Behavioral and molecular consequences of a ‘double-hit’ challenge on the pathogenesis of mouse models of depression and amyotrophic lateral sclerosis (ALS)

MAASTRICHT UNIVERSITY

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Alexander Trofimov

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DISSERTATION

to obtain the degree of Doctor at Maastricht University,
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by

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Propositions

1. Experiencing a ‘double-hit’ of chronic stress and LPS-induced systemic inflammation increases depressive-like behaviors and inhibits aggression in a mouse model of depression (*this thesis*).

2. When present simultaneously, these factors do not act in an additive way, but result in a distinct behavioral and molecular response (*this thesis*).

3. The behavioral changes arising from the ‘double-hit’ are associated with suppression of pro-inflammatory cytokines and serotonin (5-HT)-mediated mechanisms both in the brain and in the periphery (*this thesis*).

4. In the FUS[1-359]-tg model of amyotrophic lateral sclerosis (ALS), LPS-induced systemic inflammation in pre-symptomatic mice exacerbates the emotional and molecular abnormalities caused by the FUS gene mutation (*this thesis*).

5. Individual susceptibility to stress-induced depressive-like syndrome in mice is associated with elevated COX2 and Iba-1 expression, and decreased Ki67 expression in the hippocampus (*this thesis*).

6. The use of COX-2 inhibitors in the management of depressive syndromes, such as major depressive disorder (MDD), may be an example of how the manipulation of the inflammatory response may be beneficial in the treatment of neuropsychiatric disorders (*valorization*).

7. The use of the 'double-hit' model to study the underlying mechanisms of neuropsychiatric disorders is highly valid, as it allows for the modeling of clinical conditions (*impact*).

8. "In life, unlike chess, the game continues after checkmate." — Isaac Asimov, biochemist and science fiction writer.

List of abbreviations

5-HT	5-hydroxytryptamine, serotonin
ALS	Amyotrophic lateral sclerosis
ANOVA	Analysis of variance
BBB	Blood-brain barrier
cDNA	Complementary deoxyribonucleic acid
Cel	Celecoxib
Cit	Citalopram
CLRs	C-type lectin receptor
CMS	Chronic mild stress
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
CSF	Cerebral spinal fluid
DALYs	Disability-adjusted life-years
DAMPs	Danger-associated molecular patterns
FTLD	Frontotemporal lobar degeneration
FUS	Fused in sarcoma protein
FUS-tg	FUS-transgenic mice
GAPDH	Glyceraldehyde-3-phosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor

Hip (HIP)	Hippocampus
HPA	Hypothalamic–pituitary–adrenal
HPLC	High-performance liquid chromatography
IFN	Interferon
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
i.p.	intraperitoneal
JAK-STAT	Janus kinase (JAK)-signal transducer and activator of transcription
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDD	Major depressive disorder
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-B
NLRs	Nucleotide-binding and oligomerization domain NOD-like receptors
NSAIDs	Non-steroidal anti-inflammatory drugs
NT	no treatment
PAMPs	Pathogen-associated molecular patterns
PFC	Prefrontal cortex
PG	Prostaglandin

PRRs	Pattern recognition receptors
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription and quantitative polymerase chain reaction
SC	Spinal cord
SEM	Standard error of the mean
SERT	Serotonin transporter
SOD	Superoxide dismutase
TDP-43	Transactive response (TAR) DNA-binding protein 43
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Veh	Vehicle
UI	Uncertainty interval
WHO	World Health Organization

CHAPTER 1

General Introduction

Chapter 1. General introduction

The prevalence of neuropsychiatric disorders, including depressive disorders, has been increasing worldwide, resulting in a significant disease burden (GBD 2019 Mental Disorders Collaborators 2022). During the last three decades, the global number of disability-adjusted life-years (DALYs) due to neuropsychiatric disorders has increased from 80.8 million (95% uncertainty interval (UI) 59.5–105.9) to 125.3 million (93.0–163.2), and the proportion of global DALYs attributed to mental disorders has increased from 3.1% (95% UI 2.4–3.9) to 4.9% (3.9–6.1) (GBD 2019 Mental Disorders Collaborators 2022). As a result, there is growing demand for effective prevention and treatment of CNS disorders (Xia et al. 2021).

Depressive symptoms are a common feature of many neuropsychiatric disorders, including stress-associated syndromes and neurodegenerative disorders (Baquero and Martín 2015). Major depressive disorder (MDD) is one of the most prevalent neuropsychiatric disorders (Yoshino and Dwivedi 2019), and its incidence has increased substantially during the COVID-19 pandemic (Kola et al. 2022). MDD may be comorbid with many chronic diseases (Gold et al. 2020) and is among the leading causes of health loss worldwide, with a global lifetime prevalence estimated to be up to 280 million people (www.who.int/news-room/fact-sheets/detail/depression) (Lee and Giuliani 2019; GBD 2019 Mental Disorders Collaborators 2022).

Recent evidence suggests that stress and inflammation play key roles in the development of depression (Richter-Levin and Xu 2018). The iatrogenic effect of interferon, used as medical treatment for hepatitis, established an association between systemic inflammation and the

development of MDD (Dantzer et al. 2011) and continues to receive the interest of researchers. The number of PubMed publications on "Inflammation MDD" has risen from 1 in 2001 to 166 in 2022. The interaction between the immune system and stress also plays a significant role in depression development (Kim et al. 2022).

Neurodegenerative disorders like Amyotrophic lateral sclerosis (ALS)/ Frontotemporal lobar degeneration (FTLD) have a significant association with neuroinflammation, which can lead to neuronal damage, disease progression, and emotional disturbances that increase the predisposition to MDD (McCauley and Baloh 2019; de Munter et al. 2020; Masrori et al. 2022). Neuroinflammation can be initiated by diverse sources of systemic inflammation, such as 'sterile' inflammation that can be induced by non-infectious factors and 'non-sterile' pathogen-induced inflammation (Feldman et al. 2015). Although the prevalence of disease-driven/genetic inflammatory response and chronic infectious inflammation in clinical practice is well-established (Pahwa et al. 2022), the mechanisms underlying the 'double-hit' disease-driven and environmental inflammatory responses in the development of depressive symptoms remain poorly understood, highlighting the need for further research in this area.

1.1 Low-grade 'sterile' inflammation and cellular mechanisms of adaptation and maladaptation

Inflammation is a complex, local or general protective and adaptive process that occurs in response to damage or the action of a pathogenic stimulus (Nathan 2022). Inflammatory mechanisms are involved in

maintaining homeostasis under various challenges, including pathogens, tissue injury, stress, and toxic agents (Golia et al. 2019; Medzhitov 2010).

Inflammation can be infectious or 'sterile', with the main molecular mediators involved in the regulation of the inflammatory response including pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) (Chen et al. 2018; Jabbour et al. 2009). These receptors are expressed on immune and non-immune cells and activated through recognition of pathogen-associated molecular patterns (PAMPs) to activate infectious inflammation or endogenous danger-associated molecular patterns (DAMPs) to activate non-infectious 'sterile' inflammation (Chen and Nuñez 2010; Li and Wu 2021) (**Figure 1.1**). Chronic low-grade 'sterile' inflammation is initiated due to various cellular stressors, including ischemia, trauma, stress, and environmental factors leading to the extrusion of cellular components and debris, including DAMPs, such as nuclear and mitochondrial DNA, specific proteins, reactive oxygen species, and other molecules, which then bind to PRRs and activate a cascade of cellular signals promoting an inflammatory response (Byappanahalli et al. 2023).

Activation of intracellular signaling pathways including MAPK (mitogen-activated protein kinase), NF-κB (nuclear factor kappa-B), and JAK-STAT (Janus kinase (JAK)-signal transducer and activator of transcription) leads to the activation of pro-inflammatory cytokine gene expression such as interleukin (IL)-1ß, IL-6, IL-8, IL-12, tumor necrosis factor (TNF), interferon (IFN)-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hendrayani et al. 2016; Henríquez-Olguín et al. 2015; Kyriakis and Avruch 2001). These cytokines are produced by

predominantly macrophages to recruit leukocytes to the site of infection or injury (Chen et al. 2018).

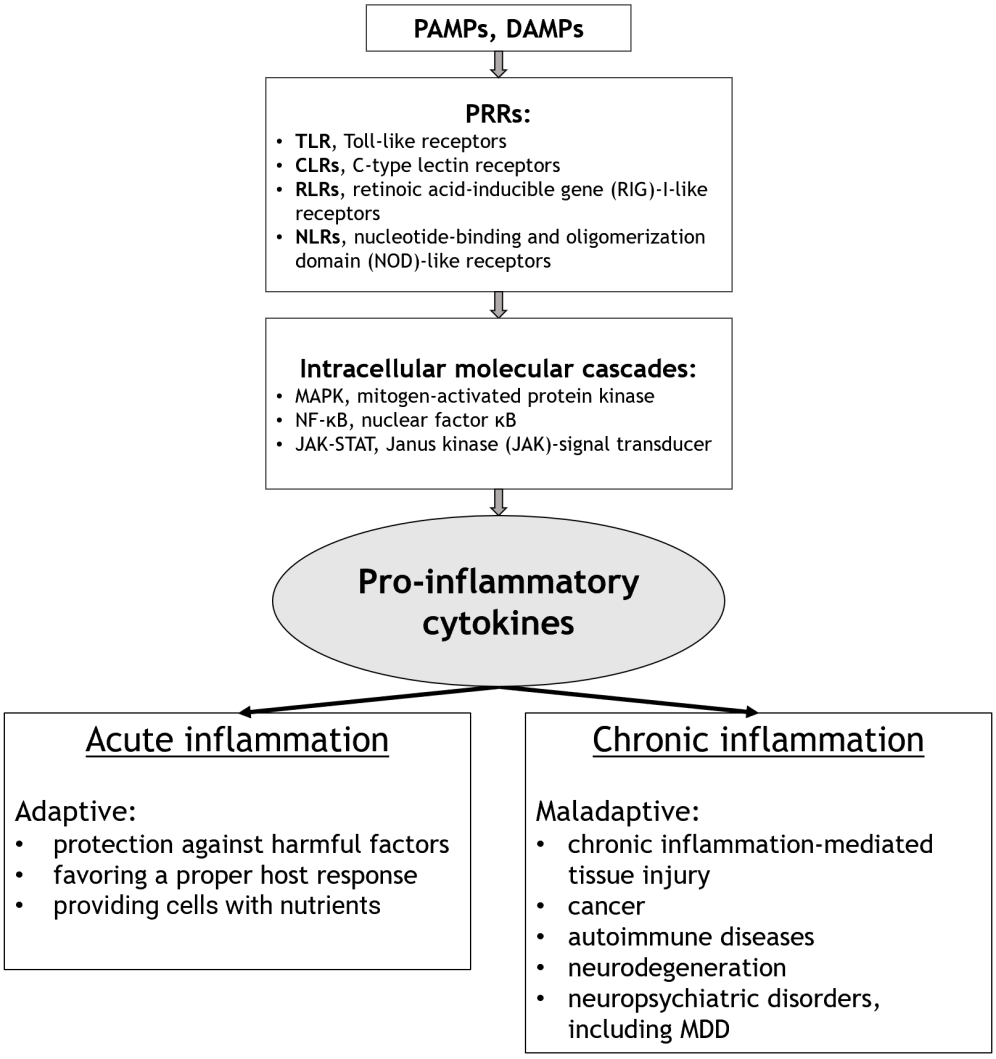

Fig. 1.1. General mechanisms of inflammatory response and role in adaptive and maladaptive outcomes (original figure; see the details in the text).

A range of inflammatory proteins such as C-reactive protein (CRP), superoxide dismutase (SOD), glutathione peroxidase (GPx), NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), and cyclooxygenase (COX)-2, as well as non-protein molecules including histamine, prostaglandins (PG), and leukotrienes, participate in the transduction of inflammatory response, restoration of homeostasis, and reduction of microbial growth, functioning independently of antibodies (Chen et al. 2018).

While inflammatory mechanisms are targeted at controlling adaptive responses to harmful effectors, uncontrolled acute inflammation, if not inhibited by anti-inflammatory cytokines such as IL-4, IL-10, IL-11, and transforming growth factor (TGF)-β, may become chronic (Kulkarni et al. 2016; Chen et al. 2018). Chronic inflammation can lead to neuroinflammation (Sun et al. 2022) primarily via the activation of microglia and the hypothalamic–pituitary–adrenal (HPA) axis (Kulkarni et al. 2016; Hara et al. 1986).

Low concentrations of pro-inflammatory cytokines are able to selectively cross the blood-brain barrier (BBB) from the blood into the brain (Banks 2015). Peripheral pro-inflammatory cytokines promote cytokine synthesis by macrophages of the circumventricular organs, with further penetration of cytokines into the brain (Dantzer 2006; Yarlagadda et al. 2009; Pan et al. 2011; Banks 2015). In the brain vascular system, COX-2 is activated by pro-inflammatory cytokines IL-1 and IL-6 to produce PGE_2 from arachidonic acid (Rawat et al. 2019). PGE_2 affects the integrity of the BBB and induces neuroinflammation via activating microglial synthesis of pro-inflammatory cytokines (Engblom et al. 2002).

Microglia represents the main form of active immune defense in the CNS (Filiano et al. 2015). In chronic neuroinflammation, microglia remains active and enhances the course of the inflammatory process (Ransohoff and El Khoury 2015). Activated microglia secretes pro-inflammatory cytokines such as TNF, IL-1ß, IL-12, and IFN-γ, as well as PGE2, COX-2, mitogenic factors chemoattractant protein (MCP-1) and macrophage colony-stimulating factor (M-CSF), which have neurotoxic effects on neurons (Kaur et al. 2017; Scheiblich et al. 2017).

Dysregulation of the immune-to-brain communication may lead to maladaptive changes of the brain response to inflammatory challenges that contribute to the development of neuropsychiatric disorders (Lasselin et al. 2018; McEwen and Gianaros 2010).

1.2 Clinical evidence of the pathogenic role of inflammation in major depressive disorder (MDD)

Clinical evidence supports the pathogenic role of inflammation in MDD (Labra Ruiz et al. 2021), which is a leading cause of mental disability globally (Yoshino and Dwivedi 2020). The inflammatory hypothesis of MDD (Rengasamy et al. 2021) is based on Smith's "macrophage" theory, which suggests that excessive cytokine production triggers depression mechanisms (Smith 1991). Maes and colleagues described various indicators of moderate activation of the inflammatory response system that accompany major depression, including increased production of cytokines such as IL-1, IL-6, and IFN-γ, as well as changes in other biomarkers such as serum Zn, erythron, high-density lipoprotein cholesterol (HDL-C), and ω3 polyunsaturated fatty acids (PUFAs) (Maes 1999). Administration of

cytokines can produce symptoms of depression in healthy volunteers (Capuron and Miller 2004).

Several meta-analyses have shown that MDD patients have elevated levels of circulating blood cytokines, including TNF (Rengasamy et al. 2021; Gkesoglou et al. 2022), IL-6 (Dowlati et al. 2010; Haapakoski et al. 2015; Liu et al. 2012), CRP (Haapakoski et al. 2015), and IL-1(Howren et al. 2009). Cerebral spinal fluid (CSF) cytokine levels may correlate with symptom severity and do not always directly mirror blood cytokine levels in MDD patients. Studies have shown that an association between depression scores and IL-6 CSF levels exists in suicide attempters compared to healthy controls, suggesting that IL-6 levels may reflect symptom severity (Lindqvist et al. 2009). This finding was supported by a later report of positive correlations of IL-1β, IL-6 and TNF levels with suicide ideation in MDD patients (Martinez et al. 2012; Ganança et al. 2021).

A relationship between immune activation and depression is often observed when depression is comorbid with somatic pathologies (Gold et al. 2020), such as rheumatoid arthritis (Nerurkar et al. 2019), coronary insufficiency (Frasure-Smith and Lespérance 2008; Wu et al. 2021), obesity (Ouakinin et al. 2018; Milaneschi et al. 2019), type 2 diabetes (Milaneschi et al. 2019), as well as aging (Straka et al. 2020). Iatrogenic depression is observed when using cytokines as medical treatment, but only in patients who initially had high levels of cytokines (Prather et al. 2009; Felger and Lotrich 2013). This risk is associated with a high reactivity of the HPA axis to the introduction of cytokines (Capuron and Dantzer 2003). Genetic factors, such as serotonin transporter (SERT) gene polymorphism, have also been predictive of a propensity to develop depression with exogenous cytokine administration (Dantzer et al. 2011). The use of endotoxin for

therapeutic purposes supports the findings that cytokines are associated with the development of depressive symptoms (Wright et al. 2005; Remus and Dantzer 2016; Kotulla et al. 2018).

1.3 Low-grade inflammation during major depression: mechanisms of development and contribution to the pathophysiology of the disease

The development of low-grade 'sterile' inflammation, which increases the risk of depression, can be triggered by various environmental and genetic factors, including stress (Fleshner et al. 2017), air pollution (Ragguett et al. 2017), exposure to allergens (Amritwar et al. 2017), pesticides and other toxins (Koh et al. 2017), chronic noise exposure (Beutel et al. 2016), exposure to heavy metals (Nguyen et al. 2022), including mercury-containing amalgam dental fillings (Kern et al. 2014; Siblerud and Mutter 2021). Even dietary factors, such as a Western diet, can trigger inflammation and increase the risk of developing depression (Veniaminova et al. 2020). Changes in the levels of pro-inflammatory and anti-inflammatory cytokines can affect mechanisms of neuroplasticity, leading to long-term impairments in mood, cognition, and behavior, including anxiety or depressive-like symptoms (Rhie et al. 2020; Yirmiya et al. 2000).

Activation of the HPA axis by pro-inflammatory cytokines stimulates the synthesis of corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) in the hypothalamus, with subsequent increases in cortisol levels observed in patients with MDD (Rengasamy et al. 2021). Furthermore, patients with MDD exhibit glucocorticoid resistance, and additional activation of CRH neurons has been observed (Slavich and Irwin

2014; Mikulska et al. 2021). AVP enhances the effect of CRH on adrenocorticotropin (ACTH) release, with vasopressin V1B receptor antagonism recently found to have an antidepressant effect (Chaki 2021). Studies have also shown decreases in peripheral AVP concentration in patients with remitted depression (Halaris et al. 2020; Goekoop et al. 2011).

Patients with MDD exhibit altered neurotransmitter levels (**Figure 1.2**), including increased SERT activity and the activation of IDO (Mikulska et al. 2021; Miller et al. 2009), reduced synaptic levels of serotonin, dopamine, norepinephrine, and GABA, and higher levels of glutamate in various brain regions (Rengasamy et al. 2021). Pro-inflammatory cytokines may trigger or contribute to these alterations. In particular, IL-1β and TNF can increase the activity of SERT (Zhu et al. 2006), while TNF may stimulate GABA receptor endocytosis, leading to reduced GABA activity while stimulating glutamate signaling (Stellwagen et al. 2005; Haroon and Miller 2017).

Pro-inflammatory cytokines also reduce monoaminergic transmission by increasing monoamine reuptake and decreasing monoamine synthesis (Hodes et al. 2015). Decreased monoamine levels are connected with the kynurenine pathway, which involves the degradation of tryptophan into kynurenine by IDO. Activation of IDO during systemic inflammation leads to a decrease in tryptophan levels, an increase in kynurenine levels, and a subsequent reduction of serotonin synthesis, resulting in depressive symptomatology (Muneer 2020). Microglia can convert kynurenine into quinolinic acid, an excitotoxic compound that acts as an NMDA receptor agonist (Jhamandas et al. 2000). Selective blockade of IDO in LPS-treated

mice revealed a suppression of depressive-like behaviors (O'Connor et al. 2009).

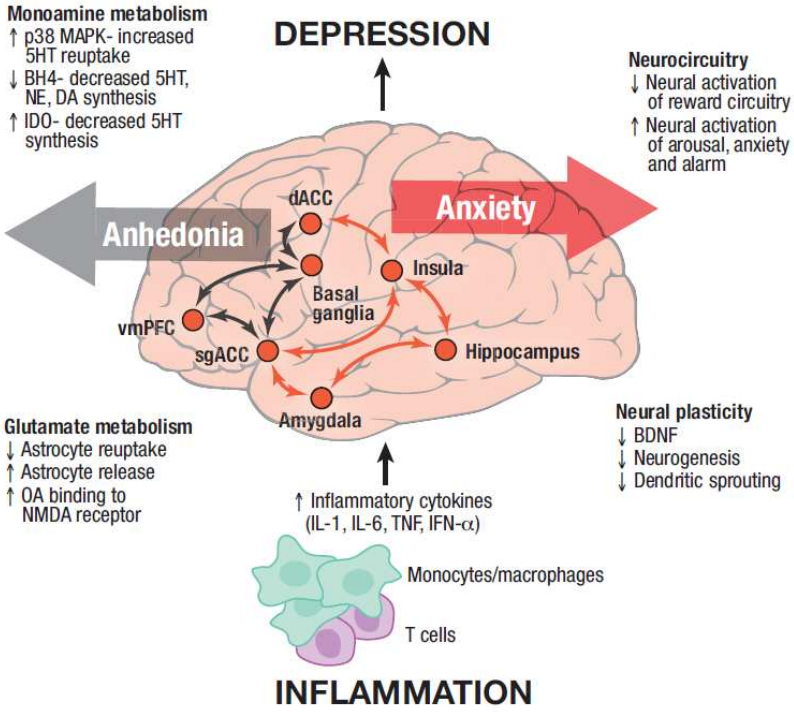

Fig. 1.2. Inflammatory mechanisms contributing to pathogenesis of depression (https://www.psychiatrictimes.com/view/five-things-know-about-inflammation-and-depression).

Studies in depressed individuals suggest that increased pro-inflammatory cytokines are associated with reduced volume of various brain areas (Baune et al. 2012; Frodl and Amico 2014) and impaired neurocircuitry, particularly in reward circuits, including the projections of dopamine neurons from the ventral tegmental area to the ventral striatum,

including the nucleus accumbens (Felger et al. 2016). Dysfunctional neurogenesis is also associated with depression (Krishnan and Nestler 2008), and pro-inflammatory cytokines may affect neurogenesis (Kohman and Rhodes 2013; Borsini et al. 2015) and cause deficits of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), which is associated with depressive symptomatology (Molendijk et al. 2014; Carvalho et al. 2008).

1.4 Animal models of MDD mimicking etiological roles of stress, systemic inflammation and their interaction

Animal models have been instrumental in studying the pathogenic mechanisms of inflammation and stress-induced affective disorders (Demin et al. 2019; Petković and Chaudhury 2022). Rodent models of depression utilize a range of pro-inflammatory agents, including lipopolysaccharide (LPS) (Remus and Dantzer 2016; Biesmans et al. 2016), viral mimetic polyriboinosinicpolyribocytidylic acid (Poly I:C) (Gibney et al. 2013), and pro-inflammatory cytokines (Orsal et al. 2008; Kentner et al. 2007) to induce depressive-like behaviors in animals. LPS administration to rodents produces depressive-like behaviors in the forced swim and tail suspension tests, reduced food intake, and decreased motor activity (Frenois et al. 2007; O'Connor et al. 2009; Yirmiya 1996). These behaviors are mediated by increased cytokine levels in the brain (Horita et al. 2020).

Stress-based models of depressive symptoms include chronic unpredictable stress, chronic social defeat stress, chronic restraint, prolonged social isolation, single prolonged stress, and ultrasound stress. Studies using these models have established an association between elevated plasma pro-inflammatory cytokines and depressive-like behaviors

(Ma et al. 2017; Couch et al. 2013). One of the most commonly used rodent models of MDD is chronic mild stress (CMS), developed in the 1980s (Katz 1981), which resembles the key symptoms of the disease (Strekalova et al. 2022), including anhedonia, i.e., a reduced sensitivity to a reward in sucrose preference test (Willner et al. 1992), behavioral despair and helplessness in forced swim and tail suspension tests (Porsolt et al. 2001; Strekalova et al. 2005), psychomotor agitation, i.e., increased anxiety and decreased exploratory behavior in novel cage test, open field test, elevated-plus maze, elevated O maze, and light-dark box (Ihne et al. 2012; Strekalova et al. 2004), apathy, such as decreased self-grooming in splash test (Planchez et al. 2019; Smolinsky et al. 2009; Petković and Chaudhury 2022), and reduced drive for sociability in social interaction tests (Biselli et al. 2019).

In a study investigating the combined effects of LPS and chronic stress, a single dose of LPS (i.p. 100 μg/kg) reversed the depressive- and anxiogenic-like behavior induced by stress (Kreisel et al. 2014). It increased locomotor activity and the time spent in the center of the open field arena, reduced floating time, and increased the latency to first float in the forced swim test. However, LPS did not reverse stress-induced reduction in sucrose preference and social interaction (Kreisel et al. 2014).

Therefore, animal models have been essential in understanding the pathogenic mechanisms of inflammation and stress-induced affective disorders. Rodent models of depression utilizing stress and inflammation-based paradigms have provided insights into the complex interplay between inflammation, stress, and depressive-like behaviors.

1.5 Neurodegenerative disorders, ALS/FTLD and molecular mechanisms of neuroinflammation

Neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and Amyotrophic lateral sclerosis (ALS) / Frontotemporal lobar degeneration (FTLD), are often characterized by inflammatory processes in both peripheral and central nervous systems. These processes involve the activation of microglial and astroglial cells, production of pro-inflammatory cytokines, disruption of the BBB, and weakened anti-inflammatory mechanisms (Hu et al. 2017; Yu et al. 2022; Kwon and Koh 2020) (**Figure 1.3**), which have been linked to the development of affective symptoms like depression and anxiety in patients with these disorders (Meyer et al. 2020; Talati et al. 2022).

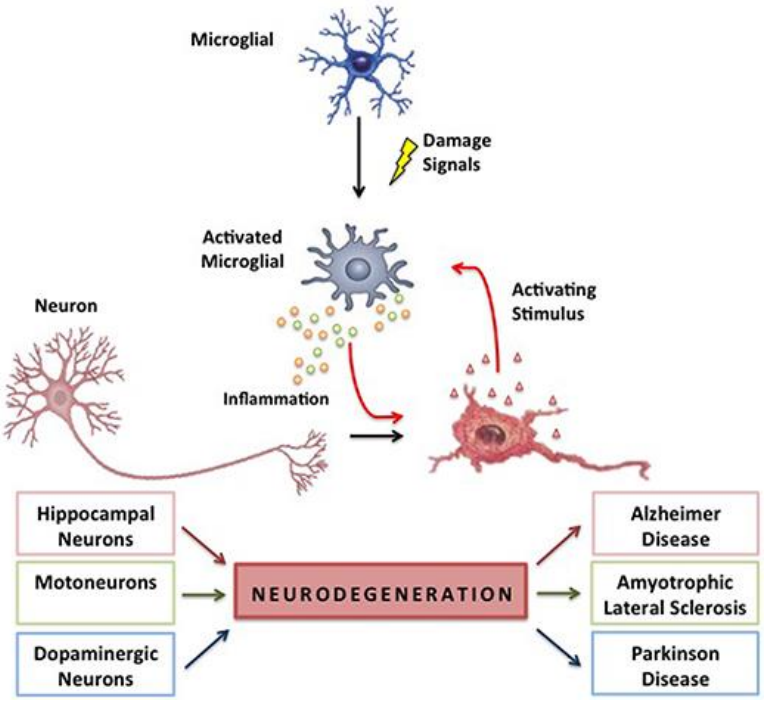

Fig. 1.3. Model of neuroinflammation and neurodegeneration (Morales et al. 2016).

