenase (IDO) in the pathophysiology of interferon-alpha-induced depression. J Psychiatry Neurosci 2004;29:11-7.

[131] Myint AM, Schwarz MJ, Muller N. The role of the kynurenine metabolism in major depression. J Neural Transm 2011.

[132] Wichers MC, Koek GH, Robaeys G, Verkerk R, Scharpe S, Maes M. IDO and interferon-alpha-induced depressive symptoms: a shift in hypothesis from tryptophan depletion to neurotoxicity. Mol Psychiatry 2005;10:538-44.

[133] Stone TW, Forrest CM, Mackay GM, Stoy N, Darlington LG. Tryptophan, adenosine, neurodegeneration and neuroprotection. Metab Brain Dis 2007;22:337-52.

[134] Schwarcz R. The kynurenine pathway of tryptophan degradation as a drug target. Curr Opin Pharmacol 2004;4:12-7.

[135] Myint AM. Kynurenines: from the perspective of major psychiatric disorders. FEBS J 2012.

[136] Maes M, Bonaccorso S, Marino V, Puzella A, Pasquini M, Biondi M et al. Treatment with interferon-alpha (IFN alpha) of hepatitis C patients induces lower serum dipeptidyl peptidase IV activity, which is related to IFN alpha-induced depressive and anxiety symptoms and immune activation. Mol Psychiatry 2001;6:475-80.

[137] Bonaccorso S, Marino V, Biondi M, Grimaldi F, Ippoliti F, Maes M. Depression induced by treatment with interferon-alpha in patients affected by hepatitis C virus. J Affect Disord 2002;72:237-41.

[138] Miller CL, Llenos IC, Dulay JR, Weis S. Upregulation of the initiating step of the kynurenine pathway in postmortem anterior cingulate cortex from individuals with schizophrenia and bipolar disorder. Brain Res 2006;1073-1074:25-37.

[139] Myint AM, Kim YK, Verkerk R, Park SH, Scharpe S, Steinbusch HW et al. Tryptophan breakdown pathway in bipolar mania. J Affect Disord 2007;102:65-72.

[140] Soczynska JK, Kennedy SH, Goldstein BI, Lachowski A, Woldeyohannes HO, McIntyre RS. The effect of tumor necrosis factor antagonists on mood and mental health-associated quality of life:

novel hypothesis-driven treatments for bipolar depression? Neurotoxicology 2009;30:497-521.

[141] Clark IA, Alleva LM, Vissel B. The roles of TNF in brain dysfunction and disease. Pharmacol Ther 2010;128:519-48.

[142] Rahman MM, McFadden G. Modulation of tumor necrosis factor by microbial pathogens. PLoS Pathog 2006;2:e4.

[143] Charles P, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C et al. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-alpha therapy in rheumatoid arthritis. J Immunol 1999;163:1521-8.

[144] Raison CL, Borisov AS, Woolwine BJ, Massung B, Vogt G, Miller AH. Interferon-alpha effects on diurnal hypothalamic-pituitary-adrenal axis activity: relationship with proinflammatory cytokines and behavior. Mol Psychiatry 2010;15:535-47.

[145] Liu Y, Ho RC, Mak A. Interleukin (IL)-6, tumour necrosis factor alpha (TNF-alpha) and soluble interleukin-2 receptors (sIL-2R) are elevated in patients with major depressive disorder: A meta-analysis and meta-regression. J Affect Disord 2011.

[146] Himmerich H, Fulda S, Linseisen J, Seiler H, Wolfram G, Himmerich S et al. Depression, comorbidities and the TNF-alpha system. Eur Psychiatry 2008;23:421-9.

[147] Grassi-Oliveira R, Brietzke E, Pezzi JC, Lopes RP, Teixeira AL, Bauer ME. Increased soluble tumor necrosis factor-alpha receptors in patients with major depressive disorder. Psychiatry Clin Neurosci 2009;63:202-8.

[148] Diniz BS, Teixeira AL, Talib LL, Mendonca VA, Gattaz WF, Forlenza OV. Increased soluble TNF receptor 2 in antidepressant-free patients with late-life depression. J Psychiatr Res 2010;44:917-20.

[149] Moorman AJ, Mozaffarian D, Wilkinson CW, Lawler RL, McDonald GB, Crane BA et al. In patients with heart failure elevated soluble TNF-receptor 1 is associated with higher risk of depression. J Card Fail 2007;13:738-43.

[150] Barbosa IG, Huguet RB, Mendonca VA, Sousa LP, Neves FS, Bauer ME et al. Increased plasma levels of soluble TNF receptor I in patients with bipolar disorder. Eur Arch Psychiatry Clin Neurosci 2011;261:139-43.

[151] Maes M. Depression is an inflammatory disease, but cell-mediated immune activation is the key component of depression. Prog Neuropsychopharmacol Biol Psychiatry 2010.

[152] Li Z, Qi D, Chen J, Zhang C, Yi Z, Yuan C et al. Venlafaxine inhibits the upregulation of plasma tumor necrosis factor-alpha (TNF-alpha) in the Chinese patients with major depressive disorder: a prospective longitudinal study. Psychoneuroendocrinology 2013;38:107-14.

[153] Janssen DG, Caniato RN, Verster JC, Baune BT. A psychoneuroimmunological review on cytokines involved in antidepressant treatment response. Hum Psychopharmacol 2010;25:201-15.

[154] O'Brien SM, Scully P, Fitzgerald P, Scott LV, Dinan TG. Plasma cytokine profiles in depressed patients who fail to respond to selective serotonin reuptake inhibitor therapy. J Psychiatr Res 2007;41:326-31.

[155] Tyring S, Gottlieb A, Papp K, Gordon K, Leonardi C, Wang A et al. Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. Lancet 2006;367:29-35.

[156] Persoons P, Vermeire S, Demyttenaere K, Fischler B, Vandenberghe J, Van OL et al. The impact of major depressive disorder on the short- and long-term outcome of Crohn's disease treatment with infliximab. Aliment Pharmacol Ther 2005;22:101-10.

[157] Uguz F, Akman C, Kucuksarac S, Tufekci O. Anti-tumor necrosis factor-alpha therapy is associated with less frequent mood and anxiety disorders in patients with rheumatoid arthritis. Psychiatry Clin Neurosci 2009;63:50-5.