ALS is a progressive neurodegenerative disease that affects the motor neurons in the frontal cortex, brainstem, and spinal cord, leading to muscle weakness and ultimately resulting in respiratory failure and death (Abramzon et al. 2020). The majority of cases are sporadic, with similar pathogenic mechanisms involving proteins that are prone to aggregation, resulting in neuroinflammation and neuronal death, while 5-10% of cases are hereditary (de Carvalho et al. 2017). FTLD is a type of neuropathology that is characterized by the selective degeneration of the frontal and temporal lobes and results in progressive frontotemporal dementia (FTD) (Neumann et al. 2009). Recent studies have shown a pathological connection between ALS and FTLD, with both disorders sharing pathological hallmarks, such as the involvement of TAR DNA-binding protein 43 (TDP-43) and FUS proteinopathies (Riku et al. 2014; Ishigaki and Sobue 2018; Carey and Guo 2022). As a result, ALS and FTLD are now considered to be a single disease entity known as ALS/FTLD (Ishigaki and Sobue 2018).

Neuroinflammation leading to neurodegeneration is a common feature of sporadic and familial ALS/FTLD (**Figure 1.4**) (Haukedal and Freude 2019). Glial cells in ALS/FTLD exhibit increased numbers of autophagic and secretory vesicles, which are characteristic of cellular stress (Liu and Wang 2017). The secretion of cytokines by microglia activates the A1 subtype of reactive astrocytes, leading to an amplification of the inflammatory response (Liddelow et al. 2017).

ALS/FTLD patients demonstrate peripheral immune abnormalities, primarily pro-inflammatory, with elevated levels of cytokines such as TNF, granulocyte-macrophage colony-stimulating factor (G-CSF), IL-2, IL-15, IL-17, MCP-1, and macrophage inflammatory protein-1α (MIP1α) in CSF (Yu et

al. 2022; Sun et al. 2022; McCombe et al. 2020; Chen et al. 2018). In addition, the levels of pro-inflammatory immune cells, including leukocytes, monocytes, granulocytes, natural killer cells, and mast cells, are typically increased in the blood of ALS/FTLD patients (Murdock et al. 2017; Rolfes et al. 2021). Elevated peripheral levels of pro-inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-18, and TNF, have also been observed in ALS/FTLD patients and may serve as diagnostic biomarkers of the disease (Hu et al. 2017; Italiani et al. 2014; Sun et al. 2021).

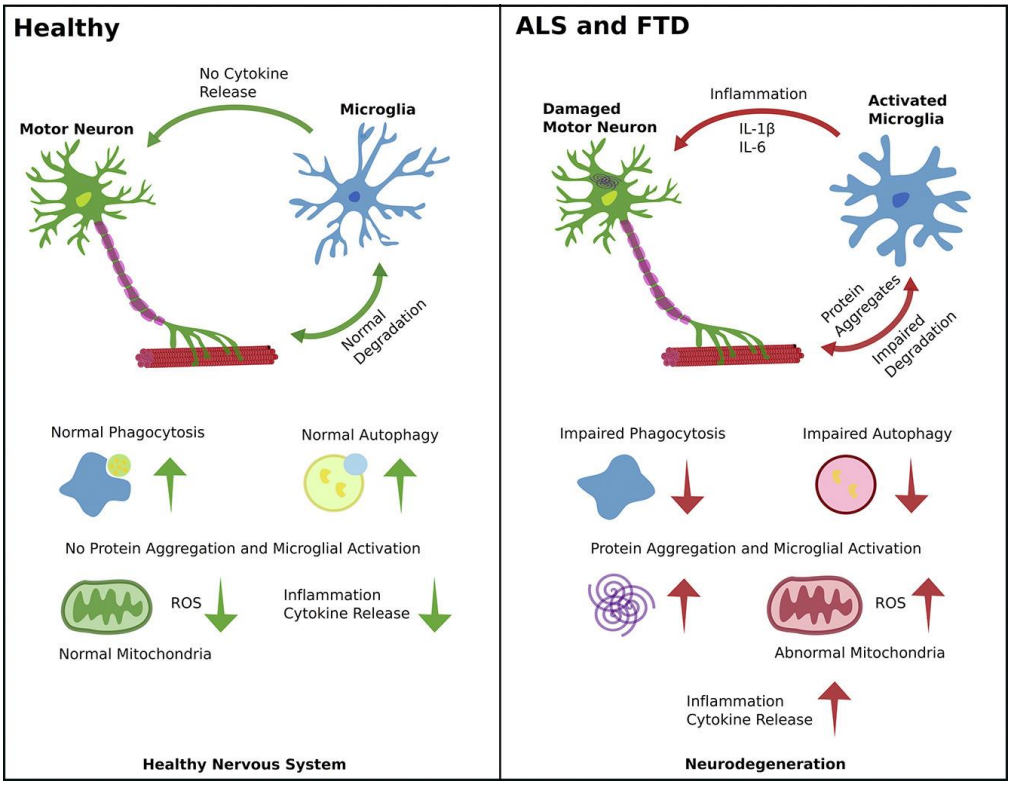

Fig. 1.4. Neuronal and microglial involvement in ALS and FTLD pathology, compared to healthy conditions. In disease conditions microglia are activated, secreting pro-inflammatory cytokines, causing neuroinflammation and neuronal damage. Phagocytosis and autophagy is impaired, leading to protein aggregation and mitochondrial dysfunction, increased reactive oxygen species production and further inflammation, overall causing neurodegeneration (Haukedal and Freude 2019).

Individuals with ALS/FTLD diagnosis and animal models of ALS/FTLD exhibit astrogliosis, an atypical proliferation of astrocytes surrounding deteriorating motor neurons, and these astrocytes express pro-inflammatory markers, such as COX-2 (Vargas and Johnson 2010). Furthermore, motor neurons grown in cell cultures show cytotoxicity when exposed to astrocytes obtained from the spinal cords of patients with familial or sporadic ALS/FTLD (Haidet-Phillips et al. 2011). Some mouse models of ALS/FTLD also show accumulations of mast cells and neutrophils around motor axons, the sciatic nerve, and ventral spinal nerves, indicating immune cell infiltration along the entire peripheral motor pathway (Trias et al. 2018).

While it remains unclear whether the inflammatory reaction develops before the onset of ALS/FTLD or during the disease progression due to a lack of pre-symptomatic studies of ALS/FTLD patients, hyperactivation of microglia and astroglia accompanied by elevated production of pro-inflammatory cytokines is found throughout the CNS during ALS/FTLD development (Tortarolo et al. 2017; Morello et al. 2017; Crisafulli et al. 2018). In a meta-analysis of 25 clinical studies comprising 812 ALS patients and 639 control subjects (Hu et al. 2017), elevated levels of peripheral pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF, TNF receptor 1, and vascular endothelial growth factor (VEGF) were described to contribute to the pathological mechanisms of ALS, particularly through endothelial cell damage and disruption of the blood-CNS barrier (B-CNS-B), leading to blood vessel leakage in motor neuron areas (Garbuzova-Davis et al. 2019).

Therefore, it is crucial to study the potential link between the FUS-related form of ALS/FTLD and neuroinflammatory processes, particularly

during the pre-symptomatic phase, as treatment during this phase is more likely to result in a positive outcome.

1.6 Animal models of ALS/FTLD that recapitulate a pro-inflammatory aspect of the disease

Numerous mouse models of ALS/FTLD are widely used to study different aspects of disease pathogenesis, as well as to screen therapeutic agents in pre-clinical trials (Philips and Rothstein 2015; Picher-Martel et al. 2016; Tosolini and Sleigh 2017; Lutz 2018; Martinez and Peplow 2022; de Munter et al. 2020a). The most widely used toxicological model of ALS/FTLD is the administration of ß-Sitosterol glucoside (BSSG), a plant sterol found in cycas, which mimics motor disorders including asymmetric limb paralysis, muscular denervation, and loss of spinal motor neurons (Wilson et al. 2002; Tabata et al. 2008; Déziel et al. 2021).

The most widely used genetic models of ALS/FTLD in mice comprise mutants in SOD1, FUS, and TDP43 proteins. Mutations in the SOD1 gene, which catalyzes the dismutation of superoxide into oxygen, are found in most cases of the disease, and animals expressing mutant SOD1 develop selective loss of spinal motor neurons, muscle atrophy, and paralysis leading to death (Rosen et al. 1993; Nardo et al. 2016; Peggion et al. 2022). This model demonstrates genetic instability, with variations in the number of mutated SOD1 gene copies that can directly impact disease severity (Zwiegers et al. 2014; Lutz 2018).

TDP-43 is a protein that is normally found in the cell nucleus but can aggregate in the cytoplasm as part of ubiquitin inclusions. Mutations in this gene have been found in 4% of patients with ALS, but it is not clear whether

these mutations result in an increase or loss of protein function, or whether cytosolic aggregation of TDP-43 is a by-product of other pathogenic mechanisms (Philips and Rothstein 2015; Taylor et al. 2016; Suk and Rousseaux 2020). The gain and loss of TDP-43 function affect RNA processing differently, suggesting that both may be involved in the development of pathology (Fratta et al. 2018). There have been nearly 20 different mouse models of TDP-43 developed, with the TDP43-Q331K model being the most extensively studied (Watkins et al. 2021). This model displays many of the characteristic features of ALS/FTLD, including progressive motor dysfunction, muscle atrophy, and motor neuron degeneration, but it does not exhibit cytoplasmic aggregation of TDP-43 (Arnold et al. 2013; Philips and Rothstein 2015; Morrice et al. 2018). Other TDP-43-based models do exhibit this feature (Stribl et al. 2014).

Mutations in the gene for the RNA-binding protein FUS are the second most common cause of ALS/FTLD. When FUS protein is dysfunctional, it leads to the development of neuroinflammation and oxidative stress, causing neurodegeneration in the brain, spinal cord, and muscle tissue atrophy (Monahan et al. 2017; Huang et al. 2022). FUS-R521C transgenic mice exhibit early onset of disease within weeks after birth and typically die within three months, displaying motor impairments such as muscle weakness and gait disturbances. By the 16th postnatal day, significant loss of spinal cord neurons is observed, while surviving motor neurons exhibit decreased dendritic branching complexity, synaptic defects, and DNA damage. Although cortical neurons are not lost, they display reduced dendritic complexity and synaptic density (Qiu et al. 2014).

The FUS[1-359]-transgenic mouse line, developed by research group of Natalia Ninkina, is a model that reproduces the main symptoms of FUS

proteinopathy in ALS/FTLD (Shelkovnikova et al. 2013). Expressing a truncated form of FUS protein (amino acids 1-359) lacking a nuclear localization signal causes a disturbance in the mRNA processing regulated by FUS. This causes severe motor dysfunctions and numerous cytoplasmic FUS aggregates in the spinal cord, leading to a rapid progression of neurodegeneration in heterozygous FUS-tg[1-359] mice, whose lifespan is around 4-4.5 months with ALS-like symptoms developing within a month (Lysikova et al. 2019; Ninkina 2020).

Pre-symptomatic FUS-tg[1-359] mice display several aberrant behaviors, including signs of helplessness, anhedonia, anxiety-like features, cognitive and social abnormalities. These mice also exhibit pro-inflammatory changes in the CNS, specifically, increased TNF and COX-1 mRNA expression in prefrontal cortex. Anti-inflammatory treatments are effective in reducing behavioral abnormalities in this model (de Munter et al. 2020b; Probert et al. 2022).

The FUS-tg[1-359] model is a reliable and valid tool for studying the pathophysiology of ALS/FTLD and exploring new therapeutic approaches (Probert et al. 2022; de Munter et al. 2020a,b; Sambon et al. 2020). This model can also be used to investigate how genetically-driven 'sterile' inflammation contributes to behavioral and mood abnormalities during the pre-symptomatic stage. Additionally, the administration of LPS to these animals can serve as a valuable ‘double-hit’ model to explore how it affects behavioral outcomes and the underlying molecular mechanisms.

Hypothesis and aims

Our hypothesis is that behavioral consequences of chronic low-grade 'sterile' inflammation overlap regardless of the etiology of inflammation, such as stress or genetically-determined neurodegeneration. We were expecting additive effects in a 'double-hit' model where pro-inflammatory challenges exacerbate stress-induced and genetically-determined behavioral disorders, while also exacerbating the molecular abnormalities that underlie these behaviors.

To investigate this, we used two mouse models to explore emotional abnormalities resulting from either CMS or genetically-determined neuropathology of ALS/FTLD. In the CMS studies, we investigated whether low-dose LPS injection as a pro-inflammatory challenge would exacerbate depressive-like and aggressive behaviors in mice exposed to various stressors. In the study on the FUS[1-359]-tg mouse model of ALS/FTLD, we aimed to evaluate emotional abnormalities during the pre-symptomatic stage of the disease in mice that received a single dose of LPS as a 'double-hit' model.

Our focus was on studying molecular and cellular changes in the hippocampus that are associated with individual susceptibility to stress-induced depressive-like behaviors in mice. Specifically, we studied the role of hippocampal COX-2, a key regulator of inflammatory response, in individual susceptibility to stress-induced syndrome. Additionally, we evaluated whether COX-2 inhibitors, coxibs, could prevent stress-induced anhedonia and depressive-like behaviors in mice.

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CHAPTER 2

Low-dose lipopolysaccharide (LPS) inhibits aggressive and augments depressive behaviours in a chronic mild stress model in mice

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Chapter 2. Low-dose lipopolysaccharide (LPS) inhibits aggressive and augments depressive behaviours in a chronic mild stress model in mice

Abstract

Background. Aggression, hyperactivity, impulsivity, helplessness and anhedonia are all signs of depressive-like disorders in humans and are often reported to be present in animal models of depression induced by stress or by inflammatory challenges. However, chronic mild stress (CMS) and clinically silent inflammation, during the recovery period after an infection, for example, are often coincident, but comparison of the behavioural and molecular changes that underpin CMS vs a mild inflammatory challenge and impact of the combined challenge is largely unexplored. Here, we examined whether stress-induced behavioural and molecular responses are analogous to lipopolysaccharide (LPS)-induced behavioural and molecular effects and whether their combination is adaptive or maladaptive.

Methods. Changes in measures of hedonic sensitivity, helplessness, aggression, impulsivity and CNS and systemic cytokine and 5-HT-system-related gene expression were investigated in C57BL/6J male mice exposed to chronic stress alone, low-dose LPS alone or a combination of LPS and stress.

Results. When combined with a low dose of LPS, chronic stress resulted in an enhanced depressive-like phenotype but significantly reduced manifestations of aggression and hyperactivity. At the molecular level, LPS was a strong inducer of TNFα, IL-1β and region-specific 5-HT2A

mRNA expression in the brain. There was also increased serum corticosterone as well as increased TNFα expression in the liver. Stress did not induce comparable levels of cytokine expression to an LPS challenge, but the combination of stress with LPS reduced the stress-induced changes in 5-HT genes and the LPS-induced elevated IL-1ß levels.

Conclusions. It is evident that when administered independently, both stress and LPS challenges induced distinct molecular and behavioural changes. However, at a time when LPS alone does not induce any overt behavioural changes per se, the combination with stress exacerbates depressive and inhibits aggressive behaviours.

Keywords: SERT, Chronic stress, LPS, Aggressive behaviour, 5-HT, Cytokines

1. Background

The association between depression and inflammation has been recognized for some time [1, 2]. Indeed, clinical trials have reported antidepressant treatment effects for anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs), and pro-inflammatory cytokine inhibitors have also shown antidepressant treatment effects compared to placebo. Tumour necrosis factor alpha (TNFα) blockade, for example, improved depressive symptoms in patients with treatment-resistant depression, but only in patients with high baseline CRP levels [3], suggesting that the anti-inflammatory therapy targets processes independent of the etiological mechanisms underlying major depressive disorder (MDD). However, the additive nature of inflammation-induced depressive-like behaviours when combined with MDD highlights that

inflammation is likely to be clinically relevant tractable target in many clinical forms of depression. However, while the impact of inflammatory challenges on the negative affect component of depression has been examined, the impact of inflammation on other accompanying behaviours has often been overlooked. Aberrant social behaviours, particularly aggression, as well as psychomotor agitation, often accompany depression and stress-related conditions in man and rodents [4–6]. Indeed, aggressive behaviour during major depression is associated with an enhanced risk of suicide [7]. Altered neuroimmune responses are also known to contribute to the neurobiology of aggression [8], and pro-inflammatory cytokine production, in particular, has been implicated in the mechanisms underpinning the stress response [5, 9] as well as aggressive behaviour [10–13].

Human and animal studies have linked aggression and impulsivity to the increased production of certain inflammatory mediators [11, 14]. In particular, aggressive traits in humans have been associated with increased serum TNFα [12], C-reactive protein [15] and other cytokines [16]. Indeed, patients in whom cytokines have been therapeutically administered often display signs of aggression [17, 18]. Furthermore, systemic expression of inflammatory mediators, such as increased systemic interleukin-1 beta (IL-1ß) and interleukin-6 (IL-6), are associated with locomotor agitation during aging [11]. Conversely, mice selectively bred for high levels of aggression also display increased cytokine levels [19] and knockout of both TNFα-receptor-1 and TNFα-receptor-2 abrogates aggressive behaviours [20] suggesting that overall, cytokines and aggressive behaviours are linked. The finding that stress is associated with the induction of inflammation [21–23] could be interpreted in evolutionary terms, as a coherent

mechanism to enhance survival. Stressors, such as predation, could potentially lead to injury and infection. Thus, pre-activation of the immune system would theoretically enhance survival and recovery [24].

In humans, parallels to the sickness behaviour observed upon systemic infection in rodents clearly exist. Interferon (IFN) therapy is known to induce transient signs of depression or malaise [25], and systemic inflammatory diseases are known to be accompanied by depressive-like signs [26, 27]. In rodents, CNS expression of pro-inflammatory cytokines IL-1β and TNFα contribute to anhedonia and behavioural measures of helplessness after chronic stress [28, 29]. Pro-inflammatory changes are associated with altered serotonergic function [30], over-expression of the 5-HT2A receptor and over-expression of the serotonin transporter (SERT) [29], which together with other 5-HT-related elements underlie mechanisms of depressive symptoms and social dominancy [31]. Despite this, it remains unclear at what level and to what extent sickness behaviours and depression converge and how similar the underlying molecular profile is. For example, the impact of inflammation-induced depressive-like behaviour compared with chronic stress on measures of aggression and impulsivity or hyperactivity has been largely overlooked. Irrespective of whether the pathways leading to such aberrant behaviours are distinct, it is clear that a 'double-hit' of stress and infection impacts on the pathogenesis of depression [32, 33].

In the current study, we sought to determine the degree to which the behaviours associated with chronic mild stress (CMS) may be influenced by a mild, low-dose lipopolysaccharide (LPS) challenge that does not normally give rise to anything other than transient and subtle changes in behaviour that persist for no more than a couple of hours. MDD is a disease that is

characterized by a recurrent episode of depression, but it is often unclear what factors might have precipitated relapse. Here, we were interested to discover how the single LPS challenge would impact on behaviour in a pre-stressed animal at a time when the effect of LPS had resolved. In this way, it is possible to evaluate the residual effects of acute inflammation on stress-induced behavioural changes. We examined behavioural parameters of aggression and impulsivity/hyperactivity, anhedonia and helplessness, as well as the expression of inflammatory and serotonergic markers of the periphery and specific brain areas, including the medial pre-frontal cortex and hippocampus as these sites are well recognized to play a crucial role in the stress response [34], and we have previously found that 5-HT2A and SERT expression levels change in response to systemic inflammation [35] and chronic stress paradigms [29] in these regions.

A 10-day stress procedure was selected in the present study because it has been previously shown to induce a depressive-like syndrome in mice, which is accompanied by changes in CNS serotonergic and pro-inflammatory genes [29, 36]. Previous work in rats has shown that repeated LPS challenges, sufficient to induce sickness behaviour, when combined with chronic mild stress can induce additive increases in plasma corticosterone and TNFα in rats will enhance depressive-like behaviour [37]. In contrast to these findings in rats, our investigations have established, using a single low-dose LPS (0.1 mg/kg) in CMS mice and a broader set of behavioural tests, that there is no simple additive effect when inflammation and stress are combined but highlight selective independent effects on a number of stress-related behaviours and on the underlying molecular biology.

2. Methods

2.1. Animals

Studies were performed using 3.5-month-old male C57BL/6J mice; 3.5-month-old male CD1 mice were used as intruders for social stress, and 2–5-month-old Wistar rats were used for predator stress. All animals were supplied by the Gulbenkian Institute of Science, Oeiras, Portugal. C57BL/6J mice were housed individually for 14 days before the start of the experiments; CD1 mice and rats were housed in groups of five before the experiment and then individually thereafter. All animals were kept under a reversed 12-h light-dark cycle (lights on: 21:00 h) with food and water ad libitum, under controlled laboratory conditions (22 ± 1°C, 55 % humidity). A minimum of six animals were used in all the behavioural experiments, and a minimum of five animals per group were used in the molecular biology experiments. All studies were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals upon approval by the Ethics Committee of Maastricht University for animal research (CPV, DEC-UM 2009-109) and permission 0421/000/000/2013 issued by the General Directory of Ethics Committee of the New University of Lisbon.

2.2. Study outline

The study design is outlined in the schematic in Fig. 1, and the animals were randomly assigned to test groups. The behavioural responses were studied in three separate cohorts. The first cohort was used to establish a subthreshold working dose of LPS that would not induce altered behaviour in the elevated O-maze and resident-intruder test in naïve mice,

so that we would be able to explore the interaction of stress and LPS (Fig. 1a). A lack of immediate behavioural effects with 0.1 mg/kg LPS and a suppression of social and locomotor behaviour with 0.5 mg/kg LPS has been previously reported [38, 39]. The animals were subjected to either an acute LPS challenge, 0.5 or 0.1 mg/kg, or treated with vehicle alone and tested 24 h post-injection or, in order to explore the delayed behavioural response to LPS [40], 48 h post-injection, in separate subgroups. The second cohort (Fig. 1b) was subjected to chronic mild stress or no stress (minimal handling). Separate subgroups of mice were subjected to the novel cage, O-maze, forced swim test and a resident-intruder test or sucrose test and tail suspension or were killed for analysis of central and peripheral changes in gene expression and blood corticosterone. The stressed and non-stressed animals were then subsequently treated with either LPS at 0.1 mg/kg or vehicle 24 h prior to testing. The number of animals per group is indicated in the figure legends. A third cohort, duplicating the first, was employed to establish the effects of 0.1 and 0.5 mg/kg LPS on behaviour in the open field at 24 or 48 h post-injection using TruScan apparatus. Resting time and average speed were recorded.

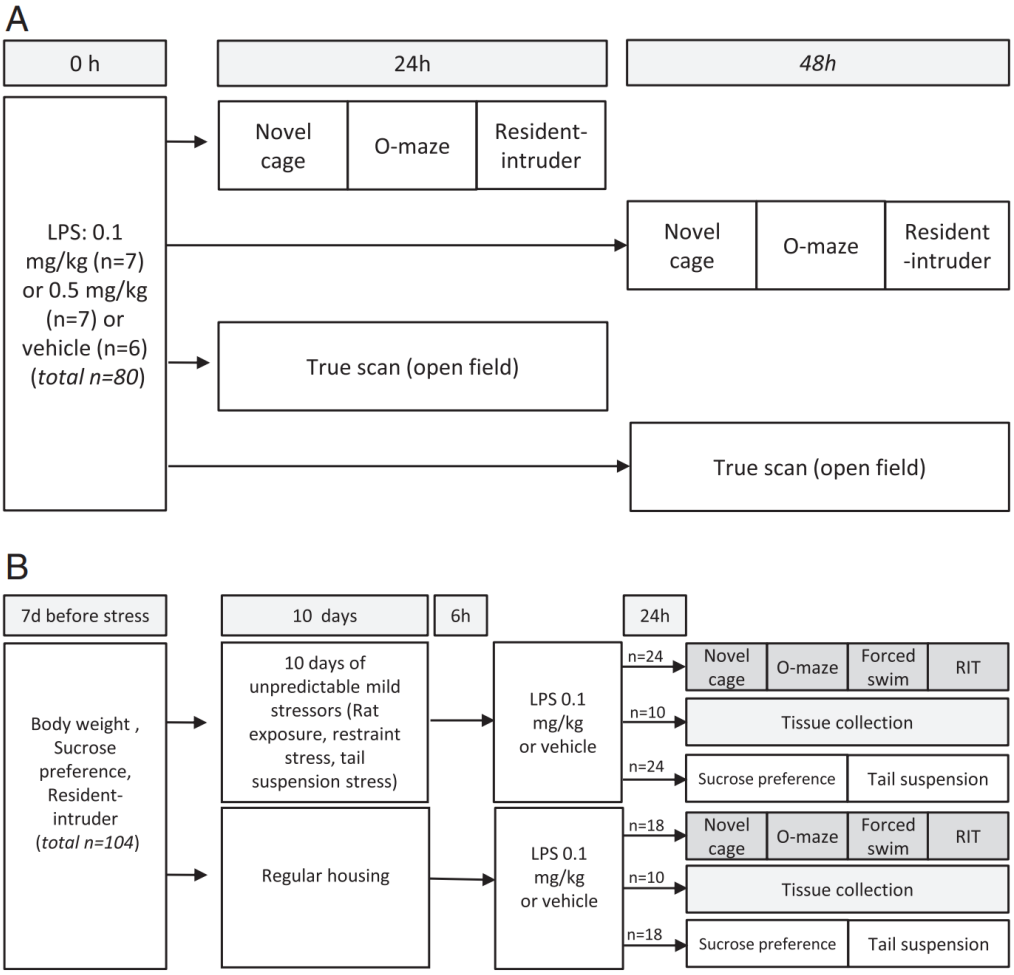

Fig. 1. Schematic outline of the behavioural studies for **a** LPS-challenged stress-naïve mice and **b** LPS-challenged or vehicle-challenged stressed/non-stressed animals compared with control animals. The numbers in each group and total numbers are shown

2.3. Acute LPS challenge

The animals were exposed to a single dose of LPS either 24 or 48 h prior to behavioural testing in the novel cage, O-maze or resident-intruder

test. LPS (E. coli 0111:B4, Sigma-Aldrich) was made as a stock solution in sterile saline (0.9 %) and injected intraperitoneally (i.p.) at 0.1 or 0.5 mg/kg in a volume of 0.1 ml. Control animals received a single i.p. dose of saline (0.1 ml) to control for injection stress.