[158] Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF et al. A Randomized Controlled Trial of the Tumor Necrosis Factor Antagonist Infliximab for Treatment-Resistant Depression: The Role of Baseline Inflammatory Biomarkers. Arch Gen Psychiatry 2012:1-11.

[159] Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. Trends Immunol 2007;28:138-45.

[160] Biesmans S, Meert TF, Bouwknecht JA, Acton PD, Davoodi N, De HP et al. Systemic immune activation leads to neuroinflammation and sickness behavior in mice. Mediators Inflamm 2013;2013:271359.

[161] Erickson MA, Banks WA. Cytokine and chemokine responses in serum and brain after single and repeated injections of lipopolysaccharide: Multiplex quantification with path analysis. Brain Behav Immun 2011.

[162] Kondo S, Kohsaka S, Okabe S. Long-term changes of spine dynamics and microglia after transient peripheral immune response triggered by LPS in vivo. Mol Brain 2011;4:27.

[163] Borges BC, Rorato R, ntunes-Rodrigues J, Elias LL. Glial cell activity is maintained during prolonged inflammatory challenge in rats. Braz J Med Biol Res 2012;45:784-91.

[164] Persoons JH, Berkenbosch F, Schornagel K, Thepen T, Kraal G. Increased specific IgE production in lungs after the induction of acute stress in rats. J Allergy Clin Immunol 1995;95:765-70.

[165] Dhabhar FS, McEwen BS. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav Immun 1997;11:286-306.

[166] Dhabhar FS. Acute stress enhances while chronic stress suppresses skin immunity. The role of stress hormones and leukocyte trafficking. Ann N Y Acad Sci 2000;917:876-93.

[167] Dhabhar FS. Stress-induced augmentation of immune function--the role of stress hormones, leukocyte trafficking, and cytokines. Brain Behav Immun 2002;16:785-98.

[168] Viswanathan K, Dhabhar FS. Stress-induced enhancement of leukocyte trafficking into sites of surgery or immune activation. Proc Natl Acad Sci U S A 2005;102:5808-13.

[169] Padgett DA, Marucha PT, Sheridan JF. Restraint stress slows cutaneous wound healing in mice. Brain Behav Immun 1998;12:64-73.

[170] Anisman H. Cascading effects of stressors and inflammatory immune system activation: implications for major depressive disorder. J Psychiatry Neurosci 2009;34:4-20.

[171] de Pablos RM, Villaran RF, Arguelles S, Herrera AJ, Venero JL, Ayala A et al. Stress increases vulnerability to inflammation in the rat prefrontal cortex. J Neurosci 2006;26:5709-19.

[172] Wohleb ES, Fenn AM, Pacenta AM, Powell ND, Sheridan JF, Godbout JP. Peripheral innate immune challenge exaggerated microglia activation, increased the number of inflammatory CNS macrophages,

and prolonged social withdrawal in socially defeated mice. Psychoneuroendocrinology 2012;37:1491-505.

[173] Weber MD, Frank MG, Sobesky JL, Watkins LR, Maier SF. Blocking toll-like receptor 2 and 4 signaling during a stressor prevents stress-induced priming of neuroinflammatory responses to a subsequent immune challenge. Brain Behav Immun 2013.

[174] Curtin NM, Mills KH, Connor TJ. Psychological stress increases expression of IL-10 and its homolog IL-19 via beta-adrenoceptor activation: reversal by the anxiolytic chlordiazepoxide. Brain Behav Immun 2009;23:371-9.

[175] Quan N, Avitsur R, Stark JL, He L, Shah M, Caligiuri M et al. Social stress increases the susceptibility to endotoxic shock. J Neuroimmunol 2001;115:36-45.

[176] Connor TJ, Brewer C, Kelly JP, Harkin A. Acute stress suppresses pro-inflammatory cytokines TNF-alpha and IL-1 beta independent of a catecholamine-driven increase in IL-10 production. J Neuroimmunol 2005;159:119-28.

[177] Goujon E, Parnet P, Laye S, Combe C, Kelley KW, Dantzer R. Stress downregulates lipopolysaccharide-induced expression of proinflammatory cytokines in the spleen, pituitary, and brain of mice. Brain Behav Immun 1995;9:292-303.

[178] Meltzer JC, MacNeil BJ, Sanders V, Pylypas S, Jansen AH, Greenberg AH et al. Stress-induced suppression of in vivo splenic cytokine production in the rat by neural and hormonal mechanisms. Brain Behav Immun 2004;18:262-73.

[179] Audet MC, Jacobson-Pick S, Wann BP, Anisman H. Social defeat promotes specific cytokine variations within the prefrontal cortex upon subsequent aggressive or endotoxin challenges. Brain Behav Immun 2011;25:1197-205.

[180] Barnum CJ, Pace TW, Hu F, Neigh GN, Tansey MG. Psychological stress in adolescent and adult mice increases neuroinflammation and attenuates the response to LPS challenge. J Neuroinflammation 2012;9:9.

[181] Zhu L, Ramboz S, Hewitt D, Boring L, Grass DS, Purchio AF. Non-invasive imaging of GFAP expression after neuronal damage in mice. Neurosci Lett 2004;367:210-2.

[182] Ridet JL, Malhotra SK, Privat A, Gage FH. Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 1997;20:570-7.

[183] Briard E, Zoghbi SS, Simeon FG, Imaizumi M, Gourley JP, Shetty HU et al. Single-step high-yield radiosynthesis and evaluation of a sensitive 18F-labeled ligand for imaging brain peripheral benzodiazepine receptors with PET. J Med Chem 2009;52:688-99.

[184] Vasey MW, Thayer JF. The continuing problem of false positives in repeated measures ANOVA in psychophysiology: a multivariate solution. Psychophysiology 1987;24:479-86.

[185] Painsipp E, Kofer MJ, Sinner F, Holzer P. Prolonged depression-like behavior caused by immune challenge: influence of mouse strain and social environment. PLoS One 2011;6:e20719.

[186] Puentener U, Booth SG, Perry VH, Teeling JL. Long-term impact of systemic bacterial infection on the cerebral vasculature and microglia. J Neuroinflammation 2012;9:146.

[187] Gibney SM, McGuinness B, Prendergast C, Harkin A, Connor TJ. Poly I:C-induced activation of the immune response is accompanied by depression and anxiety-like behaviours, kynurenine pathway activation and reduced BDNF expression. Brain Behav Immun 2013;28:170-81.