2.4. Chronic mild stress

In the second (stressed) cohort, the animals underwent a previously validated 10-day chronic stress procedure [41]. The stress procedure consisted of rat exposure between the hours of 18:00 and 09:00 h (light phase of dark-light cycle) concomitant with a combination of restraint stress for 2 h and tail suspension for 40 min, applied in a semi-random manner with an inter-session interval of at least 4 h [29]. Briefly, during predation stress, mice were introduced to a transparent glass cylinder (15 cm high × ⌀ 8 cm) and placed into the rat cage for 15 h as described and validated previously [39, 40, 42]. For a restraint stress, mice were placed into a small container (50-ml Falcon tube) with space for breathing but no space for free movement, for 2 h, and for tail suspension, they were hung by their tails during the dark phase of the animals' light cycle, as described previously [29]. Body weight, sucrose preference and previously defined social behaviour parameters were determined 1 week before the chronic stress procedure [38, 39, 41]. A further cohort of animals were killed, and tissue was collected for messenger RNA (mRNA) analysis.

2.5. Behavioural testing

Behaviour was tested after 24 h because at this point, LPS-induced behavioural changes in stress-naïve mice had returned to baseline for the low-dose LPS challenge (Fig. 2). All behavioural testing was carried out

during the dark phase of the animals' light-dark cycle. Tests were recorded on film and analysis carried out post hoc and blinded, unless otherwise stated in the text.

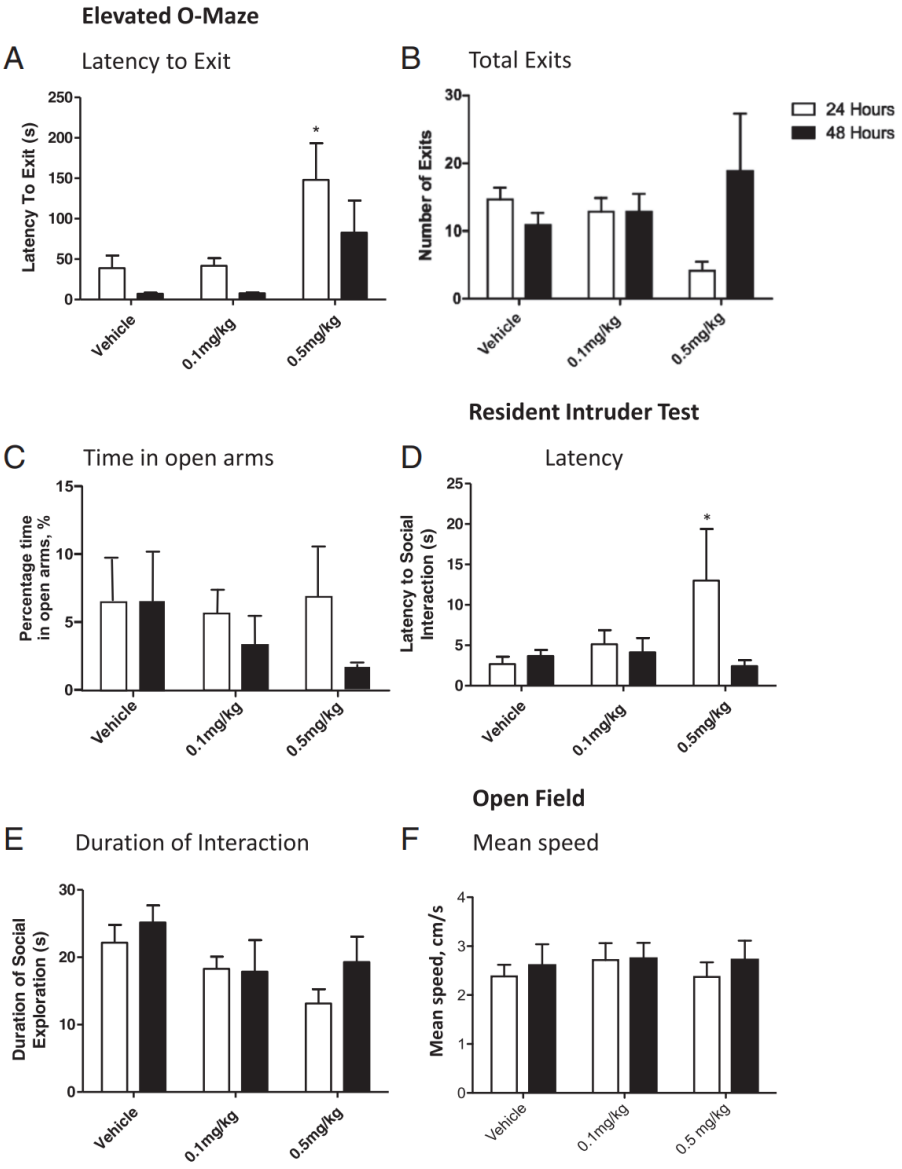

Fig. 2. The effect of low doses of LPS on behavioural outcomes at 24 and 48 hours post-challenge in naïve mice. Animals were subjected to a single dose of LPS: 0.1 mg/kg or 0.5 mg/kg (n=7 in each group)or vehicle administration (*n*=6), and were tested 24 h or 48 h thereafter in the elevated O-maze for (**a**) latency to exit to open arm, (**b**) number of exits into open arms, and (**c**) the time spent in the open arms. Subsequently, mice were observed for (**d**) latency and (**e**) duration of social interaction in a resident-intruder test. Mean speed (**f**) was also recorder in the open field. Data are mean ± SEM; **p* < 0.05 when compared to control animals

2.5.1. Elevated O-maze

The apparatus (Technosmart, Rome, Italy) consisted of a circular path (runway width 5.5 cm, diameter 46 cm) that was placed 45 cm above the floor. Two opposing arms were protected by walls (closed area, height 10 cm), and the illumination strength was 25 lx. The apparatus was placed on a dark surface in order to maintain control over lighting conditions during testing. Mice were placed in one of the closed-arm area of the apparatus. Behaviour was assessed using previously validated parameters during a 5-min observation period [36, 43]. The latency of the first exit to the 'open' compartments of the maze, the number of exits to the open arms and the percentage of time spent in the open arms were recorded [28].

2.5.2. Resident-intruder test

The resident-intruder test procedure was adapted from previously described protocols [36, 44]. In this paradigm, the C57BL/6J mice were placed individually in an observation cage (30 × 60 × 30 cm) for 30 min to acclimatize. Thereafter, a previously group-housed male CD1 mouse was introduced as an intruder to the same cage and left with the resident mouse for 8 min. During the observation period, both resident mice were

scored for the latency and duration of social interaction (nose-nose interactions) as well as the latency to attack, the number of attacks and the duration of crawl over behaviour. Crawl over time was recorded when the resident mouse positioned itself on top of the intruder mouse and often was usually associated with paw pressure on the head of the intruder [45]. During these periods, the intruder mouse showed little, if any, resistance to this mounting and displayed a submission by closing the eyes and not moving.

2.5.3. Novel cage test

The novel cage test was performed to assess exploration in a new environment as described elsewhere [44, 46]. Mice were introduced into a standard plastic cage (21 × 21 × 15 cm) filled with fresh sawdust. The number of exploratory rears was counted under red light during a 5-min period.

2.5.4. Open field TruScan

Mice were placed into TruScan activity boxes (26 × 26 × 39 cm; Coulbourn) for 10 min. The boxes were evenly illuminated with white light at 25 lx. Horizontal movements (speed) and resting time were scored automatically by red beam cells using TruScan software (Coulbourn), as described elsewhere [47]. Mean speed and total duration of resting behaviour, defined by a lack of crossing of more than three beams over 60 s, were evaluated.

2.5.5. Sucrose preference

Mice were given 24 h of free choice between two bottles of either 1 % sucrose or standard drinking water, as described elsewhere [44]. The bottles were weighed before and after conducting the sucrose preference and consumption calculated accordingly. The beginning of the test started with the onset of the dark (active) phase of the animals' cycle. To prevent the possible effects of side preference in drinking behaviour, the position of the bottles in the cage was switched at 12 h, halfway through testing. No prior food or water deprivation was applied before the test. Other conditions of the test were applied as described elsewhere [48]. Both baseline and post-stress paradigm sucrose preference tests utilized a 1 % sucrose solution. Percentage preference for sucrose was calculated at the end of the test using the following formula: Sucrose Preference = VolumeSucrose solution / (VolumeSucrose solution + VolumeWater) × 100.

2.5.6. Tail suspension test

The protocol used in this study was adapted from a previously validated procedure [43, 47]. Mice were suspended by the tails to a rod 50 cm above the floor using adhesive tape. Animals were left on this apparatus for 6 min in a dark room. The apparatus was illuminated with a single spotlight (5 lx at animal height). The trials were recorded by a video camera positioned directly in front of the mice while the experimenter observed the session from a distance in a dark area of the experimental room. The total duration of this behaviour, a putative measure of 'behavioural despair', were scored using protocols that were previously validated with automated tools [43, 47]. In accordance with the commonly

accepted criteria, immobility was defined as the absence of any movements of the animals' head and body.

2.5.7. Forced swim test

The protocol used for the Porsolt forced swim test was modified to prevent behavioural artefacts caused by stress-induced hyperlocomotion [41]. Mice were placed into a transparent pool (20 × 35 × 15 cm) lit with red light and filled with warm water (30 °C, to a depth of 9.5 cm) for 2 min. Floating behaviour, commonly interpreted as 'behavioural despair' in mice [49], was defined as the absence of directed movements of the animals' head and body. Floating was measured by visual observation which was validated previously in comparison to automated scoring with specialized software [43, 47]. The latency to begin floating was scored as the time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 s. The total time spent floating was scored for the entire duration of the test using video footage.

2.6. Tissue collection

Mice were terminally anaesthetized with an intraperitoneal injection of sodium pentobarbitone. The left ventricle was perfused in situ with 10 ml ice-cold saline; the brain and liver of each mouse were dissected. The pre-frontal cortex and striatum were collected by placing the brain, on its ventral side, on a metal plate. The olfactory bulbs were removed, and a 1-mm-thick coronal section of the most anterior cortical tissue was collected. The left and right cortical sections were further dissected to take the medial pre-frontal cortex while avoiding the motor cortex and

anterior forceps of the corpus callosum. The left and right striatum was collected by generating a coronal section at bregma 0 and bregma +1. The cortex and corpus callosum were carefully removed and the left and right striatum collected. The hippocampus was removed by generating a coronal slice at bregma −1 and bregma −3. The overlying cortex was carefully removed, and the left and right hippocampus was removed. The dorsal raphe nucleus was collected from a 1-mm-thick section from bregma −4 to bregma −5 by collecting a diamond-shaped piece of tissue under the fourth ventricle. Small segments of liver tissue were isolated and stored at −80 °C.

2.7. Quantitative RT-PCR (qPCR)

RNA extraction was performed as previously described from specifically microdissected snap-frozen brain regions and liver biopsies using the RNeasy Mini Kit (Qiagen, UK) [29]. The serotonergic genes 5-HT2A and SERT were selected for analysis based on prior observation that their expression levels change in response to systemic inflammation [35] and chronic stress paradigms [29]. Primers were custom designed and synthesized, taking basic secondary structure into account during the design process (PrimerDesign Ltd., UK). All primers were validated against a standard complementary DNA (cDNA) biobank (PrimerDesign) to ensure adequate amplification and single melt-curve products. Five hundred nanograms of whole mRNA was converted to cDNA using random primers supplied with the High Capacity Reverse Transcription cDNA Kit (Applied Biosystems, UK), and final samples were diluted to 5 ng/μl. Standard curves were generated from a mixed cohort of cDNA, and analysis was performed using SYBR green (PrimerDesign Ltd., Southampton, UK) and a

LightCycler 480 (Roche, UK). Cycle conditions were 8-min enzyme activation (95 °C), 15-s denaturation (95 °C), followed by 40 cycles of denaturation (15 s at 95 °C) and data collection (45 s at 60 °C). Total cDNA was used to enable normalization to expression of the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) using the Pfaffl method [50]. Details of primers are listed in (Supplementary file 1: Table S1). Results are expressed as relative-fold compared to control animals.

2.8. Corticosterone high-performance liquid chromatography (HPLC)

Blood was taken via cardiac puncture immediately before perfusion and stored in heparinized vials prior to centrifugation (10 k rpm, 10 min, 4 °C), and plasma was removed and immediately stored at −20 °C. Corticosterone was analysed using HPLC coupled with mass spectroscopy, based on the principles from Marwah et al. [51]. Briefly, plasma samples were diluted 1:1 with distilled water and applied to 2 ng of internal standard (5-pregnen-3b-ol-20-one-16a-carbonitrile). Diethyl ether was added to separate organic compounds into a water-free layer. Samples were vortexed and centrifuged (5 min, 1500×g) to fully separate solvent and aqueous layers. Solvent layers were removed and dried using a heated vacuum centrifuge. Organic residues were dissolved in 100 µl eluent A (see below) and applied to columns. Separations were carried out using a Waters 2695 separations module (Waters, Elstree, UK) with an ACE C18 3 µm, 100 × 2.1 mm column (Hichrom, Reading) maintained at 35 °C. The specific eluents were 2 mM acetic acid (A) and acetonitrile (B), with a linear gradient of 30–75 % of B over 8 min. The flow rate was 0.25 ml/min. The eluent was monitored using a Waters Micromass ZQ mass detector using positive electrospray ionization in single ion mode and Waters

Empower 2 software. Mass spectrometry was performed under the following conditions: capillary voltage, 2.7 kV; source temperature, 125 °C; desolvation temperature, 475 °C; desolvation gas flow, 575 l/h; and cone gas flow, 80 l/h. Corticosterone was monitored at m/z 347.1 (M + H), cone voltage 20 V. The internal standard CA4 was monitored at m/z 302.1, cone voltage 35 V.

2.9. Statistics

Data were analysed using GraphPad Prism version 6.0 for Windows (San Diego, CA) and InVivoStat software. Two-way ANOVA and RM-ANOVA were used followed by post hoc tests as appropriate (Bonferroni) and as indicated in the text. The level of confidence was set at 95 % ($p < 0.05$), and data are shown as mean ± SEM.

3. Results

3.1. A low dose of LPS of 0.1 mg/kg has no significant behavioural effects in naïve mice

Naive mice were challenged with either 0.1 or 0.5 mg/kg LPS to determine the behavioural effects of each dose 24 or 48 h thereafter [35]. In the elevated O-maze, there was a main effect of LPS dose, but not time post-challenge, on the overall latency to exit to the open arms (Fig. 2a; two-way ANOVA dose $p < 0.01$ $F_{2,34} = 7.89$; time post-challenge $p = 0.06$ $F_{1,34} = 0.55$; dose:time post-challenge $p = 0.78$ $F_{2,34} = 0.25$). Post hoc analysis demonstrates that at 24 h post-challenge, 0.5 mg/kg LPS animals have a significantly increased latency to exit to the open arms compared to controls (Fig. 2a; Bonferroni $p < 0.05$). The total number of exits from

the closed area of the O-maze was not affected by either LPS dose or time (Fig. 2b; two-way ANOVA dose $p = 0.93$ $F_{2,34} = 0.07$; time post-challenge $p = 0.28$ $F_{1,34} = 1.18$; dose:time post-challenge $p = 0.07$ $F_{2,34} = 2.90$). There was a non-significant tendency for the higher dose of LPS to affect the total number of exits at 24 h post-challenge; this was not significant (Fig. 2b; $p = 0.07$). The proportion of time spent in the open arms of the elevated O-maze was also unaffected by treatment (Fig. 2c; two-way ANOVA dose $p = 0.60$ $F_{2,34} = 0.5.14$; time post-challenge $p = 0.25$ $F_{1,34} = 1.343$; dose:time post-challenge $p = 0.63$ $F_{2,34} = 0.455$).

In the resident-intruder test, there was no overall effect of dose or time on social interaction (Fig. 2d; two-way ANOVA dose $p = 0.31$ $F_{2,34} = 1.18$; time post-challenge $p = 0.16$ $F_{1,34} = 2.06$; dose:time post-challenge $p = 0.13$ $F_{2,34} = 2.14$), but post hoc testing showed a significantly increased latency of social interaction in animals receiving 0.5 mg/kg and tested at 24 h when compared to vehicle-treated controls (Fig. 2d; Bonferroni $p < 0.05$). The total time spent interacting with the intruder was also not affected by LPS at either 24 or 48 h (Fig. 2e; two-way ANOVA dose $p = 0.07$ $F_{2,34} = 2.89$; time post-challenge $p = 0.26$ $F_{1,34} = 1.27$; dose:time post-challenge $p = 0.57$ $F_{2,34} = 0.57$). Using open field and novel cage tests, we observed no effect of either dose on locomotor activity on mean speed (Fig. 2f; two-way ANOVA dose $p = 0.77$ $F_{2,34} = 0.257$; time post-challenge $p = 0.47$ $F_{1,34} = 0.53$; dose:time post-challenge $p = 0.90$ $F_{2,34} = 0.10$) or on resting time or the number of rears (Supplementary file 1: Figure S1).

3.2. Stress-induced depressive-like behaviours tend to be exacerbated by systemic inflammation

Since the lower dose of LPS did not affect the behaviour of naïve mice at 24 h, it was used for the chronic stress study. We first assessed body weight (experimental groups were balanced at baseline) and showed that stress reduced body weight as expected (Supplementary file 1: Figure S2). Low-dose LPS (0.1 mg/kg) given 24 h prior to testing does not significantly alter parameters of sucrose preference test (Supplementary file 1: Figure S2) [41]. However, it was hypothesized that if stress increases pro-inflammatory cytokines, stimulation of the system with an inflammatory challenge may significantly alter this behaviour. All animals showed a preference of >65 % for a 1 % sucrose solution prior to testing and a consistent sucrose and water intake (Supplementary file 1: Figure S2 C–E). Control animals, and animals injected 24 h prior to testing with 0.1 mg/kg LPS, maintained a sucrose preference of >65 % and were not significantly different from each other (Fig. 3a). After 10 days of chronic stress, there was a significant main effect of stress on sucrose consumption but not of LPS, and there was no interaction between stress and LPS (Fig. 3a; two-way ANOVA; stress $p < 0.001$ $F_{1,54} = 16.62$; LPS $p = 0.28$ $F_{1,54} = 1.182$; stress:LPS $p = 0.41$ $F_{1,54} = 0.689$). Consistent with the main effects, post hoc tests showed that after 10 days of chronic stress and a single i.p. dose of saline, animals displayed a significant decrease (<65 %) preference for a sucrose solution (Fig. 3a; Bonferroni post hoc $p < 0.05$). Post hoc analysis revealed that animals undergoing 10 days of chronic stress combined with a single i.p. dose of LPS (0.1 mg/kg) 24 h prior to testing also showed a decrease in sucrose preference compared to controls ($p < 0.001$). While

there appears to be a decrease in sucrose preference for stressed animals receiving LPS compared to those without LPS, this difference is not significant ($p = 0.192$). Since sample sizes are unequal across groups, post hoc tests should be considered with caution. Total sucrose intake somewhat reflects this, here showing a main effect of both stress and LPS but no interaction (Fig. 3b; two-way ANOVA; stress $p < 0.001$ $F_{1,54} = 25.36$; LPS $p < 0.01$ $F_{1,54} = 10.27$; stress:LPS $p = 0.28$ $F_{1,54} = 1.15$). Post hoc testing revealed a significant decrease in sucrose consumption in stressed animals when compared to non-stressed controls (Fig. 3b; Bonferroni post hoc $p < 0.01$). Post hoc testing also reveals a decreased sucrose intake in stressed mice treated with LPS when compared to those treated with vehicle, suggesting a higher degree of anhedonia in these animals (Fig. 3b; Bonferroni post hoc $p < 0.0001$). Finally, stressed mice show some degree of hyperdipsia, with water consumption being affected by stress, but not by any other factors (Fig. 3c; two-way ANOVA; stress $p < 0.05$ $F_{1,54} = 5.38$; LPS $p = 0.31$ $F_{1,54} = 1.04$; stress:LPS $p = 0.35$ $F_{1,54} = 0.85$).

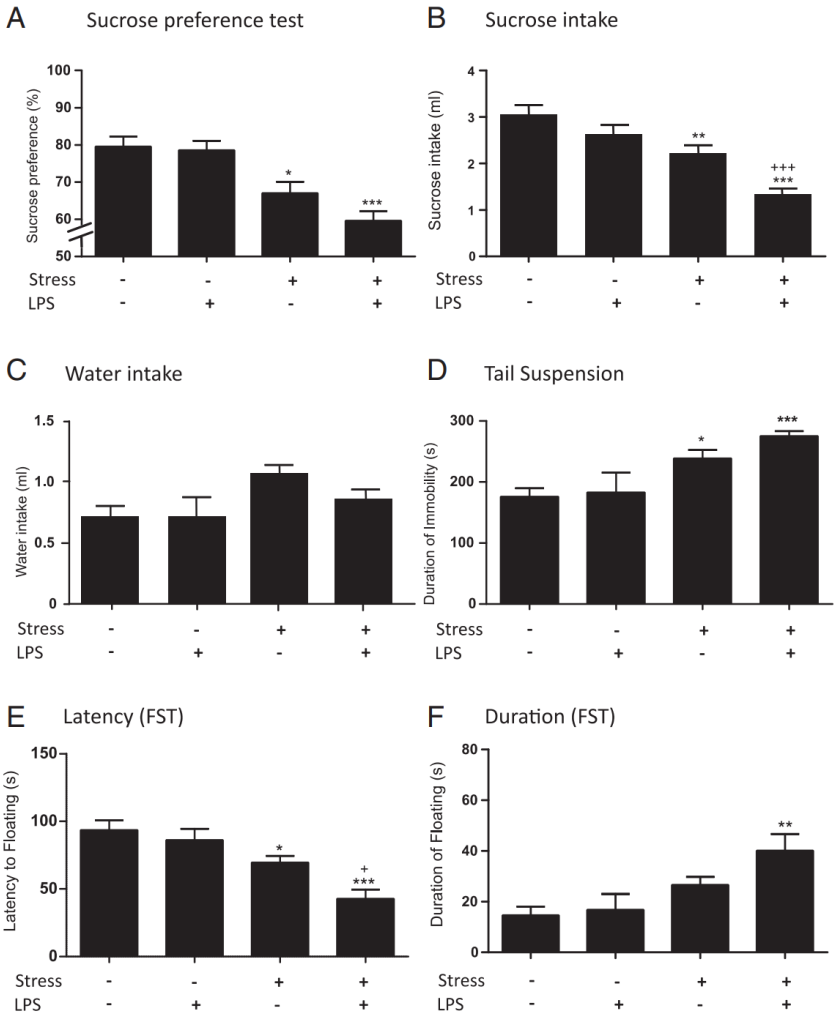

Fig. 3. The effect of low-dose LPS on depressive-like behaviours in stressed mice. Naïve and stressed animals were subjected to either a single dose of LPS (0.1 mg/kg) or vehicle injection and tested 24 h thereafter in a two-bottle sucrose preference test investigating **a** overall preference for sucrose, **b** total sucrose consumption, **c** water intake in a sucrose test, **d** the period of immobility in the tail suspension test, and in the forced swim test for **e** latency to floating and **f** total time spent floating. All animals showed >65 % preference for sucrose at baseline and similar sucrose preference prior to bolus injection of LPS or vehicle (Supplementary file **1**: Figure S2). Data are mean ± SEM; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared to controls; +$p < 0.05$ and +++$p < 0.001$ compared to stressed animals

Tail suspension is used to measure helpless behaviour, which is associated with a depressive-like state in mice [52, 53]. Analysis showed a significant effect of stress on the total time spent immobile in the test (Fig. 3d; two-way ANOVA; stress $p < 0.001$ $F_{1,40} = 24.89$; LPS $p = 0.16$ $F_{1,40} = 1.97$; stress:LPS $p = 0.35$ $F_{1,40} = 0.89$) but no other main effects and no interactions. Post hoc testing showed that all stressed animals, irrespective of treatment, were immobile for significantly longer periods than controls (Fig. 3d; Bonferroni post hoc; stress $p < 0.05$; stress and LPS $p < 0.0001$).

In the forced swim test, another test for helpless behaviour, control and LPS-alone animals showed similar values in both the latency to float and total time spent floating. Analysis showed that both stress and LPS had a main effect on the latency to floating behaviour but that there was no interaction between factors and therefore, all results should be considered with caution (Fig. 3e; two-way ANOVA; stress $p < 0.001$ $F_{1,43} = 21.46$; LPS $p < 0.05$ $F_{1,43} = 5.495$; stress:LPS $p = 0.19$ $F_{1,43} = 1.76$). In post hoc tests, chronic stress significantly decreased the latency to float compared to controls (Fig. 3e; Bonferroni post hoc; $p < 0.05$), as did chronic stress combined with LPS ($p < 0.001$). Using multiple pairwise comparisons (Bonferroni post hoc), LPS combined with stress is significantly different from stress alone ($p < 0.05$); however, as there is no interaction between these factors, this result should be interpreted with caution.

There was a main effect of stress, not LPS, on the total duration of floating behaviour, and there was no interaction between factors (two-way ANOVA; stress $F_{1,43} = 9.654$, $p < 0.01$; LPS $F_{1,43} = 1.922$, $p = 0.17$; stress: LPS $F_{1,43} = 0.99$, $p = 0.32$; Fig. 3f). In post hoc tests, the combination of chronic stress and LPS significantly increased the total time spent floating

compared to the control group in the forced swim test (Fig. 3f; Bonferroni post hoc; $p < 0.05$). While this suggests that LPS combined with stress significantly affects floating behaviour in the forced swim test, the lack of interaction makes these results difficult to interpret.

3.3. Inflammation decreases aggression and impulsivity in stressed animals

In the O-maze, stress and LPS significantly affected the latency to exit into the open arms independently and through interaction (Fig. 4a; two-way ANOVA; stress $F_{1,39} = 4.41$, $p < 0.05$; LPS $F_{1,39} = 9.84$, $p < 0.01$; stress: LPS $F_{1,39} = 4.87$, $p < 0.05$). In stressed animals, LPS reversed the stress-induced decrease in the latency to exit to the open arms, ameliorating this parameter which is an assumed sign of impulsivity (Fig. 4a; Bonferroni post hoc; $p < 0.001$). Similarly, the total number of exits to the open arms of the maze was significantly affected by stress and LPS independently and in terms of interaction (Fig. 4b; two-way ANOVA; stress $F_{1,39} = 4.55$, $p < 0.05$; LPS $F_{1,39} = 4.58$, $p < 0.05$; stress: LPS $F_{1,39} = 5.01$, $p < 0.05$). Post-hoc testing also demonstrated that the presence of LPS significantly diminished the number of exits to the open arms of the O-maze in stressed animals, thus abolishing the impulsivity/hyperlocomotion in these mice (Fig. 4b; Bonferroni post-hoc; $p < 0.01$).