[188] Dong Y, Benveniste EN. Immune function of astrocytes. Glia 2001;36:180-90.

[189] Olsen AL, Morrey JD, Smee DF, Sidwell RW. Correlation between breakdown of the blood-brain barrier and disease outcome of viral encephalitis in mice. Antiviral Res 2007;75:104-12.

[190] Casellas P, Galiegue S, Basile AS. Peripheral benzodiazepine receptors and mitochondrial function. Neurochem Int 2002;40:475-86.

[191] Ching AS, Kuhnast B, Damont A, Roeda D, Tavitian B, Dolle F. Current paradigm of the 18-kDa translocator protein (TSPO) as a molecular target for PET imaging in neuroinflammation and neurodegenerative diseases. Insights Imaging 2012;3:111-9.

[192] Imaizumi M, Briard E, Zoghbi SS, Gourley JP, Hong J, Fujimura Y et al. Brain and whole-body imaging in nonhuman primates of [11C]PBR28, a promising PET radioligand for peripheral benzodiazepine receptors. Neuroimage 2008;39:1289-98.

[193] Hannestad J, Gallezot JD, Schafbauer T, Lim K, Kloczynski T, Morris ED et al. Endotoxin-induced systemic inflammation activates microglia: [(11)C]PBR28 positron emission tomography in nonhuman primates. Neuroimage 2012.

[194] Venneti S, Wang G, Nguyen J, Wiley CA. The positron emission tomography ligand DAA1106 binds with high affinity to activated microglia in human neurological disorders. J Neuropathol Exp Neurol 2008;67:1001-10.

[195] Jones HA, Valind SO, Clark IC, Bolden GE, Krausz T, Schofield JB et al. Kinetics of lung macrophages monitored in vivo following particulate challenge in rabbits. Toxicol Appl Pharmacol 2002;183:46-54.

[196] Hatori A, Yui J, Yamasaki T, Xie L, Kumata K, Fujinaga M et al. PET imaging of lung inflammation with [18F]FEDAC, a radioligand for translocator protein (18 kDa). PLoS One 2012;7:e45065.

[197] Jones HA, Marino PS, Shakur BH, Morrell NW. In vivo assessment of lung inflammatory cell activity in patients with COPD and asthma. Eur Respir J 2003;21:567-73.

[198] Corcia P, Tauber C, Vercoullie J, Arlicot N, Prunier C, Praline J et al. Molecular imaging of microglial activation in amyotrophic lateral sclerosis. PLoS One 2012;7:e52941.

[199] Yasuno F, Kosaka J, Ota M, Higuchi M, Ito H, Fujimura Y et al. Increased binding of peripheral benzodiazepine receptor in mild cognitive impairment-dementia converters measured by positron emission tomography with [(1)(1)C]DAA1106. Psychiatry Res 2012;203:67-74.

[200] Gulyas B, Toth M, Schain M, Airaksinen A, Vas A, Kostulas K et al. Evolution of microglial activation in ischaemic core and peri-infarct regions after stroke: a PET study with the TSPO molecular imaging biomarker [((11))C]vinpocetine. J Neurol Sci 2012;320:110-7.

[201] Hardwick MJ, Chen MK, Baidoo K, Pomper MG, Guilarte TR. In vivo imaging of peripheral benzodiazepine receptors in mouse lungs: a biomarker of inflammation. Mol Imaging 2005;4:432-8.

[202] den Haan JM, Kraal G. Innate immune functions of macrophage subpopulations in the spleen. J Innate Immun 2012;4:437-45.

[203] Kaissling B, Hegyi I, Loffing J, Le HM. Morphology of interstitial cells in the healthy kidney. Anat Embryol (Berl) 1996;193:303-18.

[204] Chen MK, Guilarte TR. Translocator protein 18 kDa (TSPO): molecular sensor of brain injury and repair. Pharmacol Ther 2008;118:1-17.

[205] Foster SL, Medzhitov R. Gene-specific control of the TLR-induced inflammatory response. Clin Immunol 2009;130:7-15.

[206] Beurel E, Jope RS. Glycogen synthase kinase-3 regulates inflammatory tolerance in astrocytes. Neuroscience 2010;169:1063-70.

[207] Zhang Y, Woodruff M, Zhang Y, Miao J, Hanley G, Stuart C et al. Toll-like receptor 4 mediates chronic restraint stress-induced immune suppression. J Neuroimmunol 2008;194:115-22.

[208] Zhang Y, Foster R, Sun X, Yin Q, Li Y, Hanley G et al. Restraint stress induces lymphocyte reduction through p53 and PI3K/NF-kappaB pathways. J Neuroimmunol 2008;200:71-6.

[209] raya-Callis C, Hiemke C, Abumaria N, Flugge G. Chronic psychosocial stress and citalopram modulate the expression of the glial proteins GFAP and NDRG2 in the hippocampus. Psychopharmacology (Berl) 2012;224:209-22.

[210] Tynan RJ, Beynon SB, Hinwood M, Johnson SJ, Nilsson M, Woods JJ et al. Chronic stress-induced disruption of the astrocyte network is driven by structural atrophy and not loss of astrocytes. Acta Neuropathol 2013.

[211] Imbe H, Kimura A, Donishi T, Kaneoke Y. Chronic restraint stress decreases glial fibrillary acidic protein and glutamate transporter in the periaqueductal gray matter. Neuroscience 2012;223:209-18.

[212] Imbe H, Kimura A, Donishi T, Kaneoke Y. Effects of restraint stress on glial activity in the rostral ventromedial medulla. Neuroscience 2013;241:10-21.

[213] Glaser R, Kiecolt-Glaser JK. Stress-induced immune dysfunction: implications for health. Nat Rev Immunol 2005;5:243-51.

[214] Godbout JP, Glaser R. Stress-induced immune dysregulation: implications for wound healing, infectious disease and cancer. J Neuroimmune Pharmacol 2006;1:421-7.

[215] Shelton RC. The molecular neurobiology of depression. Psychiatr Clin North Am 2007;30:1-11.

[216] Schiepers OJ, Wichers MC, Maes M. Cytokines and major depression. Prog Neuropsychopharmacol Biol Psychiatry 2005;29:201-17.