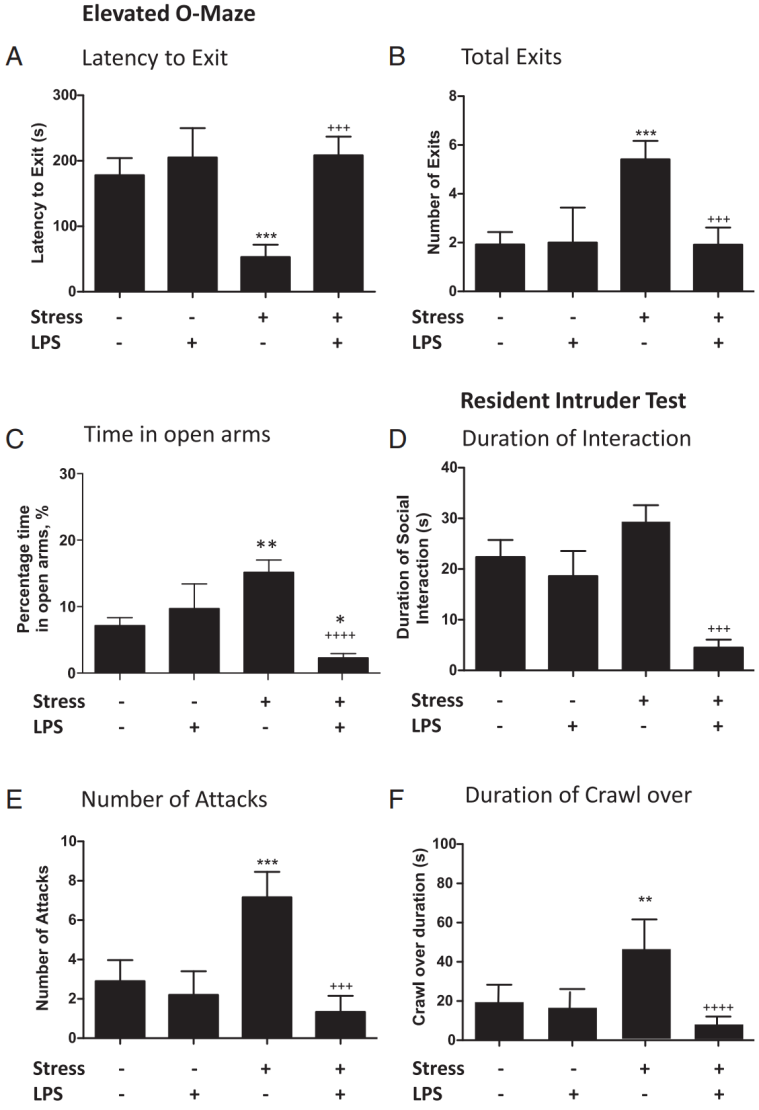

Fig. 4. The effect of low-dose LPS on anxiety and aggression-like behaviours in stressed mice. Naïve and stressed animals were challenged with a single dose of LPS (0.1 mg/kg) or vehicle (saline) and tested 24 h thereafter in the elevated O-maze for the **a** latency to exit to the open arms and **b** number of exits to the open arms; in the resident-intruder paradigm for **c** duration of social interaction and **d** latency to attack conspecific, **e** total number of attacks and **f** duration of crawl over behaviour. Data are mean ± SEM; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to control animals; +++$p < 0.001$ and ++++$p < 0.0001$ compared to stressed animals

The resident-intruder test can be used to assess both social and aggressive behaviours [54]. Resident-intruder testing was performed on all animals before undertaking the chronic stress and/or dosing procedure, and all groups were shown to be balanced at baseline (Supplementary file 1: Figure S3). We found that the duration of social exploration was significantly decreased by LPS and there was also an interaction between stress and LPS (Fig. 4d; two-way ANOVA; stress $F_{1,35} = 1.17$, $p = 0.28$; LPS $F_{1,35} = 18.81$, $p < 0.0001$; stress: LPS $F_{1,35} = 10.15$, $p < 0.01$). Post-hoc testing found that stressed animals challenged with LPS interacted with their intruders for significantly less time than those not challenged with LPS (Fig. 4d; Bonferroni post hoc; $p < 0.0001$).

When aggressive behaviour was examined, we found that the 10 days of chronic stress increased crawl over behaviour and the number of attacks and this was significantly inhibited by LPS treatment (Fig. 4). LPS treatment affected the number of the total number of attacks compared to control animals in an independent fashion, and analysis revealed a further interaction with stress (Fig. 4e; two-way ANOVA; stress $F_{1,35} = 1.89$, $p = 0.17$; LPS $F_{1,35} = 7.16$, $p < 0.01$; stress: LPS $F_{1,35} = 4.39$, $p < 0.05$). LPS significantly reduced the stress-induced rise in the number of attacks analysed with post hoc testing (Fig. 4e; Bonferroni post hoc; $p < 0.001$). Crawl over behaviour, a measure of a dominant-like interaction [45], was found to be increased in the animals exposed to stress, and this was once more significantly reduced in the stressed animals that were challenged with LPS (Fig. 4f; RM-ANOVA; stress/LPS treatment $F_{3,35} = 3.59$, $p < 0.05$; before/after $F_{1,35} = 2.85$, $p = 0.1$; stress/LPS: before/after $F_{3,35} = 6.78$, $p < 0.01$). Stressed animals showed an increased amount of crawl over behaviour when compared to controls (Fig. 4f; Bonferroni post hoc

$p < 0.01$). Furthermore, stressed animals treated with LPS showed significantly less crawl over behaviour when compared to animals that had undergone stress alone (Fig. 4f; Bonferroni post hoc $p < 0.0001$).

Behaviour in a novel cage was also examined in all animals. Those animals undergoing 10 days of chronic stress followed by either an LPS challenge or a vehicle challenge showed no significant change in rearing behaviour in this test (two-way ANOVA; stress $F_{1,32} = 1.29$, $p = 0.26$; LPS $F_{1,32} = 0.01$, $p = 0.9$; stress: LPS $F_{1,32} = 0.17$, $p = 0.67$). This suggests that the changes observed in behavioural tests for aggression or social interaction above were unlikely to be a result of confounding alterations in general locomotor activity (Supplementary file 1: Figure S2).

3.4. Inflammation and stress cumulatively increase hepatic IL-1β, but not corticosterone

Systemic inflammation has been shown to increase circulating cytokines, and stress is known to decrease pro-inflammatory cytokine expression via glucocorticoid induction [55]. As mentioned above, the levels of pro-inflammatory cytokines present 24 h after injection of 0.1 mg/kg endotoxin should be relatively low [56].

In this experiment, both LPS and stress had a significant effect on TNFα gene expression; furthermore, there was a significant interaction between the factors (Fig. 5a; two-way ANOVA; stress $p < 0.01$ $F_{1,18} = 9.259$; LPS $p < 0.001$ $F_{1,18} = 22.07$; stress:LPS $p < 0.05$ $F_{1,18} = 6.472$). At 24 h after LPS injection in non-stressed mice, the fivefold increase in hepatic *Tnf* compared to vehicle-treated controls was statistically significant (Fig. 5a; Bonferroni post hoc; $p < 0.001$). Chronic stress and LPS, combined,

appeared to the levels of TNFα mRNA compared to vehicle controls, but this change was not significant (Fig. 5a).

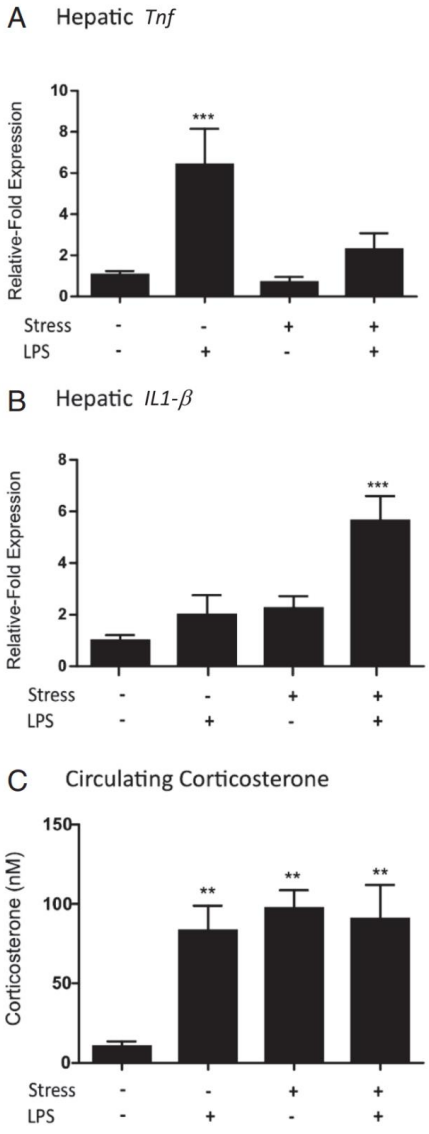

Fig. 5. Cytokine mRNA in the liver and blood corticosterone levels in control, stressed and LPS-treated animals. mRNA levels of **a** TNFα and **b** IL-1ß were measured by qPCR in the liver of animals after either 10 days of chronic stress, an acute LPS challenge (0.1 mg/kg) or a combination of both. Corticosterone levels in blood (**c**) were measured

by HPLC. qPCR data are expressed as relative-fold expression normalized to GAPDH and naïve mice. *Bars* are mean ± SEM, ($n = 5$ in each group), $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control animals

IL-1ß mRNA expression was affected by stress and LPS, but there was no significant interaction between the two factors (Fig. 5b; two-way ANOVA; stress $p < 0.001$ $F_{1,18} = 15.56$; LPS $p < 0.01$ $F_{1,18} = 12.61$; stress:LPS $p = 0.07$ $F_{1,18} = 3.711$). IL-1ß mRNA expression was slightly higher in animals treated with stress and LPS alone but in neither case are they significantly different from non-stressed, vehicle-treated controls (Fig. 5b). The combination of 10 days of chronic stress and a low-dose LPS challenge resulted in a significant sixfold increase in hepatic IL-1ß mRNA expression (Fig. 5b; Bonferroni post hoc; $p < 0.001$).

Control animals had an average of 10-nM baseline corticosterone (Fig. 5c). Both stress and LPS had a significant effect on corticosterone levels, and there was a significant interaction between these factors (Fig. 5c; two-way ANOVA; stress $p < 0.05$ $F_{1,25} = 4.605$; LPS $p < 0.01$ $F_{1,25} = 9.355$; stress:LPS $p < 0.05$ $F_{1,25} = 6.659$). More specifically, analysis showed that administration of 0.1 mg/kg LPS significantly increased circulating corticosterone when compared to controls, to an average of 90 nM (Fig. 5c; Bonferroni post hoc; $p < 0.01$). Following 10 days of stress and 10 days of stress in combination with an LPS challenge, elevated circulating corticosterone levels (100 nM) were also found and were significantly higher than controls (Fig. 5c; Bonferroni post hoc; stress alone $p < 0.01$, stress and LPS $p < 0.01$). At no point were stressed or LPS-treated animals different from each other, and stress combined with LPS did not result in an additive increase in corticosterone concentration.

3.5. Low-dose LPS-induced inflammation does not exacerbate chronic stress-induced changes in 5-HT2A and SERT expression or CNS cytokine expression

Previous work from our laboratory has demonstrated that both LPS and chronic stress are independently capable of changing the expression of the 5-HT_{2A} receptor and SERT mRNA expression [29, 35]. The data above demonstrate that LPS is capable of exacerbating certain behaviours induced by the chronic stress. Therefore, it is important to determine whether receptor expression was also cumulatively increased or whether, like corticosterone, low-level inflammation in stressed animals did not affect receptor expression. The addition of both stress and LPS into the model requires a more complex analysis with stress, LPS and brain regions as repeated factors. The general linear model applied to the earlier data remains with unstructured co-variance but with the added capacity of determining whether stress and LPS interact with each other. The number of possible interactions makes reporting this data rather excessive; therefore, only significant values are reported below.

IL-1ß mRNA levels were significantly affected by both stressors, either stress or LPS alone or combined and by brain region (Fig. 6a; RM-ANOVA brain region $p < 0.001$ $F_{4,48} = 16.91$; stress:LPS:brain region $p < 0.001$ $F_{4,48} = 13.69$). These factors also showed a significant interaction, suggesting that stress/LPS had a differential effect on IL-1ß mRNA levels in different brain regions (Fig. 6a; brain region:stressor $p < 0.001$ $F_{4,48} = 8.58$). Post hoc testing revealed significant effects of LPS alone, and stress combined with LPS, in the dorsal raphe nucleus (Fig. 6a; Bonferroni post hoc; $p < 0.001$ stress vs LPS; $p < 0.05$ control vs stress and LPS), and

these differences continued in the raphe when comparing animals that were only stressed for 10 days to animals that were stressed but also challenged with LPS (Fig. 6a; Bonferroni post hoc $p < 0.001$). Other brain regions only showed minor increases in IL-1ß receptor mRNA expression after either stress or LPS, and these did not reach significance (Fig. 6a). However, it should be cautioned that large changes in any individual brain region, such as the raphe, are likely to mask smaller changes in other brain regions.

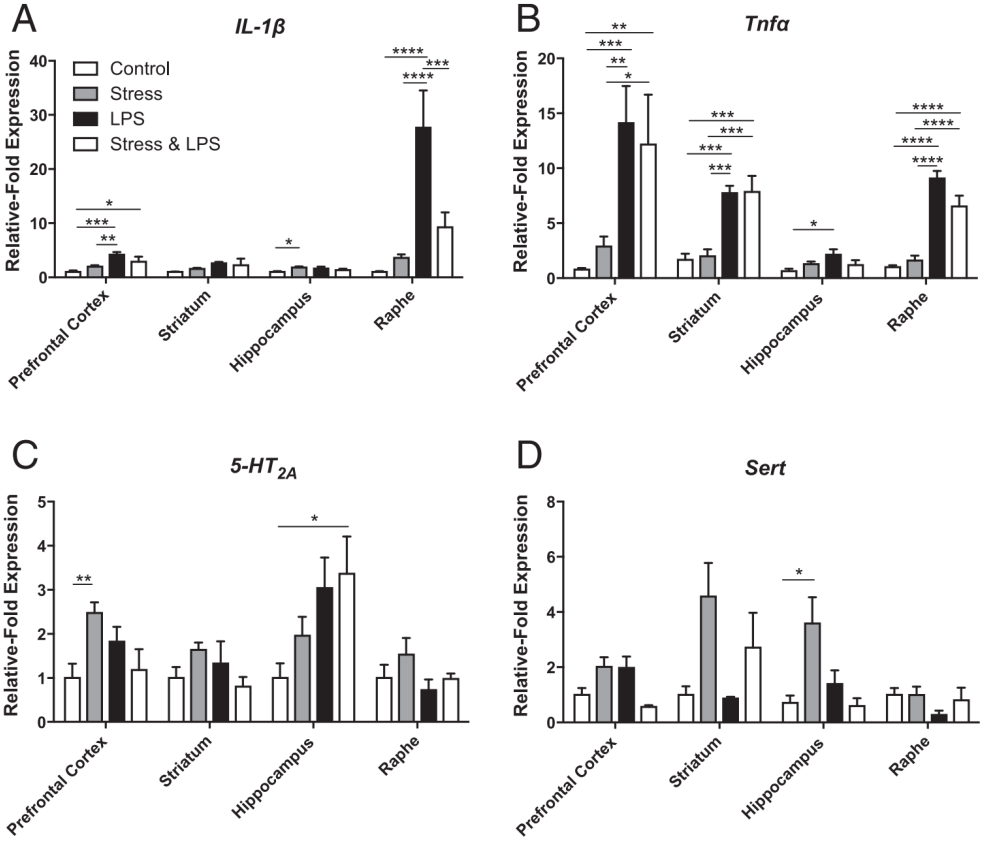

Fig. 6. L-1ß, TNFα, 5-HT_{2A} receptor and SERT mRNA expression in the brain structures of animals challenged with chronic stress, LPS or a combination of both. mRNA levels of **a** IL-1ß, **b** TNFα, **c** 5-HT_{2A} and **d** SERT were measured by qPCR in the pre-frontal

cortex, striatum, hippocampus and raphe of animals after either 10 days of chronic stress, an acute LPS challenge (0.1 mg/kg) or a combination of both. Values are expressed as relative-fold expression normalized to housekeeping gene GAPDH and to control values within each region. Data are mean ± SEM; $n = 5$ in each group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ compared to control animals

In the brain, TNFα mRNA expression was affected in a similar manner to IL-1ß mRNA expression, with significant main effects of both brain region and stressor and a significant interaction (Fig. 6b; RM-ANOVA brain region $p < 0.05$ $F_{4,48} = 15.64$; stressor $p < 0.01$ $F_{4,48} = 7.72$; stress:LPS:brain region $p < 0.05$ $F_{4,48} = 2.89$). Post hoc testing suggests that stress alone does not exacerbate TNFα mRNA expression but as with the IL-1ß results, larger changes in other regions may mask specific effects. LPS administration induced a significant increase in TNFα mRNA expression in all regions, with the exception of the hippocampus (Fig. 6b; Bonferroni post hoc; pre-frontal cortex $p < 0.0001$; striatum $p < 0.01$; raphe $p < 0.0001$). Stress combined with an inflammatory challenge results in a significant increase in TNFα expression in similar regions compared to control (Fig. 6b; Bonferroni post hoc; pre-frontal cortex $p < 0.001$; striatum $p < 0.01$; raphe $p < 0.05$). Finally, there were significant differences between stress-alone animals and animals stressed and challenged with LPS but only in the pre-frontal cortex (Fig. 6b; Bonferroni post hoc $p < 0.01$). However, there was no overt synergy between stress with LPS and LPS alone.

Analysis shows that there was only a significant main effect of brain region on 5-HT_{2A} mRNA expression, as well as a significant interaction between brain region, stress and LPS challenge (Fig. 6c; RM-ANOVA brain region $p < 0.001$ $F_{4,48} = 16.20$; stress:LPS:brain region $p < 0.01$ $F_{4,48} = 4.96$). Post hoc analysis shows 5-HT_{2A} receptor mRNA expression appeared to

increase after a single LPS injection in the pre-frontal cortex, striatum and hippocampus, compared to controls, but was only significantly different in the hippocampus (Fig. 6c; Bonferroni post hoc; $p < 0.01$). There was no difference, significant or otherwise, in 5-HT_{2A} mRNA levels in the raphe compared to controls (Fig. 6c). In a similar manner, after 10 days of chronic stress, 5-HT_{2A} mRNA appeared to be elevated in the pre-frontal cortex as well as the hippocampus but again, only reached significance in the latter when compared to control animals (Fig. 6c; Bonferroni post hoc; $p < 0.01$). Chronic stress did not change receptor expression in either the striatum or the raphe. In the CNS of animals challenged with 10 days of chronic stress and LPS, 5-HT_{2A} receptor mRNA expression was not different from controls in any region except the hippocampus, where it showed an increase of a similar magnitude to stress and LPS alone (Fig. 6c; Bonferroni post hoc; $p < 0.05$).

SERT mRNA expression showed the same main effects as for the 5-HT_{2A} receptor, but significant interactions were noted. Specifically, there was a main effect of brain region and interactions between brain region and stress, brain region and LPS challenge and all three factors (Fig. 6d; RM-ANOVA; brain region $p < 0.001$ $F_{4,48} = 22.23$; stress:brain region $p < 0.001$ $F_{4,48} = 15.46$; LPS:brain region $p < 0.001$ $F_{4,48} = 6.42$; stress:LPS:brain region $p < 0.01$ $F_{4,48} = 12.32$). Further analysis showed that SERT expression in the pre-frontal cortex after a single LPS challenge appeared to be higher than controls but did not reach significance (Fig. 6d; Bonferroni post hoc pre-frontal cortex $p = 0.081$). No other brain regions studied showed any change in SERT mRNA compared to controls after a single dose of LPS. Ten days of chronic stress did not change SERT expression in the pre-frontal cortex or the raphe compared to controls but led to significantly higher expression

in the striatum and hippocampus (Fig. 6d; Bonferroni post hoc; striatum $p < 0.001$, hippocampus $p < 0.01$). Compared to stress alone, the combination of stress and LPS did give rise to any significant increase SERT mRNA expression in any region studied (Fig. 6d).

4. Discussion

The studies reported here show that at a time when the effects of an intraperitoneal injection of LPS are no longer detectable in naïve animals, the combination of LPS with CMS increases depressive-like behaviours and inhibits the aggression and impulsivity induced by CMS. The aggressive and impulsive behaviours were accompanied by SERT induction in the hippocampus, which was ameliorated by the LPS treatment. The double-hit combination had no effect on LPS-induced TNFα expression but did suppress LPS-induced IL-1ß mRNA expression. Overall, SERT upregulation, rather than 5-HT_{2A} or the pro-inflammatory cytokines, appears to correlate with the stress-induced aggressive and impulsive behaviours. A similar independent increase in SERT was previously reported in stressed animals that become anhedonic [29]. Here, hepatic TNFα and IL-1ß mRNA levels differed between stressed mice injected with LPS compared to LPS alone in a surprising manner revealing a dissociation between the regulation of TNFα and IL-1ß mRNA expression. Moreover, these changes in hepatic cytokine expression appeared to be independent of corticosterone induction. These results are discussed in more detail below.

Using a low-dose LPS challenge after stress in both the sucrose preference test and the forced swim test, we showed that the downstream sequelae of a peripheral inflammatory response appeared to exacerbate the anhedonia and helplessness induced by stress. Indeed, there was

significant synergy for a reduction in sucrose intake. Non-stressed mice exhibit polydipsia, and this in known to be reduced by stress [57] and is further reduced by the LPS challenge, indicating that low levels of systemic inflammation that may not generate overt clinical signs per se can synergize with a stress-induced depressive illness and provoke a worsening phenotype. Thus, the diagnosis and treatment of low-grade inflammatory disease in patients may reduce some select depressive signs by mechanisms that are independent of those that are associated with major depression and those targeted by traditional antidepressants. Others have shown that the combination of endotoxin with stress in mice can result in increased mortality [58] but such severe experiments (40 mg/kg LPS compared to 0.1 mg/kg in our studies) did not set out to explore the subtle relationship between low-level infection and stress. In rats, lower levels of endotoxin were previously used to discover how inescapable shock-induced stress would be altered [59, 60]. In these experiments, the febrile response associated with inescapable shock and LPS was increased and this was associated with enhanced pro-inflammatory cytokine responses. As in our experiments, Johnson et al. [60] found the relationship between cytokine expression and the double-hit of stress and inflammation was not a straightforward relationship; enhanced pro-inflammatory cytokine responses where not necessary to observe enhanced HPA or fever responses after LPS and inescapable tailshock.

Work studying the immune response after a stressful event has suggested that stress 'primes' the inflammatory response for an immune challenge, making it more sensitive [15]. The depressive-like behaviours associated with an LPS challenge have also been shown to be ameliorated by imipramine and fluoxetine given prior to LPS administration [61], and

our results suggest that while antidepressants might target the post-infection component of the combination, anti-inflammatory therapy might also be beneficial. Indeed, celecoxib administered as an adjunctive non-steroidal anti-inflammatory drug (NSAID) appears to produce a positive therapeutic outcome in the treatment of depression [62].

In this study, although chronically stressed mice exhibit anhedonia, they also display increased rates of aggressive behaviour in the resident-intruder test where attacking and crawl over behaviours were markedly increased. Crawl overs have been investigated in rats and form part of juvenile play fighting. However, such behaviour has also been observed in aggressive encounters. In rats, crawl overs occur when the rats are unfamiliar with one another and seem to be important in establishing dominance [45]. Such stress-induced changes in attack frequency have been previously described using the same CMS regime as employed here [36]. A paradoxical 'anxiolytic-like profile', manifest as increased impulsivity in the elevated O-maze, was also observed in response to stress, in line with previously reported findings [63]. In contrast, stressed mice subjected to a low-level LPS challenge displayed a reduction in aggressive behaviour in the resident-intruder test and no signs of impulsivity/hyperlocomotion in the elevated O-maze. In studies of aggression and impulsivity, the combination of stress and low-level inflammation therefore appears to counteract, rather than exacerbate, the negative effects of stress on behaviour.

Changes in measures of aggressiveness and impulsivity/hyperactivity were accompanied by differential expression of SERT in the brain. In the hippocampus, mRNA levels of SERT were increased in chronically stressed mice. In stressed mice challenged with LPS, expression levels of SERT in

the hippocampus did not change, but they did tend towards a decrease in the pre-frontal cortex. Chronically stressed mice without exposure to LPS displayed a non-significant increase in SERT expression in the pre-frontal cortex. These data are in accordance with our previous observations [30]. Elevated SERT expression was previously reported in mice displaying aggressive behaviour induced by repeated social confrontation stress [64]. The increase in SERT in the limbic structures of the brain is frequently found after stressors of various types [65]. In contrast, a decrease in SERT expression in similar structures was shown to be a molecular correlate of clinical depression [66] and of an experimentally induced depressive-like state in animals [67]. These data, in combination with our own, suggest changes in molecular signals within specific brain regions may result in behaviourally distinct outcomes.

In vitro and in vivo studies have shown that pro-inflammatory cytokines, such as IL-1ß and TNFα, can increase SERT activity via the p38 MAPK signalling pathway [46]. Behavioural signs of helplessness resulting from circulating cytokines have been shown to be prevented by a blockade of SERT [68]. Furthermore, SERT mutant rats show abnormal behaviour (including decreased sucrose preference, decreased spontaneous activity and increased anxiety [69]) and CNS cytokine expression profiles in response to LPS [70]. In humans, however, the reverse appears to be true. Clinical studies reveal that decreased SERT function, associated with the short variant of the SERT gene and lower SERT activity, correlates with an increased risk of developing depression during IFN-α treatment [71]. Indeed, our own work has demonstrated that there is no change in the release of 5-HT in response to LPS, suggesting a post-synaptic mechanism may be more crucial to sickness behaviour [72]. Thus, the relationship

between SERT activity and responsiveness to pro-inflammatory factors in the regulation of depression pathogenesis appears to be complex and is liable to explain the differences we observed in aggressive behaviour associated with stress alone vs stress in combination with an inflammatory challenge.

The levels of 5-HT_{2A} mRNA were different in mice subjected to stress alone to those additionally challenged with LPS. Previously, elevation of 5-HT_{2A} in the limbic structures was documented as an important correlate of a depressive-like state, which represents a target for pharmacological treatment [73]. In line with our previous observations [29, 72], such changes were found in the pre-frontal cortex of stressed mice but not in naïve or stressed mice injected with LPS. However, a significant elevation of 5-HT_{2A} expression was detected in the hippocampus of the two latter groups, in line with similar findings elsewhere showing that inflammation significantly affects 5-HT_{2A} [35, 74]. The similarities in receptor expression profiles regardless of stress exposure suggest that changes in the expression of the 5-HT_{2A} receptor are unlikely to mediate the exacerbated behavioural effects observed in the double-hit mice.

Importantly, our low-dose LPS challenge in naïve animals resulted in the over-expression of TNFα in several brain structures, including the pre-frontal cortex, but this was not associated with alteration in the behaviours tested. Such findings are in accord with previously published results, showing that cytokine over-expression exerts minimal effects on social behaviour in rodents [56]. The expression of IL-1ß in the dorsal raphe nucleus was significantly elevated in both naïve and stressed LPS-treated groups. However, this effect is also unlikely to underlie behavioural differences between chronically stressed mice, with or without LPS

challenge, since naïve mice showed no obvious behavioural changes in aggression or depressive-like behaviours.

Stress is well known to increase circulating cortisol, and there is evidence linking cortisol levels and depression. Depressed patients frequently show dexamethasone non-suppression, suggesting hyperactivity of the Hypothalamic–pituitary–adrenal HPA axis [75]. Corticosterone levels are similar in animals subjected to either CMS or LPS and thus could not explain the phenotypic differences observed between stressed and LPS-challenged animals. These data are in line with previously reported findings [76] although oddly, the increase in corticosterone as a result of stress does not appear to reduce the hepatic inflammatory response. This data, and that in adrenalectomized animals, suggests that the pro-inflammatory profile during stress is independent of cortisol and may be the result of anti-inflammatory cytokines and downstream signalling pathways [77].

5. Conclusions

Here, we have shown that the effects of chronic stress and LPS are reflected by dissociated alterations in both behaviour and gene expression, with elevated SERT expression appearing to be linked to stress-induced aggression. Furthermore, we have found that the molecular and behavioural changes induced by stress or low-grade inflammatory challenges are distinct and, when the challenges were combined, some of the behaviours synergized and others, such as the aggressive behaviours, were suppressed. It seems likely that distinct mechanisms enabling the body to effectively deal separately with stress vs infection have evolved but there is no doubt that the presence of low-grade inflammation can

have a profound effect on stress-induced behaviours; the underlying mechanisms are likely to be of relevance in humans, where such combinations may precipitate depressive episodes.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YC participated in the design of the experiments, carried out the molecular studies, performed the statistical analysis and drafted the manuscript. AT, NM and VN carried out the behavioural experiments and helped with the analysis. HWS, VC, CS and K-PL advised on the experimental design and helped to draft the manuscript. DCA and TS conceived of the study, participated in its design and coordination and edited the manuscript. All authors read, edited and approved the final manuscript.

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APPENDIX A

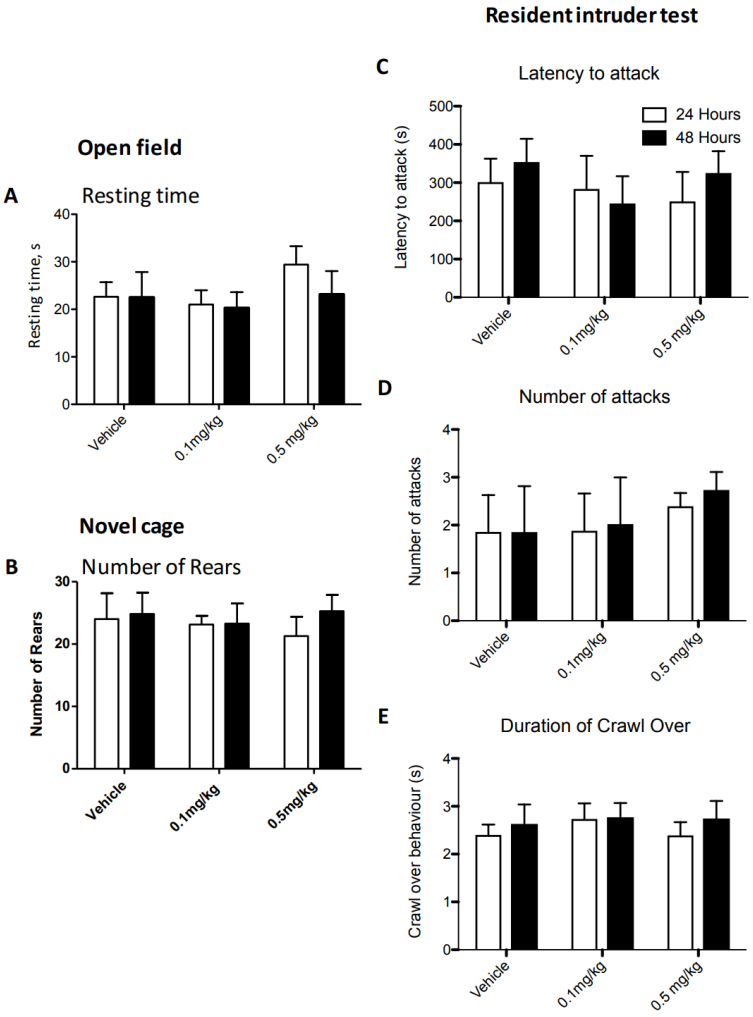
Supplementary Methods

Supplementary table (ST1): Primer sequences for qPCR. Primers were custom designed and validated by Primer Design Ltd. (Southampton, UK)

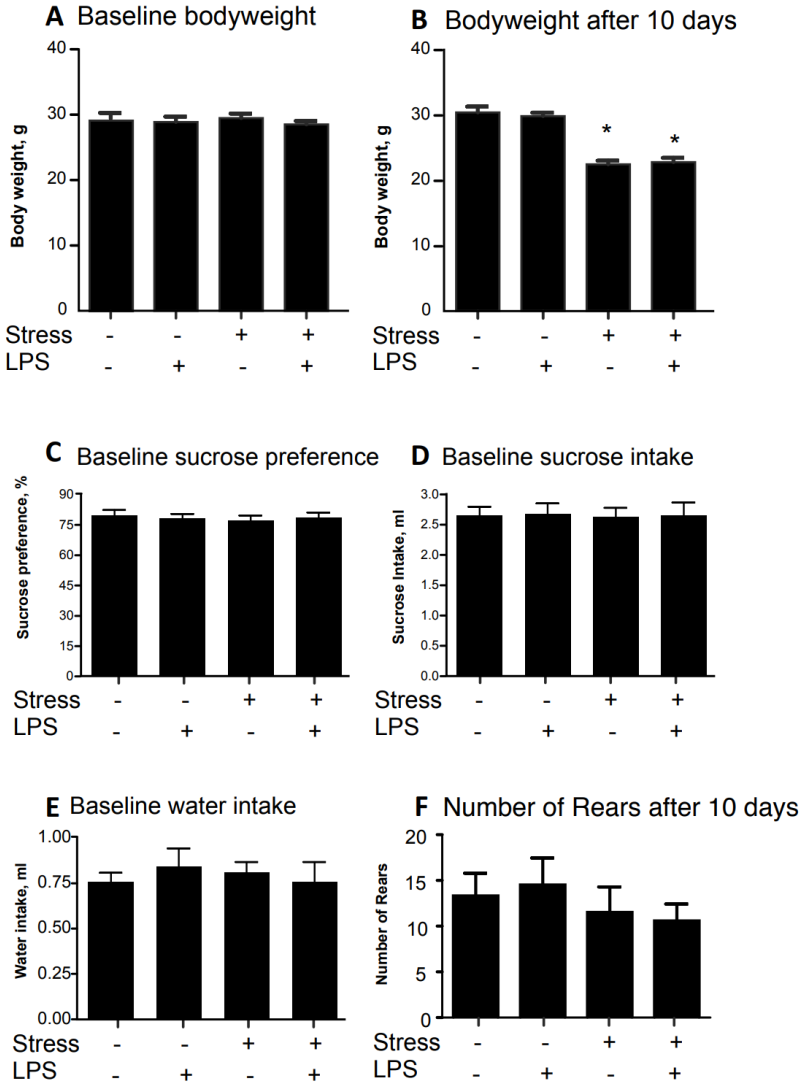
Marker	Forward primer	Reverse primer	Amplicon size (nt)
Tnf	GCTCCCTCTCATCAGTTCTAT	TTTGCTACGADCTGGGCTA	94
IL-1ß	CAACCAACAAGTGTATTCTCCAT	GTGTGCCGTCTTTCATTA	127
5-HT2A	CAGGCAAGTCACAGGATAGC	TTAAGCAGAAAGAAAATCCCACAG	93
Sert	TGCCTTTTATATCGCCTCCTAC	CAGTTGCCAGTGTTCCAAGA	127

APPENDIX B

Supplementary Data

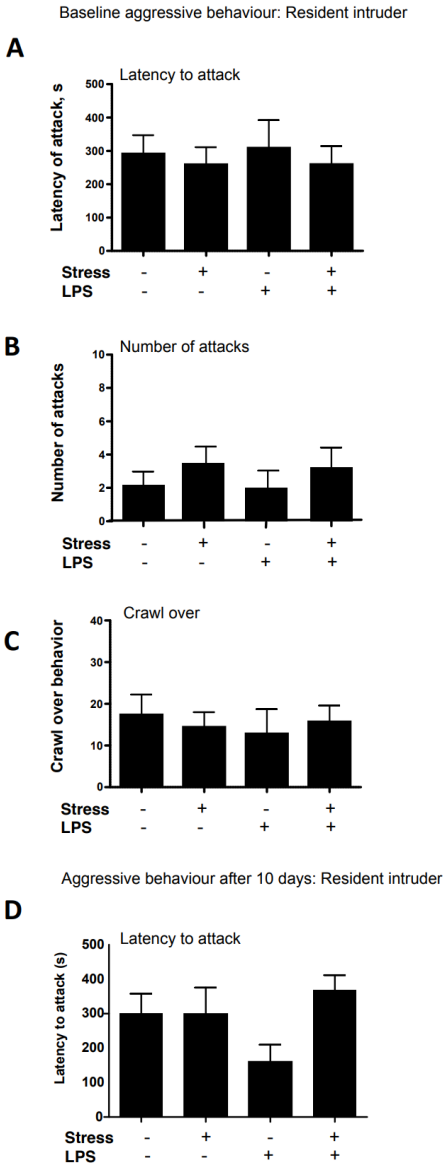


Supplementary Figure 1. The effect of low dose of LPS on locomotor activity at 24 and 48 hours post-challenge in naïve mice. Naïve animals were subjected to a single dose of LPS (0.1 mg/kg or 0.5 mg/kg) or vehicle injection, and were tested at 24 h or 48 h post-injection. (**A**) Neither the resting time was unaltered by the treatment in the Truescan open field nor (**B**) rearing in the novel cage test for total number of rear. (**C-E**) Aggressive behaviour was also unaltered. Data are mean ±SEM, 2-way ANOVA throughout.



Supplementary Figure 2. (**A&B**) Body weight in the chronic stress experiment. Experimental groups were balanced upon baseline mean values of body weight measured seven days prior the start of the chronic stress experiment and LPS challenge. Mice exposed to chronic stress had a significant reduction in body weight as compared with baseline measurements ($p^*<0.05$, pair-wise t-test). Chronically stressed mice injected either with vehicle or LPS had similar mean body weight prior LPS challenge. (**C,D&E**) Sucrose preference. Experimental groups were balanced upon baseline mean values of sucrose preference when evaluated seven days prior the experiment chronic

stress procedure and LPS challenge. Experimental groups had similar mean measures of sucrose and water intake. ($p>0.05$, one-way ANOVA and post-hoc Tukey test; see the text). (**F**) Naïve and stressed animals (10 days) were challenged with a single dose of LPS (0.1mg/kg) or vehicle (saline) and tested 24 thereafter in a novel cage test for total number of rears (see the text). Data are mean ±SEM. No differences between the groups was observed.



Supplementary Figure 3. (**A, B, &C**) Baseline behaviour in a resident-intruder test. Experimental groups were balanced upon baseline mean scores of behaviours in a resident-intruder test that were studied seven days prior the experimental chronic stress procedure and LPS challenge. Mice had similar mean measures of (**A**) latency to attack, (**B**) number of attack and duration of crawl over behaviour (**C**). ($p>0.05$, one-way ANOVA and post-hoc Tukey test; see the text). (**D**) The latency to attack after the chronic stress was not significantly altered.

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CHAPTER 3

Lipopolysaccharide triggers exacerbated microglial activation, excessive cytokine release and behavioural disturbances in mice with truncated Fused-in-Sarcoma Protein (FUS)

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Chapter 3. Lipopolysaccharide triggers exacerbated microglial activation, excessive cytokine release and behavioural disturbances in mice with truncated Fused-in-Sarcoma Protein (FUS)

Abstract

CNS inflammation, including microglial activation, in response to peripheral infections are known to contribute to the pathology of both familial and sporadic neurodegenerative disease. The relationship between Fused-in-Sarcoma Protein (FUS)-mediated disease in the transgenic FUS[1-359] animals and the systemic inflammatory response have not been explored. Here, we investigated microglial activation, inflammatory gene expression and the behavioural responses to lipopolysaccharide-induced (LPS; 0.1 mg/kg) systemic inflammation in the FUS[1-359] transgenic mice. The pathology of these mice recapitulates the key features of mutant FUS-associated familial frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS). Here, pre-symptomatic 8-week-old mutant or wild type controls were challenged with LPS or with saline and sucrose intake, novel cage exploration, marble burying and swimming behaviours were analysed. The level of pro-inflammatory gene expression was also determined, and microglial activation was evaluated. In chronic experiments, to discover whether the LPS challenge would affect the onset of ALS-like paralysis, animals were evaluated for clinical signs from 5 to 7 weeks post-injection. Compared to controls, acutely challenged FUS[1-359]-tg mice exhibited decreased sucrose intake and increased floating behaviours. The FUS[1-359]-tg mice exhibited an increase in

immunoreactivity for Iba1-positive cells in the prefrontal cortex and ventral horn of the spinal cord, which was accompanied by increased expression of interleukin-1ß, tumour necrosis factor, cyclooxygenase-(COX)-1 and COX-2. However, the single LPS challenge did not alter the time to development of paralysis in the FUS[1-359]-tg mice. Thus, while the acute inflammatory response was enhanced in the FUS mutant animals, it did not have a lasting impact on disease progression.

Keywords: Frontotemporal lobar degeneration (FTLD); Amyotrophic lateral sclerosis (ALS); Fused in sarcoma (FUS) protein; Lipopolysaccharide (LPS); Interleukin-1ß (IL-1ß); Tumour necrosis factor (TNF); Cyclooxygenase-1 (COX-1); Cyclooxygenase-2 (COX-2); Emotionality

1. Introduction

Dysfunction of Fused in Sarcoma (FUS) gene expression is associated with frontotemporal lobar degeneration (FTLD) and sporadic and familial forms of amyotrophic lateral sclerosis (ALS) (Feldman et al., 2022; Puppala et al., 2021). Both FTDL and ALS are devastating uncurable disorders and thus, their prevention and treatment remain an important unmet need (DeLoach et al., 2015; Sivasathiaseelan et al., 2019). Various forms of the FUS mutation cause RNA dysfunction and pathological protein aggregation in neurons, leading to their degeneration and death (Ling et al., 2013). Although the mechanism of cell death remains obscure.

The latest evidence suggests the non-neuronal mechanisms, such as bystander damage following glial activation, may also contribute (Vahsen et al., 2021). For example abnormal recognition and responses to toxic elements, impaired phagocytosis, or a switch to a neurotoxic cytokine over-expression by dysfunctional microglia have all been shown to be

associated with mutations of the genes associated with FDTL and ALS: SOD-1, TREM2, C9Orf72, GRN TBK1, OPTN, VCP, SQSTM1 and PFN1 (Feldman et al., 2022; McCauley and Baloh, 2019). Concerning FUS, transcriptome analysis of transgenic mice expressing the truncated highly-aggregate-prone form of human FUS has revealed an abnormal expression of microglial gene (Funikov et al., 2018). In vitro, overexpression of wild-type FUS gene in mouse and human astrocytes has been reported to increase their sensitivity to pro-inflammatory cytokine interleukin (IL)-1ß and lead to an over-expression of inflammatory mediators, microglial activation, and neuronal cell death (Ajmone-Cat et al., 2019).

The mutations also result in systemic inflammation in FTLD and ALS patients, which is a prominent feature of these pathologies and contributes to their development (Goutman et al., 2022; McCauley and Baloh, 2019). Elevated levels of pro-inflammatory cytokines tumour necrosis factor (TNF), IL-6, granulocyte-macrophage colony-stimulating factor (G-CSF), macrophage inflammatory protein 1α (MIP1α) and other inflammatory mediators in the cerebrospinal fluid and blood were found to be elevated in FTLD and ALS patients (Bright et al., 2019; Hu et al., 2017; McCauley and Baloh, 2019).

The occurrence systemic inflammation preceding FTLD or ALS has been recognized as a risk factor for these disorders. For example, bacterial and viral infections (Alam et al., 2017), occupational exposure to toxins of various kinds (Goutman et al., 2022; McCombe and Henderson, 2011), autoimmune conditions (Corzo et al., 2022; Lin et al., 2018; Sangha et al., 2020), e.g. type 1 diabetes, multiple sclerosis (Cui et al., 2021), and dysregulation of gut bacteria regulating inflammation (Nicholson et al.,

2021) have all been shown the affect the development of FTLD or ALS. However, little is known about an interaction of pre-existing pro-inflammatory status with aberrant immunity under condition of genetically determined FTLD and ALS syndromes such as the FUS mutations. Here, we used the FUS[1-359]-tg mouse line, expressing truncated highly-aggregate-prone form of human FUS, which displays the FTLD-like and ALS-like characteristics in both pre-symptomatic and symptomatic stages at 8 and 14 weeks respectively (de Munter et al., 2020a; de Munter et al., 2020b; Sambon et al., 2020; Shelkovnikova et al., 2013), in conjunction with a systemic lipopolysaccharide (LPS) challenge.

Earlier studies with the FUS[1-359]-tg mice revealed that the pathology is associated with over-expression of the pro-inflammatory cytokines IL-1ß and IL-6 in the brain, blood and spinal cord, and behavioral deficits in the symptomatic FUS[1-359]-tg mice (de Munter et al., 2020; de Munter et al., 2020; Lysikova et al., 2019). However, gene expression studies failed to reveal any signs of altered microglial function or of inflammatory markers in the CNS of naïve pre-symptomatic FUS[1-359]-tg mice (Chaprov et al., 2021). Here, we studied immunohistochemical expression of Iba-1 in the prefrontal cortex (PFC), hippocampus (HIP) and spinal cord (SC) of naïve pre-symptomatic 8-week old FUS[1-359]-tg mice 24 h after an LPS injection. We also measured gene expression for IL-1ß, TNF, cyclooxygenase(COX)-1 and COX-2 at 24 and 48h following the challenge. In a separate cohort of mice, we investigated whether an LPS administration affects the onset of paralysis in the mutants from 5 to 7 weeks after the challenge.

2. Materials and methods

2.1. Animals and Study design

8 week-old FUS[1-359]-tg (FUS-tg) and wild type (WT) male mice were housed under standard conditions *(see Supplementary File (SF))*; all protocols complied with 2010/63/EU and ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines). In the first set of experiments, ten mice of each genotype were assigned to four groups, and received an intraperitoneal (i.p.) injection of either saline or LPS (0.1 mg/kg; *see below*) and tested for sucrose intake between 18 and 24 hours following the challenge and before being culled for an immunohistochemical (IHC) study of Iba-1-positive cell density in the PFC, HIP and SC 24h post-injection (*Suppl.Fig.1A*). In a second set of animals, 6 to 14 h following the same LPS-challenge, ten mutants and ten controls were investigated in the novel cage exploration test, pellet displacement behaviour marble test and forced swim test and these animals were culled 24 h post-injection to determine the level of expression of selected pro-inflammatory mediators (*see below*) in the PFC, HIP and SC (*Suppl.Fig.1B*). In the final cohort of animals, 8-week old FUS-tg mutants were injected with LPS or saline (12 and 13 animals per group, respectively) and the onset of paralysis after the injection was recorded (Sambon et al.,2021; *Suppl.Fig.1C)*. Finally, ten 8-week-old mutants were assigned to the four groups, and were injected with either LPS or saline and culled for RT-PCR in selected CNS structures (*Suppl.Fig.1D*).

2.2. Behavioural tests

The behavioural experiments were performed as described elsewhere (J. de Munter et al., 2020; Strekalova and Steinbusch, 2010; Strekalova et al., 2022) (*see SF*).

2.3. LPS Challenge

The mice (8-week-old FUS[1-359]-tg (FUS-tg) and wild type (WT) males) were injected with LPS dissolved in sterile saline (0.9%) (E.coli 0111:B6, Sigma, St.Louis, MO, USA) at the dose 0.1 mg/kg, i.p. in 0.1 ml of the sterile saline vehicle control (Couch et al., 2016; Trofimov et al., 2017).

2.4 Culling and tissue Collection

Mice were terminally anaesthetized with an i.p. injection of sodium pentobarbitone and their left ventricle was perfused with 10 mL ice-cold saline (for PCR assay) followed by 4% paraformaldehyde solution (for IHC assay). The PFC, the HIP and lumbar segments of SC were isolated and stored as previously described (Couch et al., 2016; de Munter et al., 2020a; de Munter et al., 2020b).

2.5. Immunohistochemical analysis of Iba-1-positive cells

Immunostaining with Iba-1 antibodies and image analysis were performed as previously described (Couch et al., 2013) (*see SF*). Briefly, 10 μm-thick brain coronal sections and SC sections were cut, mounted on gelatin-coated slides, incubated with primary Iba-1 antibody and counter-stained with 4′,6-diamidino-2-phenylindole (DAPI). Cell counting was

performed using ImageJ software and the density of Iba-1-positive cells was calculated. We also examined blood-brain barrier (BBB) breakdown in the 3 month-old animals at the point at which they were culled as they began to show clinical signs. There was no evidence of any BBB breakdown at this timepoint (*Suppl.Fig.2*).

2.6. Real-time polymerase chain reaction (RT-PCR)

mRNA was extracted by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) from snap-frozen samples of PFC, HIP and SC (J. P. J. M. de Munter et al., 2020; J. de Munter et al., 2020; Trofimov et al., 2017). First strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 µg total RNA was converted into cDNA. RT-PCR of IL-1ß, TNF, COX-1, COX-2 in the samples of PFC, HIP and SC were performed; relative gene expression was calculated using the □□C_T method and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene and to the expression of the control sample (for list of primers, see *Supplementary Table S1)*.

2.7. Statistics

Data were analyzed using GraphPad Prism version 9.0 for MacOS (San Diego, CA, USA). Two-way ANOVA followed by post-hoc Tukey tests were applied to analyze the effects of treatment in the two mouse genotypes. The level of significance was set $p<0.05$. Data are Mean ± SEM.

3. Results

3.1. Iba-1-positive cell density in the CNS of FUS-tg mice challenged with LPS

A significant genotype × treatment interaction was discovered for Iba-1-positive cell density in the PFC, and a significant treatment effect was found in the ventral and dorsal horn of SC, and in the dentate gyrus of the HIP ($p<0.05$, two-way ANOVA; *SF, Table 2*). While LPS-challenged control WT mice exhibited an increase in density of Iba-1 positive cells in the dorsal horn of the SC, the LPS-challenged FUS-tg animals exhibited increases in both dorsal and ventral horn of the SC, the PFC, and in the dentate gyrus of the HIP (Fig.1A).

3.2. FUS-tg mice reveal increased baseline and LPS-induced cytokine expression 24h post-injection

In the prefrontal cortex (PFC) there was a significant genotype × treatment interaction for levels of expression of *IL-1b*, *TNF* and *COX-2*, as well as significant treatment and genotype effects; significant treatment effect was shown for *COX-1* expression ($p<0.05$, two-way ANOVA; *SF, Table 3*). LPS-challenged FUS-tg mice showed significantly higher PFC expression of transcripts for the proinflammatory mediators (*IL-1b*, *TNF* and *COX-2*) than saline-treated FUS-tg mice and the LPS-challenged wild types (Fig.2A). In the hippocampus (HIP), a significant genotype × treatment interaction for the concentration of TNFmRNA was found, and a significant treatment effect was shown in for IL-1b, TNF, COX-1 and COX-2. A significant genotype effect was found for TNF and COX-1 ($p<0.05$; *SF, Table 4*). Tukey post-hoc testing revealed significantly higher levels of *IL-1b*

mRNA and *TNF* mRNA in FUS-tg-LPS-treated mice than in saline-treated FUS-tg animals; TNF was also higher in LPS-treated wild type mice compared to saline-treated controls. Saline vehicle-treated FUS-tg mice demonstrated an increased COX-1 compared to saline vehicle-treated controls (Fig.2B), which highly the presence of evolving inflammatory pathology at an early timepoint in the FUS mutants. In the SC, there was a significant genotype × treatment interaction for the concentrations of TNF mRNA, as well as significant treatment effect in concentrations of IL-1bmRNA, TNFmRNA, COX-1mRNA and COX-2mRNA and significant genotype effect for TNFmRNA and COX-2mRNA ($p<0.05$, *SF, Table 5*). Post-hoc testing revealed that there were significant increases in TNFmRNA, COX-1mRNA and COX-2mRNA levels in FUS-tg-LPS challenged mice in comparison with the saline-treated FUS-tg mice, as well as higher levels of TNFmRNA in the FUS-tg-LPS challenged mice than in LPS-challenged controls (Fig.2C).