[217] Katz PP, Yelin EH. Prevalence and correlates of depressive symptoms among persons with rheumatoid arthritis. J Rheumatol 1993;20:790-6.

[218] Zellweger MJ, Osterwalder RH, Langewitz W, Pfisterer ME. Coronary artery disease and depression. Eur Heart J 2004;25:3-9.

[219] Capuron L, Ravaud A, Dantzer R. Early depressive symptoms in cancer patients receiving interleukin 2 and/or interferon alfa-2b therapy. J Clin Oncol 2000;18:2143-51.

[220] Bonaccorso S, Puzella A, Marino V, Pasquini M, Biondi M, Artini M et al. Immunotherapy with interferon-alpha in patients affected by chronic hepatitis C induces an intercorrelated stimulation of the cytokine network and an increase in depressive and anxiety symptoms. Psychiatry Res 2001;105:45-55.

[221] Krishnadas R, Cavanagh J. Depression: an inflammatory illness? J Neurol Neurosurg Psychiatry 2012.

[222] Kadurugamuwa JL, Modi K, Coquoz O, Rice B, Smith S, Contag PR et al. Reduction of astrogliosis by early treatment of pneumococcal meningitis measured by simultaneous imaging, in vivo, of the pathogen and host response. Infect Immun 2005;73:7836-43.

[223] Lee J, Borboa AK, Baird A, Eliceiri BP. Non-invasive quantification of brain tumor-induced astrogliosis. BMC Neurosci 2011;12:9.

[224] Tamguney G, Francis KP, Giles K, Lemus A, DeArmond SJ, Prusiner SB. Measuring prions by bioluminescence imaging. Proc Natl Acad Sci U S A 2009;106:15002-6.

[225] Cordeau P, Jr., Lalancette-Hebert M, Weng YC, Kriz J. Live imaging of neuroinflammation reveals sex and estrogen effects on astrocyte response to ischemic injury. Stroke 2008;39:935-42.

[226] Luo J, Ho P, Steinman L, Wyss-Coray T. Bioluminescence in vivo imaging of autoimmune encephalomyelitis predicts disease. J Neuroinflammation 2008;5:6.

[227] Van der Heyden JA, Zethof TJ, Olivier B. Stress-induced hyperthermia in singly housed mice. Physiol Behav 1997;62:463-70.

[228] Che Y, Wang JF, Shao L, Young T. Oxidative damage to RNA but not DNA in the hippocampus of patients with major mental illness. J Psychiatry Neurosci 2010;35:296-302.

[229] Hanson ND, Owens MJ, Nemeroff CB. Depression, antidepressants, and neurogenesis: a critical reappraisal. Neuropsychopharmacology 2011;36:2589-602.

[230] Surget A, Tanti A, Leonardo ED, Laugeray A, Rainer Q, Touma C et al. Antidepressants recruit new neurons to improve stress response regulation. Mol Psychiatry 2011;16:1177-88.

[231] Chen J, Buchanan JB, Sparkman NL, Godbout JP, Freund GG, Johnson RW. Neuroinflammation and disruption in working memory in aged mice after acute stimulation of the peripheral innate immune system. Brain Behav Immun 2008;22:301-11.

[232] Buchanan JB, Sparkman NL, Johnson RW. Methamphetamine sensitization attenuates the febrile and neuroinflammatory response to a subsequent peripheral immune stimulus. Brain Behav Immun 2010;24:502-11.

[233] de Heredia LL, Gengatharan A, Foster J, Mather S, Magoulas C. Bioluminescence imaging of the brain response to acute inflammation in living C/EBP reporter mice. Neurosci Lett 2011.

[234] Alm AS, Li K, Chen H, Wang D, Andersson R, Wang X. Variation of lipopolysaccharide-induced acute lung injury in eight strains of mice. Respir Physiol Neurobiol 2010;171:157-64.

[235] Browne CA, O'Brien FE, Connor TJ, Dinan TG, Cryan JF. Differential lipopolysaccharide-induced immune alterations in the hippocampus of two mouse strains: Effects of stress. Neuroscience 2012;225:237-48.

[236] Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. Microglia-specific localisation of a novel calcium binding protein, Iba1. Brain Res Mol Brain Res 1998;57:1-9.

[237] Ito D, Tanaka K, Suzuki S, Dembo T, Fukuuchi Y. Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke 2001;32:1208-15.

[238] Sandhir R, Onyszchuk G, Berman NE. Exacerbated glial response in the aged mouse hippocampus following controlled cortical impact injury. Exp Neurol 2008;213:372-80.

[239] Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 2007;55:453-62.

[240] Andre C, O'Connor JC, Kelley KW, Lestage J, Dantzer R, Castanon N. Spatio-temporal differences in the profile of murine brain expression of proinflammatory cytokines and indoleamine 2,3-dioxygenase in response to peripheral lipopolysaccharide administration. J Neuroimmunol 2008;200:90-9.

[241] Reyes TM, Fabry Z, Coe CL. Brain endothelial cell production of a neuroprotective cytokine, interleukin-6, in response to noxious stimuli. Brain Res 1999;851:215-20.

[242] Jaeger LB, Dohgu S, Sultana R, Lynch JL, Owen JB, Erickson MA et al. Lipopolysaccharide alters the blood-brain barrier transport of amyloid beta protein: a mechanism for inflammation in the progression of Alzheimer's disease. Brain Behav Immun 2009;23:507-17.

[243] Verma S, Nakaoke R, Dohgu S, Banks WA. Release of cytokines by brain endothelial cells: A polarized response to lipopolysaccharide. Brain Behav Immun 2006;20:449-55.

[244] Banks WA, Ortiz L, Plotkin SR, Kastin AJ. Human interleukin (IL) 1 alpha, murine IL-1 alpha and murine IL-1 beta are transported from blood to brain in the mouse by a shared saturable mechanism. J Pharmacol Exp Ther 1991;259:988-96.

[245] Bay-Richter C, Janelidze S, Hallberg L, Brundin L. Changes in behaviour and cytokine expression upon a peripheral immune challenge. Behav Brain Res 2011;222:193-9.

[246] Quan N, Whiteside M, Herkenham M. Time course and localization patterns of interleukin-1beta messenger RNA expression in brain and pituitary after peripheral administration of lipopolysaccharide. Neuroscience 1998;83:281-93.

[247] Castanon N, Medina C, Mormede C, Dantzer R. Chronic administration of tianeptine balances lipopolysaccharide-induced expression of cytokines in the spleen and hypothalamus of rats. Psychoneuroendocrinology 2004;29:778-90.

[248] Salazar A, Gonzalez-Rivera BL, Redus L, Parrott JM, O'Connor JC. Indoleamine 2,3-dioxygenase mediates anhedonia and anxiety-like behaviors caused by peripheral lipopolysaccharide immune challenge. Horm Behav 2012.

[249] Deak T, Bellamy C, D'Agostino LG, Rosanoff M, McElderry NK, Bordner KA. Behavioral responses during the forced swim test are not affected by anti-inflammatory agents or acute illness induced by lipopolysaccharide. Behav Brain Res 2005;160:125-34.

[250] Kubera M, Curzytek K, Duda W, Leskiewicz M, Basta-Kaim A, Budziszewska B et al. A new animal model of (chronic) depression induced by repeated and intermittent lipopolysaccharide administration for 4months. Brain Behav Immun 2013.

[251] Berton O, Nestler EJ. New approaches to antidepressant drug discovery: beyond monoamines. Nat Rev Neurosci 2006;7:137-51.

[252] Krishnan V, Nestler EJ. The molecular neurobiology of depression. Nature 2008;455:894-902.

[253] Der-Avakian A, Markou A. The neurobiology of anhedonia and other reward-related deficits. Trends Neurosci 2012;35:68-77.

[254] Goble KH, Bain ZA, Padow VA, Lui P, Klein ZA, Romeo RD. Pubertal-related changes in hypothalamic-pituitary-adrenal axis reactivity and cytokine secretion in response to an immunological stressor. J Neuroendocrinol 2011;23:129-35.

[255] Friedman JM. Leptin, leptin receptors, and the control of body weight. Nutr Rev 1998;56:s38-s46.

[256] Rummel C, Inoue W, Poole S, Luheshi GN. Leptin regulates leukocyte recruitment into the brain following systemic LPS-induced inflammation. Mol Psychiatry 2010;15:523-34.

[257] Sarraf P, Frederich RC, Turner EM, Ma G, Jaskowiak NT, Rivet DJ, III et al. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. J Exp Med 1997;185:171-5.

[258] Faggioni R, Fantuzzi G, Fuller J, Dinarello CA, Feingold KR, Grunfeld C. IL-1 beta mediates leptin induction during inflammation. Am J Physiol 1998;274:R204-R208.

[259] Silva SM, Madeira MD. Effects of chronic alcohol consumption and withdrawal on the response of the male and female hypothalamic-pituitary-adrenal axis to acute immune stress. Brain Res 2012;1444:27-37.

[260] Shen Y, Connor TJ, Nolan Y, Kelly JP, Leonard BE. Differential effect of chronic antidepressant treatments on lipopolysaccharide-induced depressive-like behavioural symptoms in the rat. Life Sci 1999;65:1773-86.

[261] Spijker J, De GR, Bijl RV, Beekman AT, Ormel J, Nolen WA. Duration of major depressive episodes in the general population: results from The

Netherlands Mental Health Survey and Incidence Study (NEMESIS). Br J Psychiatry 2002;181:208-13.

[262] Broad A, Jones DE, Kirby JA. Toll-like receptor (TLR) response tolerance: a key physiological "damage limitation" effect and an important potential opportunity for therapy. Curr Med Chem 2006;13:2487-502.

[263] Karin M, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. Cell 2006;124:823-35.

[264] Kendler KS, Thornton LM, Gardner CO. Genetic risk, number of previous depressive episodes, and stressful life events in predicting onset of major depression. Am J Psychiatry 2001;158:582-6.

[265] Hayase T. Depression-related anhedonic behaviors caused by immobilization stress: a comparison with nicotine-induced depression-like behavioral alterations and effects of nicotine and/or "antidepressant" drugs. J Toxicol Sci 2011;36:31-41.

[266] Eiland L, Ramroop J, Hill MN, Manley J, McEwen BS. Chronic juvenile stress produces corticolimbic dendritic architectural remodeling and modulates emotional behavior in male and female rats. Psychoneuroendocrinology 2012;37:39-47.

[267] Naert G, Ixart G, Maurice T, Tapia-Arancibia L, Givalois L. Brain-derived neurotrophic factor and hypothalamic-pituitary-adrenal axis adaptation processes in a depressive-like state induced by chronic restraint stress. Mol Cell Neurosci 2011;46:55-66.

[268] Haenisch B, Bilkei-Gorzo A, Caron MG, Bonisch H. Knockout of the norepinephrine transporter and pharmacologically diverse antidepressants prevent behavioral and brain neurotrophin alterations in two chronic stress models of depression. J Neurochem 2009;111:403-16.

[269] Chiba S, Numakawa T, Ninomiya M, Richards MC, Wakabayashi C, Kunugi H. Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. Prog Neuropsychopharmacol Biol Psychiatry 2012;39:112-9.

[270] Gibb J, Hayley S, Gandhi R, Poulter MO, Anisman H. Synergistic and additive actions of a psychosocial stressor and endotoxin challenge:

Circulating and brain cytokines, plasma corticosterone and behavioral changes in mice. Brain Behav Immun 2008;22:573-89.

[271] Gibb J, Hayley S, Poulter MO, Anisman H. Effects of stressors and immune activating agents on peripheral and central cytokines in mouse strains that differ in stressor responsivity. Brain Behav Immun 2011;25:468-82.

[272] Lepine JP, Briley M. The increasing burden of depression. Neuropsychiatr Dis Treat 2011;7:3-7.

[273] Bluthe RM, Pawlowski M, Suarez S, Parnet P, Pittman Q, Kelley KW et al. Synergy between tumor necrosis factor alpha and interleukin-1 in the induction of sickness behavior in mice. Psychoneuroendocrinology 1994;19:197-207.

[274] Kaster MP, Gadotti VM, Calixto JB, Santos AR, Rodrigues AL. Depressive-like behavior induced by tumor necrosis factor-alpha in mice. Neuropharmacology 2012;62:419-26.

[275] Manosso LM, Neis VB, Moretti M, Daufenbach JF, Freitas AE, Colla AR et al. Antidepressant-like effect of alpha-tocopherol in a mouse model of depressive-like behavior induced by TNF-alpha. Prog Neuropsychopharmacol Biol Psychiatry 2013.