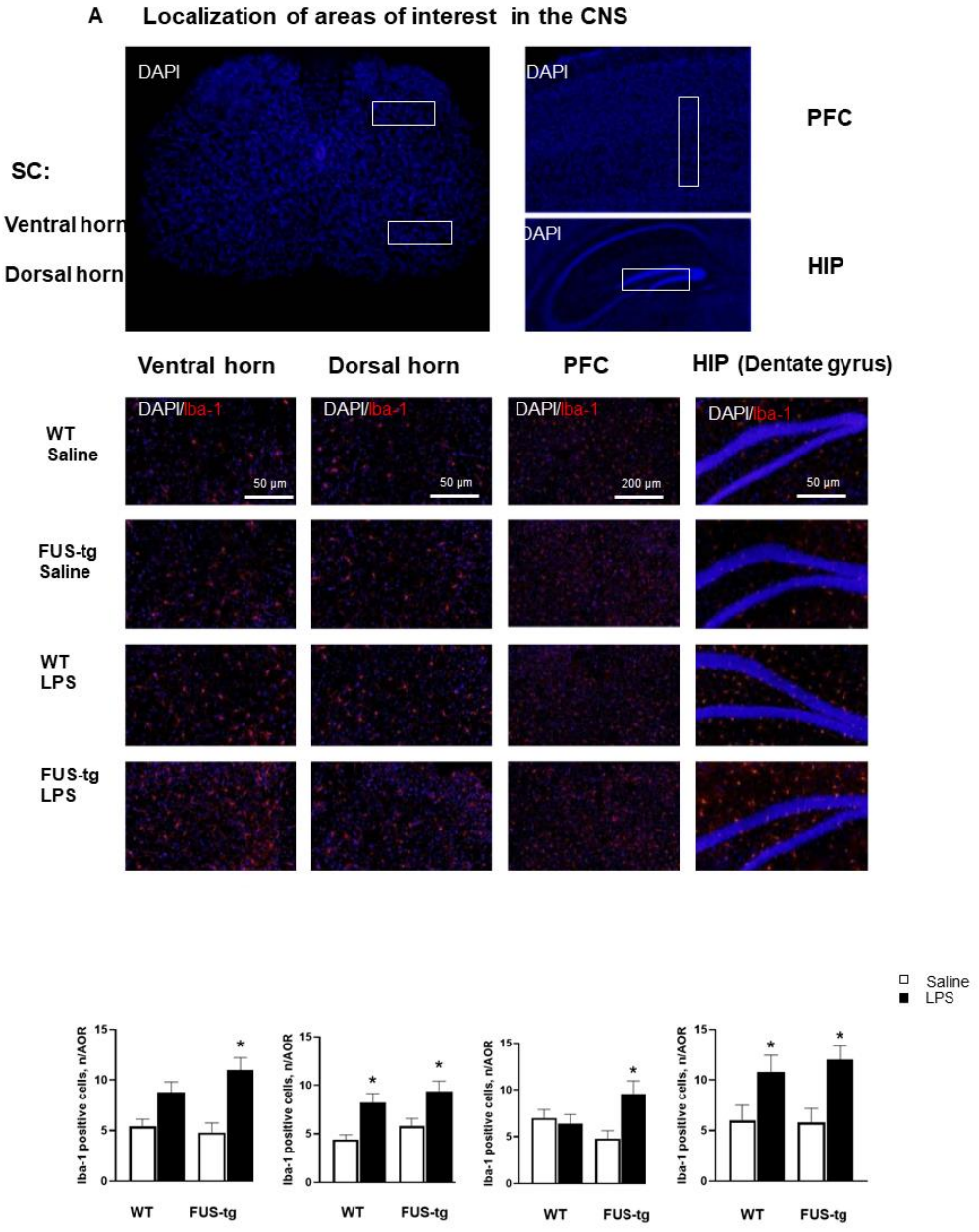

Figure 1. Iba-1-staining in CNS of FUS-tg mice. (A) Areas of interest in CNS. Representative image of Iba-1 and DAPI-staining in WT-saline, FUS-

tg-saline, WT-LPS and FUS-tg-LPS mice. **(B)** LPS-induced increases of the density of Iba-1-positive cells in WT and FUS-tg mice (two-way ANOVA and Tukey's test; *p<0.05 vs. respective control group).

3.3. Exacerbated behavioural responses in LPS-challenged FUS-tg mice

A two-way ANOVA did reveal a genotype × treatment interaction for sucrose intake, but there was a significant effect of both the genotype and treatment on sucrose intake ($p<0.05$, two-way ANOVA; SF, Table 6). Post-hoc testing revealed the presence of a significant decrease in sucrose intake in the LPS-challenged FUS-tg mice compared to both non-treated FUS-tg mice and LPS-challenged wild types (Fig.2D). In the novel cage test, there was a significant effect of treatment on the number of rears ($p<0.05$, SF, Table 6). A post-hoc Tukey test demonstrated a reduction of the number of rears in both LPS-treated groups in comparison with the saline-treated animals for each minute of observation and for the 5-min value (Fig.2E). In the marble test, there was a significant effect of genotype and the treatment on the number of displaced pellets ($p<0.05$, *SF, Table 6*). Post-hoc testing showed a reduced number of displaced pellets in LPS-challenged wild types and saline-treated FUS-tg mice both in comparison with saline-injected controls (Fig.2F). In the forced swim test, there was a significant treatment effect on the latency to float, the number of floating episodes, but not on the duration of floating; a significant effect of genotype was found in first two parameters ($p<0.05$, *SF, Table 7*). Tukey's post hoc test showed a significant reduction in the latency to float and an increase of the duration of floating in FUS-tg LPS-challenged mice in comparison with wild type LPS-challenged mice (Fig.2G).

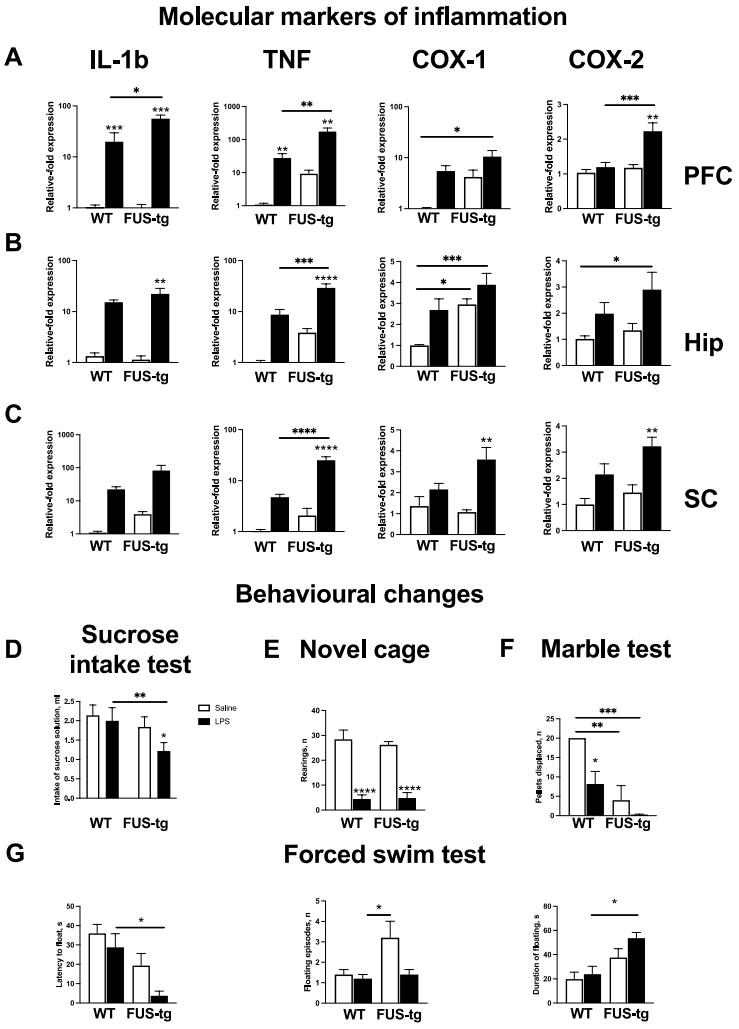

Figure 2. LPS-induced molecular and behavioral changes in FUS-tg mice. Following 24 h post-LPS-challenge, there were increases in mRNA of IL-1β, TNF, COX-1 and COX-2 in both genotypes, in **(A)** PFC, **(B)** HIP and **(C)** SC, where these changes were more pronounced in mutants. Note that COX-1 mRNA level was increased in Hip of FUS-tg mice prior to the LPS injection. Molecular changes were accompanied by **(D)** altered sucrose intake in FUS-tg-LPS group, **(E)** decreased total rearing scores in the novel cage and counted per minute in both genotypes, **(F)** lowered total number of displaced pellets in marble test and counted each 15 min in both

genotypes, **(G)** shortened latency to float, prolonged duration of floating in mutants and unchanged number of floating episodes in FUS-tg-LPS group (two-way ANOVA and post-hoc Tukey's test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Comparison without bar = saline versus LPS & bridging lines indicate other comparisons).

3.4. Lack of genotype differences in LPS-induced gene expression 48h post injection and unchanged onset of the ALS-like paralysis in LPS-challenged mutants

At time point 48h post-challenge, no genotype × treatment interaction differences were found in the mRNA concentrations of investigated genes (p>0.05, two-way ANOVA; *SF, Tables 8-10*). There was a significant effect of treatment on this measure, no significant effect of genotype (p<0.05 and p>0.0,05, respectively); Tukey's test revealed no significant differences between LPS-treated groups (p<0.05) suggesting a rapid decay of the exacerbated sensitivity to LPS administration of FUS-tg mice.

Finally, we found similar age in days at which the groups of LPS-challenged and unchallenged FUS-tg-mice displayed the first signs of paralysis after the injection (25.58 ±2.11 and 25.38 ±2.20, respectively, p=0.93, *t*-test), which was at approximately 3 months of age.

4. Discussion

Following the LPS injection, two-way analysis also revealed suppression of hippocampus-dependent performance in the marble test and elevated gene expression of IL-1ß, TNF, COX-1 and COX-2 in the hippocampus and spinal cord, and the response was always greater in the

FUS-tg mice. It is noteworthy, that COX-1 mRNA was elevated in the hippocampus of FUS-tg mice prior to the LPS injection, indicating the presence of evolving inflammatory process in the pre-symptomatic FUS-tg mice, which is in keeping with our previous data (de Munter et al., 2020a). Together, the data show that there is an exacerbated response to LPS in the young pre-symptomatic FUS-tg mice, and the effect is most obvious in the PFC. However, by 48h after the LPS injection, no genotype-associated differences in the immune response remained, suggesting a rapid decay of the increased pro-inflammatory response in the mutants. This may explain why the challenge failed to affect the onset of the ALS-like motor syndrome in FUS-tg mice subjected to a single LPS challenge found in our work.

Multiple genetic mutations are associated with familial forms of ALS, among which mutations in the FUS gene represent one subtype. The FUS[1-359] transgenic mouse model has been designed to carry a truncated form of the FUS protein to study the pathogenesis of ALS related to FUS mutations and the FUS[1-359] transgenic mouse model is primarily tailored to study FUS-mediated ALS pathogenesis. However, the insights gained here from this model are likely be relevant to understanding ALS from a broader perspective as the same molecular pathways are commonly disrupted in various genetic forms of ALS. For instance, mutant FUS can lead to mislocalization of the protein, and similar protein mislocalization or aggregation is observed in other ALS-related genes like TDP-43. Clearly, while there are shared pathways, each genetic mutation will also have its own unique pathology. It is important to note that the FUS[1-359] exhibits hallmark neuropathological features of ALS, such as motor neuron loss, protein aggregates, and gliosis. Even if the FUS[1-359] model primarily

represents FUS-related pathology, potential treatments that prove effective in this model may have broader applicability. Conversely, treatments targeting very specific FUS mechanisms might not be relevant for other forms of ALS, but the insights gained are important for those with FUS mutations. As there is growing interest in understanding how various ALS-related genes might interact with other challenges, and here we sought to explore the interaction between a proinflammatory challenge (LPS) and the FUS mutation. The LPS challenge is a somewhat reductionist approach to mimicking a bacterial infection, which would be expected to last for much longer, but our experiments reveal that the brain of the FUS-tg mouse is primed and is more susceptible to such systemic inflammatory challenges and gave rise to elevated production of cytokines and sickness behaviors. We employed a LPS challenge of 0.1mg/kg, that is not considered sufficient to cause BBB breakdown or give rise to long-lasting pathological or behavioral changes. We have previously shown that the administration of 0.5mg/kg LPS, which was used to reactivate a quiescent MS-like lesion in the murine brain, does not cause any BBB breakdown despite increasing local rCBV and provoking new leukocyte recruitment to the lesion (Serres et al., 2009). Others have used 3mg/kg to increase the permeability of the BBB, which induces sepsis like conditions in rodent (Xaio et al., 2001). We accept that the addition of the FUS pathology with a low dose of LPS used here might have a synergistic effect on blood-brain barrier integrity, a factor that might contribute to elevated cytokine production in the brain, and we will investigate this possibility in future studies. However, we chose the 0.1mg/kg dose to try avoid such complications. It is of note that we have recently shown that LPS is able to gain access across an intact BBB by piggybacking on HDL, and thus the

BBB may not represent the barrier that it is perceived to be in for such a lipophilic compound as LPS is known to be (Radford-Smith et al., 2023). Indeed, while our observations highlight the prominent role of neuro- and systemic inflammation observed by many in ALS/FTLD (McCauley and Baloh, 2019), they may also account for why therapeutic approaches to decrease inflammation have thus far failed to alter disease course in humans. Insomuch that if anti-inflammatory, antiviral, or antibiotic therapy is to be trialed, it should be timed with infections/injuries rather than as a generalized ongoing treatment regimen.

The rapid increase in Iba-1 staining in the PFC is most likely to represent activation rather than proliferation and was accompanied by depressive-like behaviors which are held to be largely regulated by this brain structure, where such behaviors can be induced by pro-inflammatory cytokine administration (Hayley et al., 2013). The deficits in the marble test and increased floating behavior in naive mutants are likely to be associated with increased baseline TNF and COX-1 expression in the PFC and HIP (Puppala et al., 2021). The suppression of exploratory novel cage activity in both genotypes after LPS challenge might be explained by the short delay between the inflammatory challenge and the assay, and a ceiling pro-inflammatory effects on mouse behavior (Schapovalova et al., 2022; Yates et al., 2022). The lack of these group differences also rules out possible confounds in measuring reported LPS-induced behavioral responses of two genotypes in other tests, which might have, potentially, been caused by distinct genotype changes in general activity after LPS injection.

5. Conclusions

Our study has revealed the presence of an increased sensitivity of microglia to inflammatory challenges at in early pre-symptomatic stages of the ALS/FTLD -like syndrome in mice with compromised FUS function. It is accompanied by elevated CNS cytokine production under unchallenged conditions and is present prior any signs of neurodegeneration in FUS-tg mutants (Chaprov et al., 2021), thus suggesting these pro-inflammatory processes to be independent from neuronal cell death. Finally, the FUS[1-359]-tg mouse line used here can be a useful model to address the role of microglia and inflammation in the mechanisms of ALS/FTLD syndrome further.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

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FZWG-2021-2016). Molecular studies were the Minobrnauki No.075-15-2021-1346.

APPENDIX A — *Supplementary File*

Animals and housing conditions

Mouse colony of FUS[1-359]-tg (FUS-tg) mice and their wild type littermates (WT) were bred and housed in the FDA-certified SPF facilities of the IPAC Center of Pre-clinical Trials (http://www.spf-animals.ru/about/providers/animals). 8-weeks-old male mice of both genotypes were single housed in standard plastic cages (27x22x15) and maintained on a 12-hour light/dark cycle (lights on at 21:00), under controllable laboratory conditions (22 ± 1°C, 55% humidity, room temperature 22-24ºC), food and water were available ad libitum. Experimental procedures were set up in accordance with a Directive 2010/63/EU and ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-animal-research-reporting-vivo-experiments) and approved by the local veterinarian Committee for Bioethics of IPAC (N19-16.06.2017) and MSMU (22/10/17-MSMU-35). All efforts were undertaken to ensure compliance with above-mentioned regulations concerning human endpoint in animal research.

Generation of FUS Transgenic Mice

The generation of FUS-tg mice was performed as describe elsewhere (Robinson et al., 2015). Briefly, a fragment of human FUS[1–359] cDNA including 9 bp of 5′-UTR was cloned into Thy-1 promoter plasmid 323-pTSC21k. A gel-purified fragment obtained by digestion of the resulting plasmid DNA with NotI was used for microinjection of mouse oocytes. Transgenic animals were identified by PCR analysis of DNA from ear biopsies by the presence of 255-bp product (primers 5′-TCTTTGTGCAAGGCCTGGGT-3′ and 5′-AGAAGCAAGACCTCTGCAGAG-3′).

Originally produced transgenic line on C57Bl6/CBA genetic background was backcrossed with CD1 wild type mice by several (>7) generations.

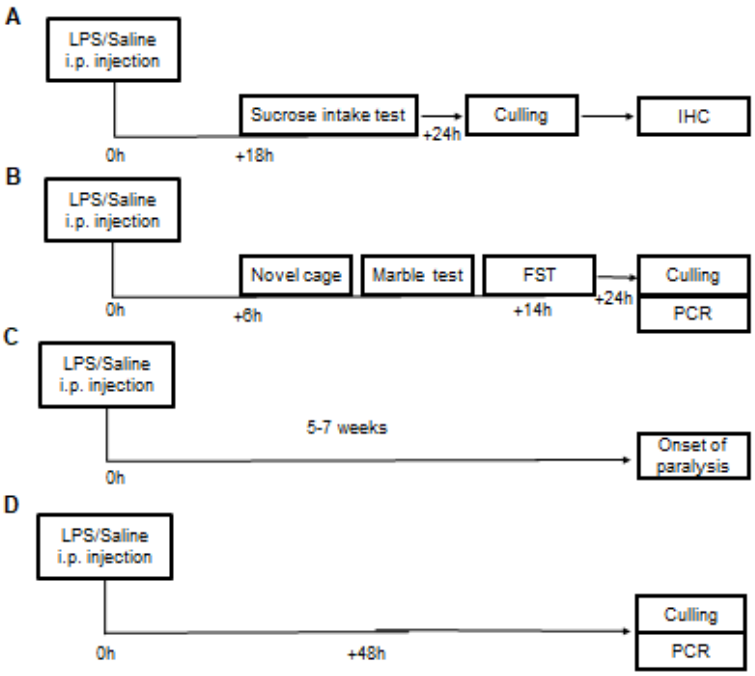


Figure S1

Supplementary Figure 1: Experimental design. Study **(A)** was performed to examine sucrose preference and the immunohistochemical investigation of Iba-1- and neuronal numbers in the CNS of saline and LPS-challenged mice. Study **(B)** comprised of behavioural tests and RT-PCR for inflammatory gene expression in the CNS saline and LPS-challenged mice at 24 h post-injection. Study **(C)** involved assessment of the onset of ALS-like paralysis in the LPS or saline-injected mutants. In study **(D)** we investigated, by RT-PCR, inflammatory gene expression in the CNS of saline and LPS-challenged mice at 48 h post-injection. **IHC**-immunohistochemistry study, **FST** – forced swim test, **LPS** – lipopolysaccharide.

General conditions of behavioural studies

Experiments were performed during the dark period of light cycle, followed by offline analysis. The person performing the experiments was blind for the genotype and treatment until the end of the behavioral tests. Experiments were carried out in the same rooms by the same persons between 09:00 and 17:00 h. Animals were allowed to adapt to experimental room for at least 1 h before testing.

Sucrose test

Mice were given 6 hours of free choice between two bottles of either 1% sucrose or standard drinking water, as described elsewhere (Strekalova et al., 2022). Bottles were weighed before and after conducting the sucrose intake test, and consumption calculated accordingly. The beginning of the test started with the onset of the dark (active) phase of animals' cycle. To prevent the possible effects of side preference in drinking behaviour, the position of the bottles in the cage was switched at 3 hours, halfway through testing. No previous food or water deprivation was applied before the test. Other conditions of the test were applied as described elsewhere (Strekalova and Steinbusch 2010).

Novel Cage Test

The 5-min long novel cage test was carried out to assess exploration of a new environment as described elsewhere (Strekalova et al., 2004; Couch et al., 2016; Veniaminova et al., 2020). Mice were introduced into a standard plastic cage (21 cm x 21 cm x 15 cm) filled with fresh sawdust. The number of exploratory rears was counted under red light per each minute, and summed up for minutes 1-5 of the test.

Pellet displacement marble test

All experimental groups were tested for pellet displacement in a marble test as described elsewhere (Strekalova and Steinbusch 2010; Veniaminova et al., 2017, 2020). A tendency to displace small objects, e.g. small stones or food pellets, from a tube inside the cage, is species-specific in mice and has been demonstrated to depend on an intact hippocampal formation. Using a paper tube (internal diameter 4 cm, length 10 cm), filled with 20 food pellets and placed in the middle of a home cage (21 cm × 27 cm × 14 cm), the number of food pellet displaced by each mouse was assessed every 15 min during 1 h and 45 min.

Forced Swim Test

The Porsolt forced swim test (FST) was used as described elsewhere (Strekalova et al., 2011; Malatynska et al., 2012). Mice were subjected to a 6-min swimming session in a transparent cylinder (Ø 17 cm) filled with water (+23 °C, water height 13 cm, height of cylinder 20 cm, illumination intensity 25 Lux). Floating behaviour was defined by the absence of any directed movements of the animals' head and body and was scored off-line. Using this method, the latency of the first episode of floating, the number of floating episodes and the duration of floating behaviour were recorded. Latency to begin floating was scored as time between introduction of the animal into the pool and the first moment of complete immobility of the entire body for a duration of >3 seconds. The total time spent floating was scored for the entire duration of the test using post-test video footage.

Immunohistochemical analysis of Iba-1 -positive cells

Immunostaining with Iba-1 and DAPI-staining and image analysis in the CNS of mice were performed as described elsewhere (Couch et al., 2016; Veniaminova et al., 2020). Coronal 10 µm-thick sections were cut on a cryostat microtome (Leica Biosystems, Nussloch, Germany) and mounted on gelatin-coated slides. Prefrontal sections were taken between 1.4 and 2.8 anterior-posterior axis; hippocampal sections were taken from lateral 3.6 to lateral 0.4 mm along the medial-lateral axis up to bregma (Paxinos and Franklin, 2001). Spinal cord sections were taken from the lumbar part. Slides were washed in PBS for 30 min and blocked for non-specific protein binding with 10% goat serum in PBS for 1 hour. Then, sections were incubated with primary antibody (Iba-1: 1:800, ab5076, Abcam, Cambridge, UK) in 1% normal goat serum at 4°C for 12 h. Visualization was performed using secondary antibodies anti-chicken-Alexa Fluor 647 (1:500, ThermoFisher, Abingdon, UK) in 1% serum in PBS (Vector Laboratories, Burlingame, CA, USA) for four hours at room temperature. To visualize the nuclei of the cells, sections were co-stained with 4′,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunostaining of ventral and dorsal horns of lumbar part of spinal cord (SC), prefrontal cortex (PFC) and dentate gyrus of the hippocampus (HIP) was examined using a microscope Leitz Dialux 20 (Leica, Wetzlar, Germany) and digital camera Basler ACE (Basler Group, Ahrensburg, Germany). These areas were specifically delineated according to the Paxinos atlas. Cell counting was carried out using ImageJ software. Three sections per each structure per animal were analyzed.

Quantitative RT-PCR (qPCR)

RNA extraction was performed as previously described from specifically microdissected snap-frozen brain regions and lumbar section of spinal cord (Couch et al., 2016, de Munter et al., 2020a,b). mRNA was extracted by using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). First strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 µg total RNA was converted into cDNA. Standard curves were generated using total cDNA to enable normalization to three housekeeping genes glyceraldehyde-3-phosphate (GAPDH), TATA-binding-protein (Tbp), and beta-Actin (ActB). The latter two genes were excluded due to their less stable expression than that of GAPDH. qPCR was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA) and the CFX96 Real-time System (Bio-Rad Laboratories, Philadelphia, , USA) for IL-1 beta, TNF, COX1, COX2 genes. Details of primers can be found in below (*Table 1*). Data were calculated as relative-fold changes compared to control mice as described elsewhere (Couch et al., 2016). Results of qRT-PCR measurement were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05% of normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and the expression of housekeeping gene GAPDH in each cDNA sample ($2^{-\Delta\Delta Ct}$ method). Data are given as expression-folds compared to the mean expression values in control mice. Results are expressed as relative-fold compared to control animals.

Table 1. Sequences of primers used

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
IL-1β	AACCTGCTGGTGTGTGACGTTTC	CAGCACGAGGCTTTTTTGTTGT
TNF	GCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
COX-1	GCCTGAGCCCAGATATAGCA	TTTCCGGCTAGAGGTGGGTA
COX-2	CCGTGCTGCTCTGTCTTAAC	TTGGGAACCCTTCTTTGTTC

Iba-1-positive cell density in the CNS of mice challenged with LPS

Iba-1-positive cell density was increased in all investigated brain structures of LPS-challenged FUS-tg animals.

Table 2. Summary of genotype and treatment effects and group comparisons in the Iba-1-positive cell density in the CNS. 2-way ANOVA revealed significant genotype x treatment interaction for the density of Iba-1-positive cells in the PFC ($F_{1,16}$=6.688, p=0.0199), but not for the ventral ($F_{1,16}$=1.941, p=0.1827) and dorsal horn of SC ($F_{1,16}$=0.0137, p=0.9080) and the dentate gyrus of the HIP ($F_{1,16}$=0.2207, p=0.6448). There was a significant treatment effect in the ventral ($F_{1,16}$=22.81, p=0.0002) and dorsal horn of SC ($F_{1,16}$=18.88, p=0.0005), and in the dentate gyrus of the HIP ($F_{1,16}$=13.63, p=0.0020), but not PFC ($F_{1,16}$=4.046, p=0.0614). No significant genotype effect was found for density of Iba-1-positive cells in the ventral ($F_{1,16}$=0.6337, p=0.4377) and dorsal horn of SC ($F_{1,16}$=2.331, p=0.1463), PFC ($F_{1,16}$=0.229, p=0.639), and dentate gyrus of the HIP ($F_{1,16}$=0.1126, p=0.7415). Tukey analysis revealed significant group differences (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑ - increase of Iba-1-positive cell density.

Groups	Ventral horn of SC	Dorsal horn of SC	PFC	Dentate gyrus of the HIP
WT-Sal vs FUS-tg-Sal	p=0.974, ns	p=0.658, ns	p=0.466, ns	p=0.999, ns
WT-LPS vs FUS-tg-LPS	p=0.434, ns	p=0.754, ns	p=0.145, ns	p=0.939, ns
WT-Sal vs -WT LPS	p=0.119, ns	**p=0.028↑**	p=0.977, ns	p=0.145, ns
FUS-tg-Sal vs FUS-tg-LPS	**p=0.025↑**	**p=0.039↑**	**p=0.0233↑**	**p=0.0427↑**

Increased LPS-induced cytokine expression 24 h post injection

At time point 24 h post-challenge, mRNA concentrations of investigated cytokines were increased in CNS structure of LPS-challenged FUS-tg mice.

Table 3. Summary of genotype and treatment effects and group comparisons in 24h post-injection gene expression of inflammatory markers in the PFC. There was a significant genotype x treatment interaction in mRNA concentrations of IL-1β ($F_{1,33}$=4.927, p=0.0334), TNF ($F_{1,33}$=4.593, p=0.0396) and COX-2 ($F_{1,33}$=5.865, p=0.0211), but not COX-1 ($F_{1,31}$=0.1674, p=0.6852). Significant genotype effect was found for IL-1β ($F_{1,33}$=4.931, p=0.0334), TNF ($F_{1,33}$=5.75, p=0.0223) and COX-2 ($F_{1,33}$=10.17, p=0.0031), but not COX-1 ($F_{1,31}$=3.524, p=0.0699) Significant treatment effect was revealed for IL-1β ($F_{1,33}$=20.48, p<0.0001), TNF ($F_{1,33}$=8.691, p=0.0058), COX-1 ($F_{1,31}$=5.995, p=0.0202) and COX-2 ($F_{1,33}$=10.85, p=0.0024. Tukey analysis revealed significant group differences (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑- increase of gene expression.