[276] Liu H, Luiten PG, Eisel UL, Dejongste MJ, Schoemaker RG. Depression after myocardial infarction: TNF-alpha-induced alterations of the blood-brain barrier and its putative therapeutic implications. Neurosci Biobehav Rev 2013.

[277] Gautron L, Lafon P, Chaigniau M, Tramu G, Laye S. Spatiotemporal analysis of signal transducer and activator of transcription 3 activation in rat brain astrocytes and pituitary following peripheral immune challenge. Neuroscience 2002;112:717-29.

[278] Bossu P, Cutuli D, Palladino I, Caporali P, Angelucci F, Laricchiuta D et al. A single intraperitoneal injection of endotoxin in rats induces long-lasting modifications in behavior and brain protein levels of TNF-alpha and IL-18. J Neuroinflammation 2012;9:101.

[279] Diz-Chaves Y, Astiz M, Bellini MJ, Garcia-Segura LM. Prenatal stress increases the expression of proinflammatory cytokines and exacerbates the inflammatory response to LPS in the hippocampal formation of adult male mice. Brain Behav Immun 2013;28:196-206.

[280] Novotny GW, Lundh M, Backe MB, Christensen DP, Hansen JB, Dahllof MS et al. Transcriptional and translational regulation of cytokine

signaling in inflammatory beta-cell dysfunction and apoptosis. Arch Biochem Biophys 2012;528:171-84.

[281] Hamilton T, Li X, Novotny M, Pavicic PG, Jr., Datta S, Zhao C et al. Cell type- and stimulus-specific mechanisms for post-transcriptional control of neutrophil chemokine gene expression. J Leukoc Biol 2012;91:377-83.

[282] Munford RS. Murine responses to endotoxin: another dirty little secret? J Infect Dis 2010;201:175-7.

[283] Eisenberger NI, Berkman ET, Inagaki TK, Rameson LT, Mashal NM, Irwin MR. Inflammation-induced anhedonia: endotoxin reduces ventral striatum responses to reward. Biol Psychiatry 2010;68:748-54.

[284] Eisenberger NI, Inagaki TK, Mashal NM, Irwin MR. Inflammation and social experience: an inflammatory challenge induces feelings of social disconnection in addition to depressed mood. Brain Behav Immun 2010;24:558-63.

[285] Zhou X, Fragala MS, McElhaney JE, Kuchel GA. Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. Curr Opin Clin Nutr Metab Care 2010;13:541-7.

[286] Grigoleit JS, Oberbeck JR, Lichte P, Kobbe P, Wolf OT, Montag T et al. Lipopolysaccharide-induced experimental immune activation does not impair memory functions in humans. Neurobiol Learn Mem 2010;94:561-7.

[287] Grigoleit JS, Kullmann JS, Wolf OT, Hammes F, Wegner A, Jablonowski S et al. Dose-dependent effects of endotoxin on neurobehavioral functions in humans. PLoS One 2011;6:e28330.

[288] Henry CJ, Huang Y, Wynne AM, Godbout JP. Peripheral lipopolysaccharide (LPS) challenge promotes microglial hyperactivity in aged mice that is associated with exaggerated induction of both pro-inflammatory IL-1beta and anti-inflammatory IL-10 cytokines. Brain Behav Immun 2009;23:309-17.

[289] Saijo K, Winner B, Carson CT, Collier JG, Boyer L, Rosenfeld MG et al. A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. Cell 2009;137:47-59.

[290] Rajkowska G, Miguel-Hidalgo JJ. Gliogenesis and glial pathology in depression. CNS Neurol Disord Drug Targets 2007;6:219-33.

[291] Niciu MJ, Henter ID, Sanacora G, Zarate CA, Jr. Glial abnormalities in substance use disorders and depression: Does shared glutamatergic dysfunction contribute to comorbidity? World J Biol Psychiatry 2014;15:2-16.

[292] Liberto CM, Albrecht PJ, Herx LM, Yong VW, Levison SW. Pro-regenerative properties of cytokine-activated astrocytes. J Neurochem 2004;89:1092-100.

[293] Jones KA, Thomsen C. The role of the innate immune system in psychiatric disorders. Mol Cell Neurosci 2013;53:52-62.

[294] Goshen I, Yirmiya R. Interleukin-1 (IL-1): a central regulator of stress responses. Front Neuroendocrinol 2009;30:30-45.

[295] Hayley S, Scharf J, Anisman H. Central administration of murine interferon-alpha induces depressive-like behavioral, brain cytokine and neurochemical alterations in mice: a mini-review and original experiments. Brain Behav Immun 2013;31:115-27.

[296] Castanon N, Bluthe RM, Dantzer R. Chronic treatment with the atypical antidepressant tianeptine attenuates sickness behavior induced by peripheral but not central lipopolysaccharide and interleukin-1beta in the rat. Psychopharmacology (Berl) 2001;154:50-60.

[297] Lawson MA, Parrott JM, McCusker RH, Dantzer R, Kelley KW, JC OC. Intracerebroventricular administration of lipopolysaccharide induces indoleamine-2,3-dioxygenase-dependent depression-like behaviors. J Neuroinflammation 2013;10:87.

[298] Bennabi D, Vandel P, Papaxanthis C, Pozzo T, Haffen E. Psychomotor retardation in depression: a systematic review of diagnostic, pathophysiologic, and therapeutic implications. Biomed Res Int 2013;2013:158746.

[299] Carlezon WA, Jr., Chartoff EH. Intracranial self-stimulation (ICSS) in rodents to study the neurobiology of motivation. Nat Protoc 2007;2:2987-95.

[300] Malkesman O, Scattoni ML, Paredes D, Tragon T, Pearson B, Shaltiel G et al. The female urine sniffing test: a novel approach for assessing reward-seeking behavior in rodents. Biol Psychiatry 2010;67:864-71.

[301] Brydon L, Harrison NA, Walker C, Steptoe A, Critchley HD. Peripheral inflammation is associated with altered substantia nigra activity and psychomotor slowing in humans. Biol Psychiatry 2008;63:1022-9.

[302] Capuron L, Pagnoni G, Demetrashvili MF, Lawson DH, Fornwalt FB, Woolwine B et al. Basal ganglia hypermetabolism and symptoms of fatigue during interferon-alpha therapy. Neuropsychopharmacology 2007;32:2384-92.

[303] Capuron L, Pagnoni G, Demetrashvili M, Woolwine BJ, Nemeroff CB, Berns GS et al. Anterior cingulate activation and error processing during interferon-alpha treatment. Biol Psychiatry 2005;58:190-6.

[304] Gunther J, Vogt N, Hampel K, Bikker R, Page S, Muller B et al. Identification of Two Forms of TNF Tolerance in Human Monocytes: Differential Inhibition of NF-kappaB/AP-1- and PP1-Associated Signaling. J Immunol 2014.

[305] Esposito E, Cuzzocrea S. TNF-alpha as a therapeutic target in inflammatory diseases, ischemia-reperfusion injury and trauma. Curr Med Chem 2009;16:3152-67.

[306] Lopresti AL, Maker GL, Hood SD, Drummond PD. A review of peripheral biomarkers in major depression: The potential of inflammatory and oxidative stress biomarkers. Prog Neuropsychopharmacol Biol Psychiatry 2014;48:102-11.

[307] Liukkonen T, Silvennoinen-Kassinen S, Jokelainen J, Rasanen P, Leinonen M, Meyer-Rochow VB et al. The association between C-reactive protein levels and depression: Results from the northern Finland 1966 birth cohort study. Biol Psychiatry 2006;60:825-30.

[308] Pasco JA, Nicholson GC, Williams LJ, Jacka FN, Henry MJ, Kotowicz MA et al. Association of high-sensitivity C-reactive protein with de novo major depression. Br J Psychiatry 2010;197:372-7.

[309] Wium-Andersen MK, Orsted DD, Nielsen SF, Nordestgaard BG. Elevated C-reactive protein levels, psychological distress, and depression in 73, 131 individuals. JAMA Psychiatry 2013;70:176-84.

[310] Lanquillon S, Krieg JC, ing-Abu-Shach U, Vedder H. Cytokine production and treatment response in major depressive disorder. Neuropsychopharmacology 2000;22:370-9.

[311] Eller T, Vasar V, Shlik J, Maron E. Pro-inflammatory cytokines and treatment response to escitalopram in major depressive disorder. Prog Neuropsychopharmacol Biol Psychiatry 2008;32:445-50.

[312] Lotrich FE, El-Gabalawy H, Guenther LC, Ware CF. The role of inflammation in the pathophysiology of depression: different treatments and their effects. J Rheumatol Suppl 2011;88:48-54.

[313] Simen BB, Duman CH, Simen AA, Duman RS. TNFalpha signaling in depression and anxiety: behavioral consequences of individual receptor targeting. Biol Psychiatry 2006;59:775-85.

[314] Monje FJ, Cabatic M, Divisch I, Kim EJ, Herkner KR, Binder BR et al. Constant Darkness Induces IL-6-Dependent Depression-Like Behavior through the NF-{kappa}B Signaling Pathway. J Neurosci 2011;31:9075-83.

[315] Chourbaji S, Urani A, Inta I, Sanchis-Segura C, Brandwein C, Zink M et al. IL-6 knockout mice exhibit resistance to stress-induced development of depression-like behaviors. Neurobiol Dis 2006;23:587-94.

[316] Mesquita AR, Correia-Neves M, Roque S, Castro AG, Vieira P, Pedrosa J et al. IL-10 modulates depressive-like behavior. J Psychiatr Res 2008;43:89-97.

[317] Miyaoka T, Wake R, Furuya M, Liaury K, Ieda M, Kawakami K et al. Minocycline as adjunctive therapy for patients with unipolar psychotic depression: an open-label study. Prog Neuropsychopharmacol Biol Psychiatry 2012;37:222-6.

[318] Muller N, Schwarz MJ, Dehning S, Douhe A, Cerovecki A, Goldstein-Muller B et al. 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 2006;11:680-4.

[319] Akhondzadeh S, Jafari S, Raisi F, Nasehi AA, Ghoreishi A, Salehi B et al. Clinical trial of adjunctive celecoxib treatment in patients with major depression: a double blind and placebo controlled trial. Depress Anxiety 2009;26:607-11.

[320] Mendlewicz J, Kriwin P, Oswald P, Souery D, Alboni S, Brunello N. Shortened onset of action of antidepressants in major depression using acetylsalicylic acid augmentation: a pilot open-label study. Int Clin Psychopharmacol 2006;21:227-31.

[321] Martins JG. EPA but not DHA appears to be responsible for the efficacy of omega-3 long chain polyunsaturated fatty acid supplementation in depression: evidence from a meta-analysis of randomized controlled trials. J Am Coll Nutr 2009;28:525-42.

[322] Appleton KM, Rogers PJ, Ness AR. Updated systematic review and meta-analysis of the effects of n-3 long-chain polyunsaturated fatty acids on depressed mood. Am J Clin Nutr 2010;91:757-70.

[323] Cavaillon JM, Adrie C, Fitting C, dib-Conquy M. Endotoxin tolerance: is there a clinical relevance? J Endotoxin Res 2003;9:101-7.

[324] O'Connor JC, Lawson MA, Andre C, Briley EM, Szegedi SS, Lestage J et al. Induction of IDO by bacille Calmette-Guerin is responsible for development of murine depressive-like behavior. J Immunol 2009;182:3202-12.

[325] Meszaros ZS, Perl A, Faraone SV. Psychiatric symptoms in systemic lupus erythematosus: a systematic review. J Clin Psychiatry 2012;73:993-1001.

[326] Vattakatuchery JJ, Rickards H, Cavanna AE. Pathogenic mechanisms of depression in multiple sclerosis. J Neuropsychiatry Clin Neurosci 2011;23:261-76.

[327] Matcham F, Rayner L, Steer S, Hotopf M. The prevalence of depression in rheumatoid arthritis: a systematic review and meta-analysis. Rheumatology (Oxford) 2013;52:2136-48.

[328] Haji N, Mandolesi G, Gentile A, Sacchetti L, Fresegna D, Rossi S et al. TNF-alpha-mediated anxiety in a mouse model of multiple sclerosis. Exp Neurol 2012;237:296-303.