Groups	IL-1β	TNF	COX-1	COX-2
WT-Sal vs FUS-tg-Sal	p>0.999, ns	p=0.998, ns	p=0.752, ns	p=0.956, ns
WT-LPS vs FUS-tg-LPS	**p=0.0103↑**	**p=0.084↑**	p=0.346, ns	**p=0.0009↑**
WT-Sal vs -WT LPS	p=0.402, ns	p=0.954, ns	p=0.494, ns	p=0.932, ns
FUS-tg-Sal vs FUS-tg-LPS	**p=0.0001↑**	**p=0.0039↑**	p=0.194, ns	**p=0.0011↑**

Table 4. Summary of genotype and treatment effects and group comparisons in 24h post-injection gene expression of inflammatory markers in the HIP. 2-way ANOVA revealed significant genotype x treatment interaction in mRNA concentration of TNF ($F_{1,32}$=6.473, p=0.016), but not IL-1β ($F_{1,33}$=0.7142, p=0.04041), COX-1 ($F_{1,31}$=0.7245, p=0.4012), and COX-2 ($F_{1,33}$=0.3408, p=0.5633). Significant treatment effects were revealed in mRNA concentration of IL-1β ($F_{1,33}$=15.59, p=0.0003), TNF ($F_{1,32}$=23.29, p<0.0001), COX-1 ($F_{1,31}$=8.9, p=0.0055), and COX-2 ($F_{1,33}$=6.217, p=0.0178). Significant genotype effect was shown in mRNA concentration of TNF ($F_{1,32}$=11.59, p=0.0018), COX-1 ($F_{1,31}$=12.77, p=0.0012) but not IL-1β ($F_{1,33}$=0.636, p=0.431), and COX-2 ($F_{1,32}$=1.524,

p=0.2257). Tukey analysis revealed significant group differences (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑- increase of gene expression.

Groups	IL-1β	TNF	COX-1	COX-2
WT-Sal vs FUS-tg-Sal	p>0.999, ns	p=0.934, ns	**p=0.0246↑**	p=0.972, ns
WT-LPS vs FUS-tg-LPS	p=0.605, ns	**p=0.0008↑**	p=0.209, ns	p=0.524, ns
WT-Sal vs -WT LPS	**p=0.014↑**	p=0.0409, ns	p=0.0619, ns	p=0.563, ns
FUS-tg-Sal vs FUS-tg-LPS	**p=0.0055↑**	**p<0.0001↑**	p=0.4122, ns	p=0.132, ns

Table 5. Summary of genotype and treatment effects and group comparisons in 24h post-injection gene expression of inflammatory markers in the SC. There was a significant genotype x treatment interaction in mRNA concentrations of TNF ($F_{1,33}$=11.49, p=0.0018), but not IL-1β ($F_{1,34}$=1.524, p=0.2254), COX-1 ($F_{1,33}$=3.648, p=0.0649), and COX-2 ($F_{1,33}$=0.8148, p=0.3733). Significant treatment effect was shown in mRNA concentration of IL-1β ($F_{1,34}$=4.587, p=0.0395), TNF ($F_{1,33}$=22.05, p<0.0001), COX-1 ($F_{1,33}$=13.41, p=0.0009), and COX-2 ($F_{1,33}$=17.91, p=0.0002). Significant genotype effect was revealed in mRNA concentration of TNF ($F_{1,33}$=14.12, p=0.0007) and COX-2 ($F_{1,33}$=4.909, p=0.0337) ,but not IL-1β ($F_{1,34}$=1.845, p=0.1833), and COX-1 ($F_{1,33}$=1.594, p=0.2156) Tukey analysis revealed significant group differences (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑- increase of gene expression.

Groups	IL-1β	TNF	COX-1	COX-2
WT-Sal vs FUS-tg-Sal	p=0.998, ns	p=0.995, ns	p=0.973, ns	p=0.818, ns
WT-LPS vs FUS-tg-LPS	p=0.211, ns	**p<0.0001↑**	p=0.0972, ns	p=0.106, ns
WT-Sal vs -WT LPS	p=0.922, ns	p=0.807, ns	p=0.63, ns	p=0.123, ns
FUS-tg-Sal vs FUS-tg-LPS	p=0.089, ns	**p<0.0001↑**	**p=0.015↑**	**p=0.0036↑**

Exacerbated behavioural responses in LPS-challenged FUS-tg mice

LPS-challenged FUS-tg mice demonstrated decreased sucrose intake, explorative behaviour in the novel cage and marble test, as well as prolonged floating in the forced swim test.

Table 6. Summary of genotype and treatment effects and group comparisons in behavioural responses to LPS the in sucrose test, novel cage and marble test. 2-way ANOVA revealed a significant effect of the genotype ($F_{1,16}$=19.25, p=0.0005) and treatment ($F_{1,16}$=9.531, p=0.0071) on the sucrose intake, but no significant effect of their interaction ($F_{1,16}$=3.802, p=0.0689). In the novel cage test, significant effect of the treatment was found in the number of rearings ($F_{1,16}$=88.28, p=0.0001), but no significant effect of genotype ($F_{1,16}$=0.1385, p=0.7147) or genotype x treatment interaction ($F_{1,16}$=0.2889, p=0.5983). In the marble test, significant effect of genotype ($F_{1,16}$=23.53, p=0.0002) and the treatment ($F_{1,16}$=9.941, p=0.0062) in the number of displaced pellets were demonstrated, but no significant effect of their interaction ($F_{1,16}$=2.614, p=0.1254). Tukey analysis revealed significant group differences (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↓- decrease of behavioral score.

Groups	Sucrose intake	Novel cage	Marble test
WT-Sal vs FUS-tg-Sal	p=0.344, ns	p=0.916, ns	**p=0.0016↓**
WT-LPS vs FUS-tg-LPS	**p=0.0019↓**	p=0.999, ns	p=0.143, ns
WT-Sal vs -WT LPS	p=0.852, ns	**p<0.0001↓**	**p=0.0183↓**
FUS-tg-Sal vs FUS-tg-LPS	**p=0.0125↓**	**p<0.0001↓**	p=0.703, ns

Table 7. Summary of genotype and treatment effects and group comparisons behavioural responses to LPS in the forced swim test scores in LPS-challenged FUS-tg mice. 2-way ANOVA revealed no significant treatment x genotype interaction in the latency of floating ($F_{1,16}$=0.6144, p=0.4446), the number of floating episodes ($F_{1,16}$=3.200, p=0.0926) and the duration of floating ($F_{1,16}$=0.9357, p=0.3478). A significant effect of genotype was found in the latency of floating

($F_{1,16}$=15.07, p=0.0013), the number of floating episodes ($F_{1,16}$=5.000, p=0.0399) and the duration of floating ($F_{1,16}$=14.72, p=0.0015). There was a treatment effect on the latency to float ($F_{1,16}$=4.527, p=0.0493), the number of floating episodes ($F_{1,16}$=5.000, p=0.0399), but not the duration of floating ($F_{1,16}$=2.599, p=0.1265). Tukey analysis revealed significant group differences (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑- increase of behavioral score, ↓ - decrease of behavioral score.

Groups	**Latency of floating**	**Number of floating episodes**	**Duration of floating**
WT-Sal vs FUS-tg-Sal	p=0.168, ns	p=0.515, ns	p=0.219, ns
WT-LPS vs FUS-tg-LPS	**p=0.0212↓**	p=0.989, ns	**p=0.0174↑**
WT-Sal vs -WT LPS	p=0.778, ns	p=0.989, ns	p=0.967, ns
FUS-tg-Sal vs FUS-tg-LPS	p=0.209, ns	p=0.515, ns	p=0.299, ns

Lack of genotype differences in LPS-induced cytokine expression 48 h post injection

At time point 48 h post-challenge, no genotype differences were found in the mRNA concentrations of investigated cytokines in any of investigated CNS structures

Table 8. Summary of genotype and treatment effects and group comparisons in 48 h post-injection gene expression of inflammatory markers in the PFC. 2-way ANOVA revealed no significant genotype x treatment interaction for mRNA expression of IL-1β ($F_{1,16}$=0.3172, p=0.1412), TNF ($F_{1,16}$=3.069, p=0.1192), COX-1 ($F_{1,16}$=0.1002, p=0.1569) and COX-2 ($F_{1,16}$=1.4923, p=0.7244). There was no significant genotype effect for mRNA expression of IL-1β ($F_{1,16}$=0.2731, p=0.9729), TNF ($F_{1,16}$=1.969, p=0.3418), COX-1 ($F_{1,16}$=0.9253, p=0.1725) and COX-2 ($F_{1,16}$=0.1239, p=0.9212). There was no significant treatment effect for mRNA expression of IL-1β ($F_{1,16}$=0.7823, p=0.1238), TNF ($F_{1,16}$=2.761, p=0.3316), COX-1 ($F_{1,16}$=0.1909, p=0.1333) and COX-2 ($F_{1,16}$=0.7319, p=0.092). Tukey analysis revealed significant group differences in target molecules (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑ - increase of gene expression.

Groups	IL-1β	TNF	COX-1	COX-2
WT-Sal vs FUS-tg-Sal	p=0.515, ns	p=0.342, ns	p=0.115, ns	p=0.216, ns
WT-LPS vs FUS-tg-LPS	p=0.301, ns	p=0.629, ns	p=0.449 ns	p=0.545 ns
WT-Sal vs -WT LPS	**p=0.001↑**	**p=0.03↑**	p=0.431, ns	p=0.602, ns
FUS-tg-Sal vs FUS-tg-LPS	**p=0.001↑**	p=0.565, ns	p=0.404, ns	p=0.328, ns

Table 9. Summary of genotype and treatment effects and group comparisons in 48 h post-injection gene expression of inflammatory markers in the PFC. 2-way ANOVA revealed no significant genotype x treatment interaction for mRNA expression of IL-1β ($F_{1,16}$=0.5129, p=0.0041), TNF ($F_{1,16}$=2.7926, p=0.1256), COX-1 ($F_{1,16}$=0.1907, p=0.1334) and COX-2 ($F_{1,16}$=0.8921, p=0.2406). There was no significant genotype effect for mRNA expression of IL-1β ($F_{1,16}$=0.5418, p=0.2345), TNF

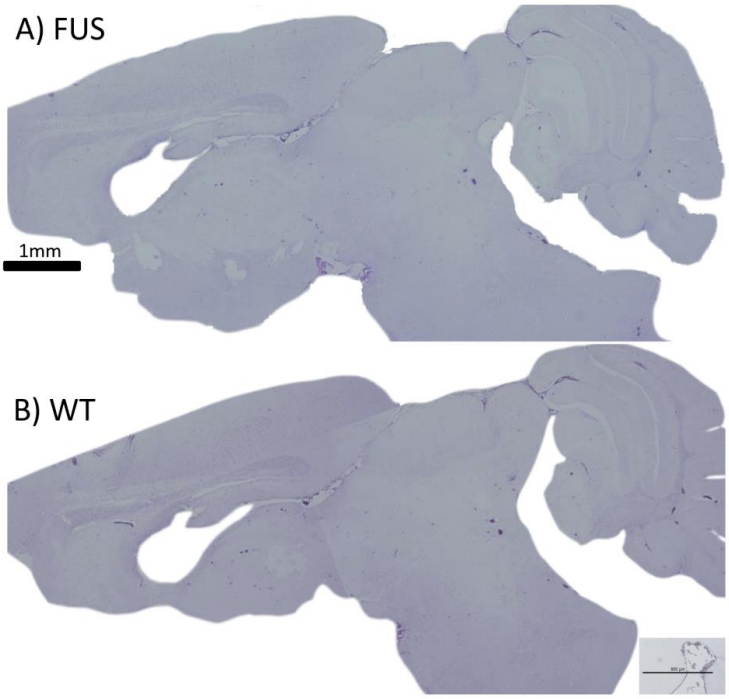
($F_{1,16}$=2.9025, p=0.2759), COX-1 ($F_{1,16}$=0.9229, p=0.9193) and COX-2 ($F_{1,16}$=0.4519, p=0.2209). There was no significant treatment effect for mRNA expression of IL-1β ($F_{1,16}$=1.1816, p=0.7189), TNF ($F_{1,16}$=1.721, p=0.2725), COX-1 ($F_{1,16}$=0.1972, p=0.1193) and COX-2 ($F_{1,16}$=0.9087, p=0.5644). Tukey analysis revealed significant group differences in target molecules (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide, ↑- increase of gene expression.

Groups	IL-1β	TNF	COX-1	COX-2
WT-Sal vs FUS-tg-Sal	p=0.605, ns	p=0.391, ns	p=0.218, ns	p=0.5776, ns
WT-LPS vs FUS-tg-LPS	p=0.311, ns	p=0.132, ns	p=0.116, ns	p=0.392 ns
WT-Sal vs -WT LPS	**p=0.03↑**	p=0.427, ns	p=0.374, ns	p=0.460, ns
FUS-tg-Sal vs FUS-tg-LPS	**p=0.001↑**	p=0.219, ns	p=0.692, ns	p=0.531, ns

Table 10. Summary of genotype and treatment effects and group comparisons in 48h post-injection gene expression of inflammatory markers in the SC. 2-way ANOVA revealed no significant genotype x treatment interaction for mRNA expression of IL-1β ($F_{1,16}$=0.5073, p=0.7142), TNF ($F_{1,16}$=2.746, p=0.1132), COX-1 ($F_{1,16}$=0.1982, p=0.1199) and COX-2 ($F_{1,16}$=0.6439, p=0.2904). There was no significant genotype effect for mRNA expression of IL-1β ($F_{1,16}$=0.5331, p=0.2109), TNF ($F_{1,16}$=2.026, p=0.2119), COX-1 ($F_{1,16}$=0.2963, p=0.9123) and COX-2 ($F_{1,16}$=0.4939, p=0.2402). There was no significant treatment effect for mRNA expression of IL-1β ($F_{1,16}$=0.7013, p=0.2182), TNF ($F_{1,16}$=2.761, p=0.3292), COX-1 ($F_{1,16}$=0.1012, p=0.1563) and COX-2 ($F_{1,16}$=0.6909, p=0.4742). Tukey analysis revealed significant group differences in target molecules (**in bold**); ns: not significant. Sal: saline, LPS: lipopolysaccharide.

Groups	IL-1β	TNF	COX-1	COX-2
WT-Sal vs FUS-tg-Sal	p=0.518, ns	p=0.318, ns	p=0.682, ns	p=0.724 ns
WT-LPS vs FUS-tg-LPS	p=0.413, ns	p=0.522, ns	p=0.528, ns	p=0.462 ns
WT-Sal vs -WT LPS	p=0.397, ns	p=0.372, ns	p=0.334, ns	p=0.549, ns
FUS-tg-Sal vs FUS-tg-LPS	p=0.439, ns	p=0.312, ns	p=0.416, ns	p=0.295, ns

Supplementary Figure 2:



BBB status in the FUS[1-359] transgenic mice. The photomicrographs are representative of immunohistochemistry for mouse IgG in the animals killed at 3 month of age at the time they developed clinical signs. 10um-thick paraffin sagittal sections were cut through the brains (ML+0.5mm) and mounted on gelatinized slides, which were then rehydrated to stain. The presence of IgG in the brain, as a marker of BBB dysfunction, was detected with a biotinylated horse anti-mouse IgG (H+L) (BA-2000 Vector Laboratories 1:1000) after an incubation for 24h at 4deg C in 10% normal horse serum blocking. Positivity was identified using standard ABC (Vector Laboratories, Peterborough, UK), and immunoreactivity was revealed with DAB. All sections were processed, immunolabelled and assessed for BBB breakdown in the same batch. Sections were then lightly counterstained with cresyl violet. There was no evidence of IgG extravasation in the WT control animals or the 3-month-old FUS mutants. The choroid plexus, serving as a positive control, was positively stained for IgG as expected. Thus the BBB is intact in these animals at this stage in the pathogenesis.

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CHAPTER 4

Hippocampal over-expression of cyclooxygenase-2 (COX-2) is associated with susceptibility to stress-induced anhedonia in mice

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Chapter 4. Hippocampal over-expression of cyclooxygenase-2 (COX-2) is associated with susceptibility to stress-induced anhedonia in mice

Abstract

The phenomenon of individual variability in susceptibility/resilience to stress and depression, in which the hippocampus plays a pivotal role, is attracting increasing attention. We investigated the potential role of hippocampal cyclooxygenase-2 (COX-2), which regulates plasticity, neuroimmune function, and stress responses that are all linked to this risk dichotomy. We used a four-week-long chronic mild stress (CMS) paradigm, in which mice could be stratified according to their susceptibility/resilience to anhedonia, a key feature of depression, to investigate hippocampal expression of COX-2, a marker of microglial activation Iba-1, and the proliferation marker Ki67. Rat exposure, social defeat, restraints, and tail suspension were used as stressors. We compared the effects of treatment with either the selective COX-2 inhibitor celecoxib (30 mg/kg/day) or citalopram (15 mg/kg/day). For the celecoxib and vehicle-treated mice, the Porsolt test was used. Anhedonic (susceptible) but not non-anhedonic (resilient) animals exhibited elevated COX-2 mRNA levels, increased numbers of COX-2 and Iba-1-positive cells in the dentate gyrus and the CA1 area, and decreased numbers of Ki67-positive cells in the subgranular zone of the hippocampus. Drug treatment decreased the percentage of anhedonic mice, normalized swimming activity, reduced behavioral despair, and improved conditioned fear

memory. Hippocampal over-expression of COX-2 is associated with susceptibility to stress-induced anhedonia, and its pharmacological inhibition with celecoxib has antidepressant effects that are similar in size to those of citalopram.

Keywords: major depression; inducible cyclooxygenase-2 (COX-2); hippocampus; anhedonia; chronic stress; stress resilience; fear conditioning; celecoxib; citalopram; mouse

1. Introduction

Major depressive disorder (MDD) is a common mental illness that markedly diminishes quality of life and has a profound medical and socioeconomic burden [**1**,**2**,**3**]. While MDD was identified by the World Health Organization (WHO) as a "global crisis" a decade ago [**4**], the COVID-19 outbreak has aggravated the situation [**5**,**6**]. The ongoing pandemic has been projected to have impact on the incidence of MDD that affects not only the patient, but also their relatives, caregivers, and the wider community [**7**]. Despite the variety of therapeutic regimens available for depression, many of them appear to be effective in half of patients or less [**8**,**9**,**10**,**11**] and cause significant side effects [**12**,**13**,**14**]. At the same time, the development of new, effective antidepressant treatment strategies is an ongoing need in neuropsychopharmacology [**15**,**16**].

Currently, the predominant treatment for MDD remains monotherapy with classic antidepressants - i.e., targeting monoaminergic neurotransmission in the brain [**14**,**17**]. Many treatment options based on other mechanisms have been proposed, with targets ranging from

neurotrophin- and immune-related molecules, to neurodevelopmental, glutamatergic, GABAergic, metabolic mechanisms, and more recently, gut microbiota [**18**]. The need for a new mechanistic framework for treating MDD is urgently required, but it has been challenging, as translation from clinically relevant animal models to clinical application has been problematic [**19**,**20**,**21**,**22**]. Among the novel compounds with antidepressant activities, the use of anti-inflammatory drugs, targeting low-level inflammation, a well-established feature of MDD [**23**,**24**,**25**,**26**,**27**], might be of particular value owing to the long-established clinical experience with these widely prescribed drugs [**28**].

Increased cyclo-oxygenase (COX) activity is a well-established feature of neuroinflammation, and the inducible isoform COX-2 in particular seems to play the predominant role in the CNS [**29**,**30**,**31**]. COX-2 is also constitutively expressed throughout the forebrain in discrete populations of neurons and is particularly enriched in the hippocampus and cortex [**32**], where it appears to contribute to fundamental brain functions, such as synaptic activity and memory consolidation [**33**,**34**]. Under resting conditions, however, it is not expressed by glial or endothelial cells [**35**,**36**].

COX converts arachidonic acid to prostaglandin (PG) G2, which, in turn, is converted to PGH2 and then to prostaglandins, prostacyclins, and thromboxanes, among which PGE2 regulates many physiological and pathological functions [**30**,**37**]. COX-2 expression is regulated by synaptic plasticity and depends on glucocorticoids, and is, therefore, regarded as important for dendritic remodeling as part of the stress response and associated with neuropsychiatric disorders [**32**,**36**,**38**]. In the hippocampus, COX-2 basal expression is positively regulated by NMDA

receptor-dependent synaptic plasticity and is restricted to the CA3 area, but under stressful conditions or global ischemia, COX-2 can also be upregulated in the CA1 area and the dentate gyrus, causing neuronal death in those regions, which is prevented by the administration of either glucocorticoids or COX-2 selective inhibitors [**32**,**39**,**40**]. Under pathological conditions, the over-expression of COX-2 results in increased synthesis of prostaglandins, including PGE2 [**38**], which, in turn, increases the sensitivity of tissues to catecholamines, stimulates the activity of the HPA axis via corticotropin-releasing factor [**41**,**42**,**43**], and leads to a surge in the production of pro-inflammatory cytokines, e.g., interleukin (IL)-1ß, IL-6, and tumor necrosis factor (TNF) [**44**,**45**]. The latter changes can elevate the activity of the serotonin transporter SERT [**46**], increase the affinity of the serotonin receptor 5-HT1A [**47**], and alter tryptophan metabolism via indoleamin-2,3-dioxigenase (IDO) mechanism [**48**] resulting in depressive-like "sickness behavior" [**49**,**50**].

COX-2 upregulation was shown to be implicated in several neuropsychiatric diseases, including MDD, schizophrenia, brain ischemia, and neurodegenerative disorders [**51**,**52**,**53**]. A number of findings suggest a role of altered COX-2-mediated molecular cascades in MDD. Increased expression and turnover of COX-2 protein, COX-2 activity, and elevated PGE2 were found to be associated with MDD symptoms, whereas COX-1 protein remained unaltered [**51**,**54**]. Earlier studies suggested that the stimulation of prostaglandin synthesis by prolactin or other hormones can contribute to mood disorders [**55**]. PGE2 is reported to be increased in the plasma and cerebrospinal fluid of depressed patients [**56**,**57**].

Furthermore, pre-clinical genetic and pharmacological studies have implicated PG- PGE2, PGD2, PGF2a, PGI2, and thromboxane-A2, all

synthesized downstream of COX-2, in the mechanisms of the depressive syndrome [**58**,**59**,**60**,**61**]. Brain over-expression of PGE2 has been associated with depressive-like behavior in a chronic mild stress (CMS) model [**59**,**61**], in a model of systemic inflammation [**61**], following a forced swim (Porsolt) test [**62**], and in the rat bulbectomy model of depression [**60**,**63**]. Roles for COX-2-mediated brain increases of PGE2, dendritic dysfunction, and neuronal injury were reported in the rat bulbectomy model [**61**].

Recent meta-analyses of clinical studies in depressed patients, including retrospective cohort studies (RHSs), randomized controlled trials (RCTs), and nested case-control studies (NCCSs) have demonstrated the therapeutic efficacy of pharmacological inhibition of COX-2 with its selective inhibitor celecoxib. Combined treatment with celecoxib increases the effectiveness of established antidepressant compounds in patients with various forms of depression when used as an augmentation strategy together with reboxetine, fluoxetine, and other antidepressants [**50**,**64**,**65**,**66**,**67**]. Treatment with celecoxib was specifically shown to normalize dysregulated cortisol secretion in MDD patients [**68**,**69**].

While the meta-analysis on the use of COX-2 inhibitors in MDD found an overall benefit of celecoxib add-on therapy, some studies have failed to support these findings [**70**,**71**]. Similarly, pre-clinical studies have generated controversies on the effects of COX-2 inhibition. For example, suppression of COX-2 in rodent studies resulted in increased Th1 immune responses and glial cell activation [**72**,**73**,**74**]. Mice genetically deficient for COX-2 revealed increased rates of neuronal damage, microglia, and astrocyte activation; over-production of markers of inflammation;

abnormal oxidative and nitrosative stress; and an abnormal response to celecoxib [**72**,**73**].

The controversial effects of COX-2 inhibition are attributed to the complex roles of this enzyme in normal brain functions and the stress response, and the broad spectrum of COX-2 activities [**38**]. While the function of COX-2 has been investigated in rodent depression models, its role in individual susceptibility to MDD-like syndrome precipitated by stress has not been not addressed. Concurrently, the phenomenon of individual differences in susceptibility versus resilience to stress and depression is attracting increasing attention [**75**,**76**,**77**]. Several important molecular and cellular mechanisms constituting the biological basis of these phenomena have been described [**78**,**79**], and the hippocampus has been argued to be the structure within the brain that plays the most important role in governing an individual's susceptibility or resilience to stress-induced depression and mental disorders in general [**80**,**81**,**82**,**83**].

To address the potential role of hippocampal COX-2 in the mechanisms of the susceptibility to MDD-like behavior, we used a variant of the original CMS model [**84**,**85**] that is based on the induction of decreased sensitivity to reward (anhedonia) as the core depressive symptom [**86**,**87**], and on the previously observed individual susceptibility of 50–70% of C57BL6 mice to this condition [**88**,**89**,**90**,**91**]. In this model, the anhedonic (susceptible) state in stressed mice is defined by a decrease in sucrose preference that is not exhibited by non-stressed control animals; typically, it is not displayed by all stressed mice. As such, the non-anhedonic (resilient) mice can be regarded as an internal control for the effects of stress that are not related to depressive-like changes [**22**,**88**,**92**].

C57BL6 mice underwent rat exposure, restraints, tail suspension, and social defeat for four weeks and were assigned to the susceptible or resilient to anhedonia groups as described elsewhere [**88**,**93**,**94**]. They were studied for floating behavior and hippocampal expression of COX-2, using PCR and immunohistochemical methods (**Figure 1**A). Additionally, Iba-1, as a marker of microglial cells, and Ki67, as a marker of cell proliferation, along with the markers for neurons and for cell nuclei were investigated, as pro-inflammatory changes are known to accompany suppressed neurogenesis under conditions of stress [**95**,**96**]. In a downstream CMS study, mice received celecoxib (30 mg/kg/day), or citalopram, an antidepressant of SSRI class (15 mg/kg/day), or DMSO-vehicle via i.p. injections for one week prior to the onset of the stress and then for the entire stress period, or they were not treated (Fig. 1 B; [**93**,**97**]). To assess hippocampus-dependent functions, contextual fear conditioning memory was investigated [**96**,**98**]. Finally, a group of mice received a single i.p. injection of celecoxib (30 mg/kg/day) prior to (**Figure 1** C) or following (**Figure 1** D) swim session in the Porsolt paradigm [**99**,**100**].