[329] Musgrave T, Benson C, Wong G, Browne I, Tenorio G, Rauw G et al. The MAO inhibitor phenelzine improves functional outcomes in mice with experimental autoimmune encephalomyelitis (EAE). Brain Behav Immun 2011;25:1677-88.

[330] Gao HX, Sanders E, Tieng AT, Putterman C. Sex and autoantibody titers determine the development of neuropsychiatric manifestations in lupus-prone mice. J Neuroimmunol 2010;229:112-22.

[331] Gao HX, Campbell SR, Cui MH, Zong P, Hee-Hwang J, Gulinello M et al. Depression is an early disease manifestation in lupus-prone MRL/lpr mice. J Neuroimmunol 2009;207:45-56.

[332] Mousa A, Bakhiet M. Role of cytokine signaling during nervous system development. Int J Mol Sci 2013;14:13931-57.

[333] Boksa P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. Brain Behav Immun 2010;24:881-97.

[334] Ratnayake U, Quinn T, Walker DW, Dickinson H. Cytokines and the neurodevelopmental basis of mental illness. Front Neurosci 2013;7:180.

[335] Michaud M, Balardy L, Moulis G, Gaudin C, Peyrot C, Vellas B et al. Proinflammatory cytokines, aging, and age-related diseases. J Am Med Dir Assoc 2013;14:877-82.

[336] Norden DM, Godbout JP. Microglia of the Aged Brain: Primed to be Activated and Resistant to Regulation. Neuropathol Appl Neurobiol 2012.

[337] Sibille E. Molecular aging of the brain, neuroplasticity, and vulnerability to depression and other brain-related disorders. Dialogues Clin Neurosci 2013;15:53-65.

[338] Quintana A, Erta M, Ferrer B, Comes G, Giralt M, Hidalgo J. Astrocyte-specific deficiency of interleukin-6 and its receptor reveal specific roles in survival, body weight and behavior. Brain Behav Immun 2013;27:162-73.

[339] Butterweck V, Prinz S, Schwaninger M. The role of interleukin-6 in stress-induced hyperthermia and emotional behaviour in mice. Behav Brain Res 2003;144:49-56.

[340] Juhasz C, Muzik O, Chugani DC, Chugani HT, Sood S, Chakraborty PK et al. Differential kinetics of alpha-[(1)(1)C]methyl-L-tryptophan on PET in low-grade brain tumors. J Neurooncol 2011;102:409-15.

[341] Juhasz C, Chugani DC, Barger GR, Kupsky WJ, Chakraborty PK, Muzik O et al. Quantitative PET imaging of tryptophan accumulation in gliomas and remote cortex: correlation with tumor proliferative activity. Clin Nucl Med 2012;37:838-42.

Curriculum Vitae

Steven Biesmans was born in Tongeren on the 22nd of August 1986. In 2004 he graduated from secondary school, earning a Mathematics-Economics-Sciences degree at the Heilig Hartcollege in Lanaken. Next, he began a Biomedical Sciences bachelor's degree program at Hasselt University/transnational University Limburg (tUL) and graduated in 2007. Subsequently, he followed a Clinical Molecular Life Sciences program at the tUL, which he completed in 2009 *magna cum laude*. His graduation project focused on neurodegeneration in cellular models of Parkinson's disease and was performed at the Neuroscience Department at Janssen Research & Development.

After obtaining a Master of Science degree, Steven worked for 6 months at RWTH Aachen University studying disease mechanisms of amyotrophic lateral sclerosis. In 2010 he started a PhD project on rodent models of inflammation-associated depression at the Biomedical Research Institute (BIOMED) at Hasselt University, in collaboration with the Neuroscience Department at Janssen R&D. The first year of his PhD project was spent at BIOMED studying immunological responses to systemic inflammation. The final 3 years were spent at Janssen R&D where Steven combined behavioral studies with the analysis of ex vivo parameters in rodents. Moreover, he visited the Janssen R&D Small Rodent Imaging group in Springhouse (PA, USA) for 3 months where he validated and optimized non-invasive imaging techniques in order to characterize immunological processes in the brain.

Bibliography

Systemic immune activation leads to neuroinflammation and sickness behavior in mice.
Mediators of Inflammation 2013; 2013:271359 (epub).
Biesmans S, Meert TF, Bouwknecht JA, Acton PD, Davoodi N, De Haes P, Kuijlaars J, Langlois X, Matthews LJR, Ver Donck L, Hellings H, Nuydens R.

Effect of stress and peripheral immune activation on astrocyte activation in transgenic bioluminescent Gfap-luc mice.
Submitted for publication
Biesmans S, Acton PD, Cotto Carlos, Langlois X, Ver Donck L, Bouwknecht JA, Aelvoet S-A, Hellings N, Meert TF, Nuydens R.

Systematic analysis of the cytokine and anhedonia response to peripheral immune activation in rats.
Submitted for publication
Biesmans S, Matthews LJR, Bouwknecht JA, De Haes P, Meert TF, Hellings N, Nuydens R, Ver Donck L.

Peripheral administration of tumor necrosis factor-alpha induces neuroinflammation and sickness but not depressive-like behavior in mice.
Submitted for publication
Biesmans S, Bouwknecht JA, Ver Donck L, Langlois X, Acton PD, De Haes P, Davoodi N, Meert TF, Hellings H, Nuydens R.

Disease progression, muscle contractile properties and cytokine response during EAE: impact of different running intensities.
Submitted for publication
Broekmans T, Hellings N, **Biesmans S**, Stinissen P, Eijnde B.

Human foot inversion prior to toe-off: an analysis by means of functional morphology and comparative anatomical observation.
Journal of Vibroengineering 2009; 11 (3):530-535
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European School of NeuroImmunology Meeting 2013, Porto, Portugal
Talk: When the immune system overpowers the brain.

International Neuroinformatics Coordinating Facility 2013, Antwerp, Belgium
Poster: Peripheral LPS administration induces neuroinflammation and sickness behavior in mice.

Belgian Molecular Imaging Congress 2013, Leuven, Belgium
Poster: When the immune system overpowers the brain - A multi-technique study in lipopolysaccharide-challenged mice.

Knowledge for Growth 2013, Ghent, Belgium
Poster: Peripheral LPS administration induces neuroinflammation and sickness behavior in mice.

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Steven, April 2014

"Difficulties mastered are opportunities won."
Winston S. Churchill, 1874-1965