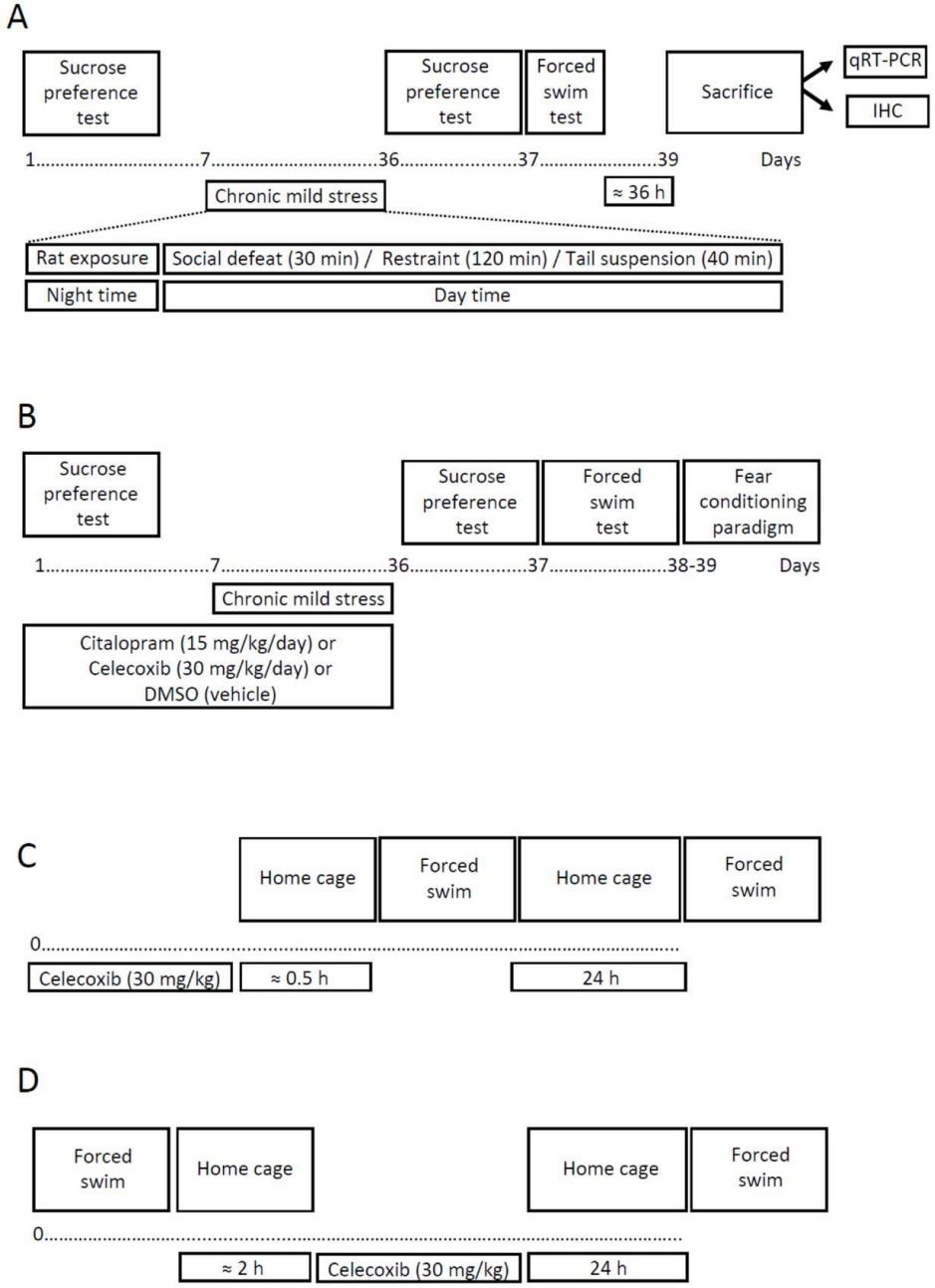

Figure 1. Experiment design. Chronic mild stress without pharmacological interventions (**A**) or with citalopram or celecoxib (**B**) involved 4 weeks with semi-

random alternations of stressors. Forced swimming studies with celecoxib treatment 0.5 h before (**C**) or 2 h after (**D**) the first swimming session were carried out within 24 h. In experiments **A** and **B**, groups were balanced by mouse preference for sucrose before the chronic mild stress procedure. Following the post-stress behavioral test battery, mouse brains from experiment **A** were used for qRT-PCR and immunohistochemical studies. qRT-PCR—quantitative reverse transcription polymerase chain reaction; IHC- immunohistochemistry; see also the ms text.

2. Results

2.1. Expression of COX-2 in the Hippocampi of CMS Mice

In the CMS study, 20 mice were assigned to the chronic stress procedure and 12 animals formed a non-stressed control group. The 4-week stress procedure caused a significant reduction in sucrose preference in the stressed mouse group, as shown by two-way ANOVA ($F_{1,90}$ = 6.029; p = 0.016) and Tukey post hoc test (p = 0.028; **Figure 2**A). According to the 65% criterion for sucrose preference that was applied [**88**,**92**], nine out of 20 stressed mice (45%) showed a sucrose preference below 65% and were defined as exhibiting anhedonia. The rest of the stressed animals, 11 out of 20 (55%), were considered to be non-anhedonic. Anhedonic mice displayed lower latency before floating ($F_{2,41}$ = 51.66, p < 0.0001) and elevated duration of floating in the forced swim test as compared to control and non-anhedonic animals, as shown by one-way ANOVA ($F_{2,29}$= 65.54, p < 0.0001) and Tukey test (p < 0.0001 for all the cases; **Figure 2**C). One-way ANOVA revealed a significant difference in the relative expression of COX-2 mRNA in the hippocampus of stressed mice ($F_{2,27}$ = 6.9; p = 0.038; **Figure 2**D). Anhedonic mice exhibited overexpression of COX-2 in comparison with non-anhedonic stressed and control animals (p = 0.035 and p = 0.03, respectively, Tukey test).

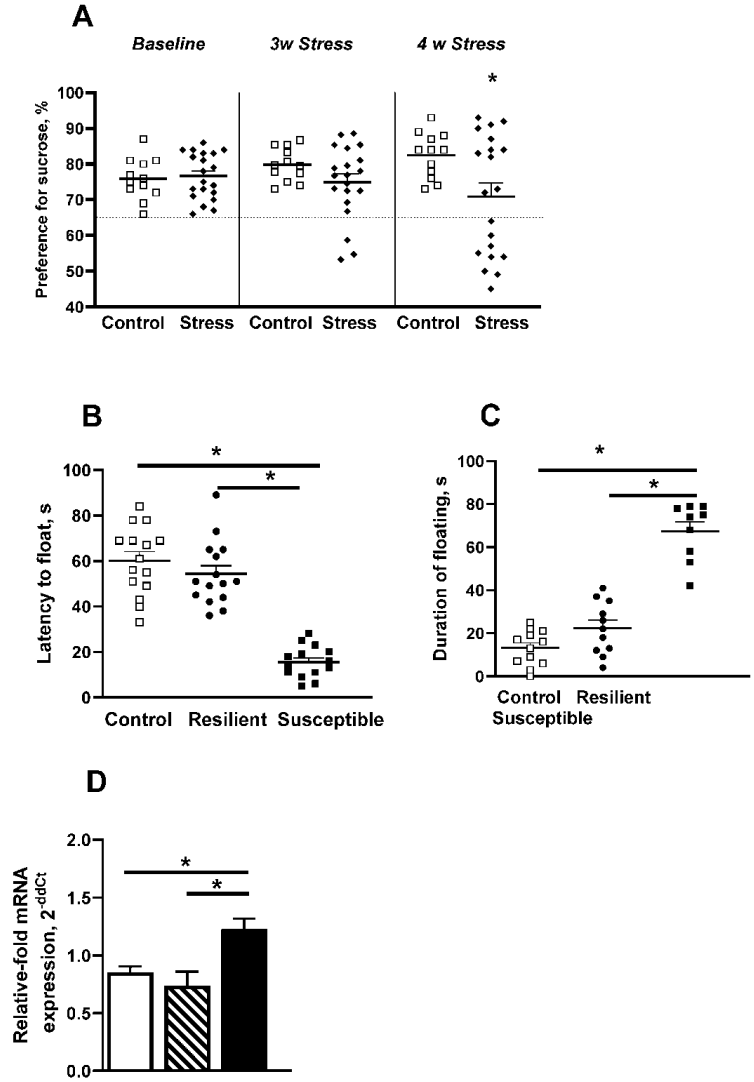

Figure 2. Behavioral and biochemical stratification of chronically stressed animals. (**A**) Preference for sucrose, measured one week before the start of chronic mild stress and 3 and 4 weeks later. A 65% preference was set as a criterion of anhedonia. Fourth week of stress is an optimum adversity duration to stratify animals into two distinct phenotypes, susceptible and resilient to anhedonia (* $p < 0.05$ vs. control, two-way ANOVA and post hoc Tukey test). (**B**) Susceptible-to-anhedonia animals had decreased latency before floating and (**C**) increased duration of floating (* $p < 0.05$ vs. control and resilient mice, one-way ANOVA and post hoc Tukey test). (**D**) COX-2 mRNA expression in hippocampus was upregulated in susceptible animals (* $p < 0.05$ vs. control, one-way

ANOVA and post hoc Tukey test). Bars are mean ± SEM. 'Open square' symbols stand for non-stressed group, 'diamond' symbols indicate stressed mice, 'circle' symbols indicate stressed resilient animals, 'filled squares' stand for anhedonic stressed group.

2.2. Immunohistochemistry for COX-2, Iba-1, and Ki67 Expression in the Hippocampi of Mice Resilient and Susceptible to CMS-Induced Anhedonia

Mice that underwent CMS exhibited a significant group difference in hippocampal COX-2 content according to one-way ANOVA in the hilus area ($F_{2,15}$ = 6.89, p = 0.075, **Figure 3**B) and subgranular zone ($F_{2,15}$ = 22.91, p < 0.0001, **Figure 3**C). COX-2 upregulation in both hippocampal areas was observed in the anhedonic group as compared to the non-anhedonic and control animals (p = 0.0104 and p = 0.023 in hilus, p = 0.0005 and p < 0.0001 in the subgranular zone, Tukey post hoc test). For the CA1 hippocampal zone, one-way ANOVA revealed significant group differences ($F_{2,15}$ = 4.23, p < 0.0001, **Figure 3**D), but for the CA3 area no differences were observed ($F_{2,15}$ = 1.38, p = 0.281, **Figure 3**E). Subsequently we examined population of Iba-1-positive microglial cells in the same areas, and one-way ANOVA revealed group differences for hilus area ($F_{2,15}$= 11.34, p = 0.001, **Figure 3**F), subgranular zone ($F_{2,15}$ = 3.65, p < 0.0508, **Figure 3**G), CA1 ($F_{2,15}$ = 6.55, p < 0.009, **Figure 3**H), and CA3 area ($F_{2,15}$ = 9.32, p < 0.0023, **Figure 3**I). In the hilus and CA1 zone, anhedonic mice had outnumbered microglia in comparison with non-anhedonic and control mice (p = 0.0076 and p = 0.0011 for hilus; p = 0.0039 and p = 0.0099 for CA1 area); and in the subgranular zone there was a significant increase in the microglial population as compared to non-anhedonic mice, but not compared to control animals (p = 0.046 and p = 0.185). In the CA3 zone, we observed a significant increase in microglial cells as compared to

control mice, but not compared to non-anhedonic ones (p = 0.0017 and p = 0.106). To examine hippocampal neurogenesis in the subgranular zone, we used Ki67, whose expression significantly varied across CMS groups ($F_{2,15}$ = 11.19, p = 0.0011, one-way ANOVA; **Figure 3**J). The lowest Ki67 content was observed in the anhedonic group (non-anhedonic and control animals, p = 0.0468 and p = 0.0008).

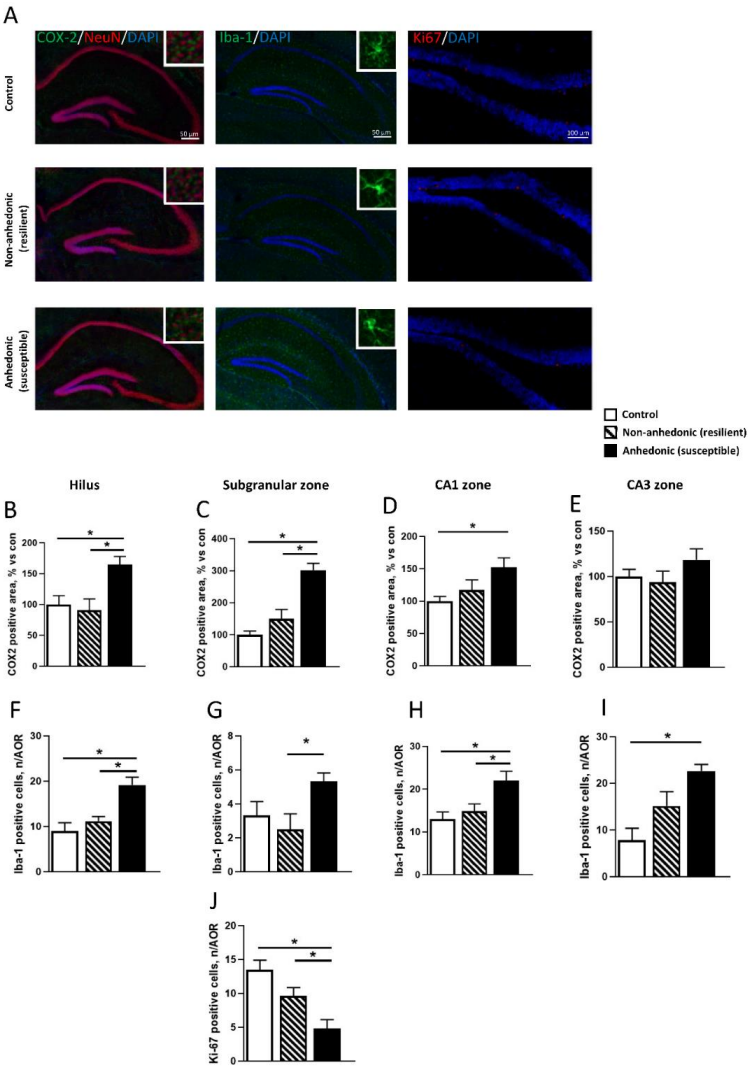

Figure 3. Immunohistochemical analysis of COX-2 and markers of microgliosis and neurogenesis in the hippocampal formation of susceptible and resilient mice. (**A**) Immunohistochemical staining of COX-2. NeuN, a neuronal marker; Iba-1, a marker of microglia; and Ki67, a marker of cellular proliferation. DNA-labeling dye DAPI was used to detect nuclei. Susceptible animals had greater COX-2 positive areas in the (**B**) hilus, (**C**) subgranular zone, and (**D**) CA1 region, but not in the (**E**) CA3 zone. Iba-1 positive cells were outnumbered in the (**F**) hilus, (**G**) subgranular zone, (**H**) CA1 zone, and (**I**) CA3 zone of hippocampus of susceptible animals. (**J**) Chronic stress diminished the number of Ki67 positive cells in the subgranular zone in susceptible animals. * $p < 0.05$ vs. control and resilient mice, one-way ANOVA and post hoc Tukey test. Bars are mean ± SEM.

2.3. Effects of Chronic Treatment with Celecoxib and Citalopram on the Development of Stress-Induced Anhedonia and Depressive-like Syndrome

After the termination of stress procedure, all stressed mice were classified as either non-anhedonic or anhedonic (*see below*). In the sucrose preference test, two-way ANOVA revealed a significant group difference ($F_{15,305} = 8.729$, $p < 0.0001$, **Figure 4**A). Post hoc analysis revealed a significant decrease in sucrose preference in the vehicle-treated group ($p = 0.018$, Tukey test) in comparison with untreated animals. The administration of citalopram or celecoxib prevented this decline ($p = 0.028$ and $p = 0.032$) as compared with the stressed untreated and stressed vehicle-treated animals, respectively. For the forced swim test, two-way ANOVA revealed significant group differences in the duration of floating ($F_{7,133} = 15.228$, $p < 0.0001$, **Figure 4**B). Post hoc analysis revealed a significant increase in floating duration in the untreated group ($p = 0.002$). Citalopram- and celecoxib-treated mice ameliorated the increased floating duration in this test ($p = 0.518$ and $p = 0.455$), as compared with the control untreated and control vehicle-treated animals, respectively. A two-tailed exact Fisher test showed that percentages of anhedonic mice in

the citalopram-treated and celecoxib-treated stressed groups were significantly lower than those of vehicle-treated and untreated groups, respectively ($p < 0.0001$ and $p < 0.0001$, respectively). In the untreated group, 15 out of 26 mice were anhedonic (57.69%); in the citalopram-treated stressed group, 5 out of 32 mice were anhedonic (15.62%); in the celecoxib-treated mice, 4 out of 26 animals were anhedonic (15.38%); and in the vehicle-treated stress group, 12 out of 20 mice were anhedonic (60%; **Figure 4**C).

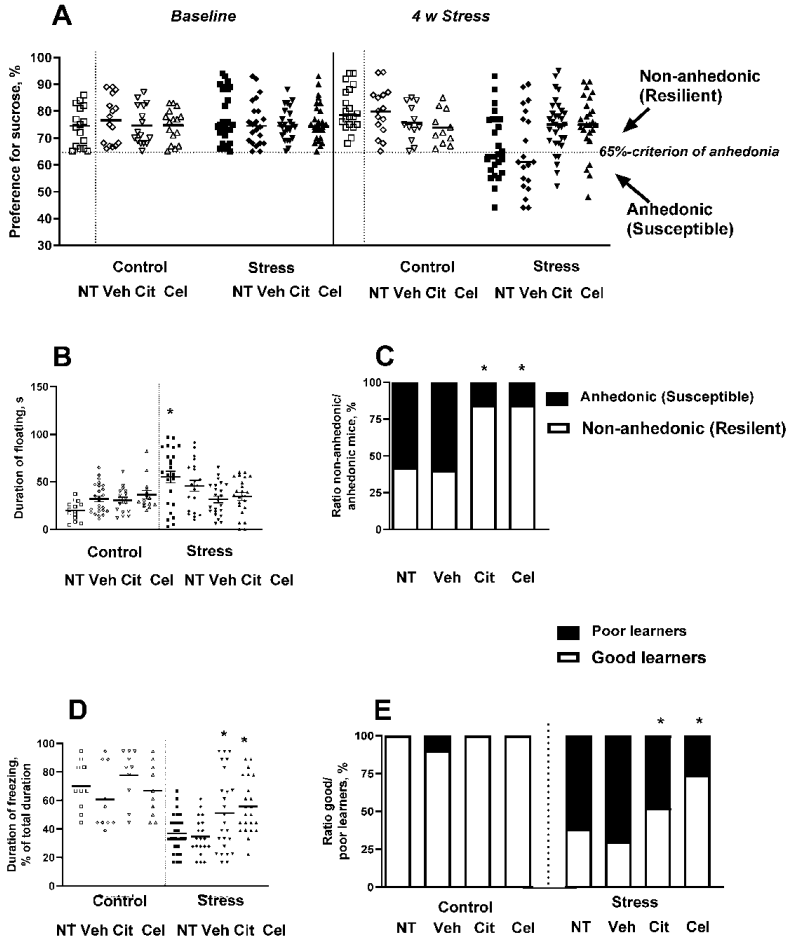

Figure 4. Effects of pharmacological intervention with citalopram or celecoxib on stratification of chronically stressed animals. (**A**) Preference for sucrose, measured one week before the start of chronic mild stress and 4 weeks thereafter, with and without citalopram and celecoxib. A 65% preference was set as a criterion of anhedonia. Both pharmacological agents did not affect sucrose preference in naïve or vehicle-treated non-stressed mice. The population of susceptible animals was decreased in both treated groups as compared with untreated and vehicle-treated stressed groups (* $p < 0.05$ vs. respective control, two-way ANOVA and post hoc Tukey test). Fourth week of stress is an optimum adversity duration to stratify animals into two distinct phenotypes, susceptible and resilient to anhedonia (* $p < 0.05$ vs. control, two-way ANOVA and post hoc Tukey test). (**B**) Citalopram- and celecoxib-treated groups of stressed animals did not demonstrate increased duration of floating as untreated and vehicle-treated did (* $p < 0.05$ vs. control and resilient mice, two-way ANOVA and post hoc Tukey test). (**C**) Ratio of non-anhedonic to anhedonic animals was reversed by both citalopram and celecoxib treatments (* $p < 0.05$ vs. respective control, two-tailed Fisher's exact test). (**D**) In the fear conditioning paradigm, citalopram and celecoxib prevented a decrease in freezing duration observed in stressed not treated or vehicle treated mice (* $p < 0.05$ vs. control and resilient mice, two-way ANOVA and post hoc Tukey test). (E) Ratio of good and poor learners was reversed by both citalopram and celecoxib treatments (* $p < 0.05$ vs. respective control, two-tailed Fisher's exact test). NT- no treatment, Veh- vehicle, Cit- citalopram, Cel- celecoxib.

In the fear conditioning paradigm, two-way ANOVA revealed significant group differences in the duration of freezing ($F_{7,126} = 4.15$, $p < 0.0001$, **Figure 4**D). Post hoc analysis revealed a significant decrease in freezing duration in both untreated and vehicle-treated groups ($p = 0.042$ and $p = 0.023$) in comparison with control untreated and vehicle-treated animals. Citalopram and celecoxib counteracted this effect ($p = 0.76$ and $p = 0.65$), as compared with the stressed untreated and stressed vehicle-treated animals, respectively. Two-way ANOVA revealed significant group differences in the ratio of good to poor learners ($F_{7, 126} = 2.39$, $p < 0.0001$, **Figure 4**E). Two-tailed exact Fisher tests showed that the percentages of poor learners, defined as mice with freezing scores below 40%, were significantly lower in the citalopram-treated stressed group and celecoxib-treated stressed group than in the vehicle-treated group and untreated stressed group, respectively ($p = 0.024$ and $p <$

0.0001). In the citalopram-treated stressed group, 12 out of 25 mice were poor learners, 48%; in the celecoxib-treated stressed group, 5 out of 23 mice were poor learners, 22%; in the untreated stressed group, 16 out of 26 were poor learners, 62%; and in the vehicle-treated stressed group, 14 out of 20 mice were poor learners, 70%; **Figure 4**E). Post hoc analysis revealed a significant reduction in the number of poor learners in both citalopram-treated and celecoxib-treated groups (p = 0.028 and p = 0.009) in comparison with stressed untreated and stressed vehicle-treated animals.

2.4. Acute Administration of Celecoxib Reduces Floating in the Porsolt Test

One-way ANOVA revealed significant effects of celecoxib injected 30 min prior to the first session on the latency before floating ($F_{2,32}$ = 7.509, p = 0.0021) and the duration of floating in the day 2 session ($F_{2,32}$ = 9.46, p = 0.006 **Figure 5**A). This treatment significantly affected latency before floating ($F_{2,32}$ = 7.835, p = 0.0017, **Figure 5**B).

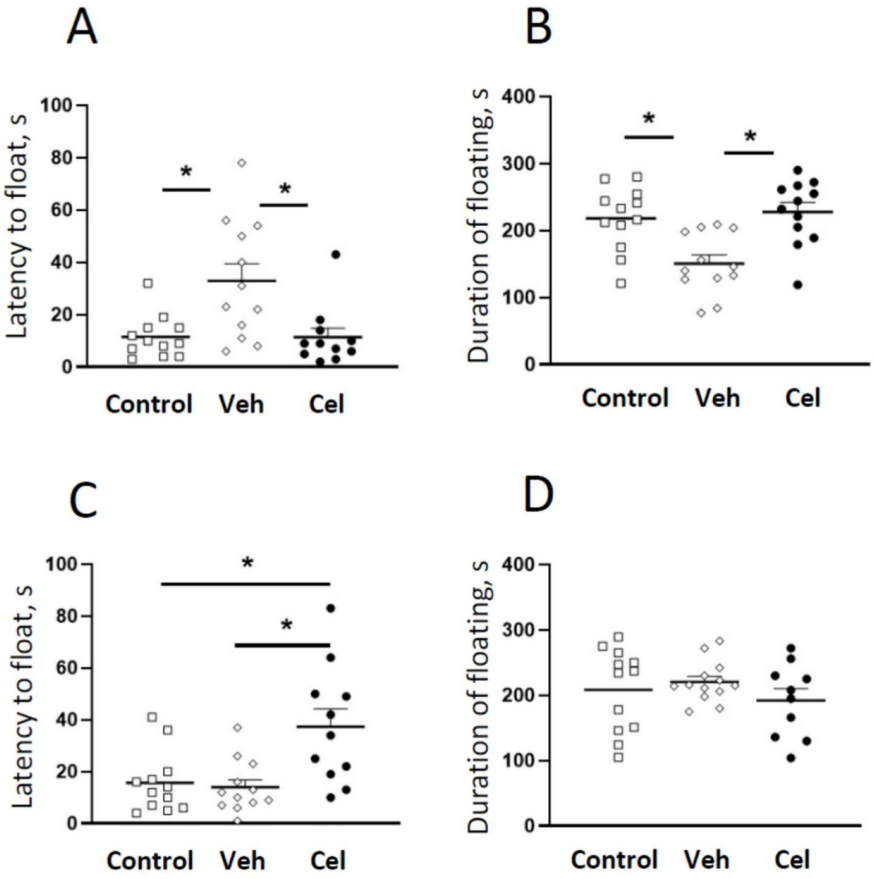

Figure 5. Effects of celecoxib treatments on behavior in the second session of Porsolt test. Celecoxib pre-treatment carried out 0.5 h before the first swimming session, prevented a decrease in latency to float (**A**) and the increase in duration of floating (**B**) in the second swimming session (* $p < 0.05$ vs. vehicle-treated group, one-way ANOVA and post hoc Tukey test). Celecoxib pre-treatment carried out 2 h after the first swimming session prevented the increase in latency before floating (**C**), and did not alter the duration of floating (**D**) in the second swimming session (* $p < 0.05$ vs. vehicle-treated group, one-way ANOVA and post hoc Tukey test).

No significant difference was observed when the treatment was applied 120 min after the first session on the duration of floating in the second session ($F_{2,32} = 0.84$, $p = 0.439$). Celecoxib treatment given between the two sessions had no effect on the duration of floating ($p =$

0.406 vs. vehicle-treated group, Tukey post hoc test, **Figure 5**C) but increased the latency before floating (p = 0.032 vs. vehicle-treated group, **Figure 5**D).

3. Discussion

The present work revealed the over-expression of COX-2 and Iba-1 in the dentate gyrus and CA1 area and downregulation of Ki67 in the subgranular zone in the hippocampi of anhedonic (susceptible), but not non-anhedonic (resilient), mice, suggesting that these changes are may underpin the mechanisms of susceptibility to stress-induced anhedonia. We found a significant decrease in the percentage of anhedonic animals among celecoxib-treated stress mice, and a shortened duration of floating in celecoxib-treated animals in the Porsolt test, which further highlights a potential role for COX-2 in the mechanisms of depression and points to the therapeutic potential of its inhibition.

The results showed that susceptibility, but not resilience to stress-induced anhedonia, a core symptom of depression, is associated with an over-expression of COX-2 in neurons in the CA1 area and dentate gyrus, but not in the CA3 area, of the hippocampus in chronically stressed mice. The changes were also coincident with the increases in the numbers of Iba-1-positive cells in the hippocampus and a reduction of Ki67-positive cell number in the subgranular zone, suggesting increased microglial activation and suppressed cell proliferation in mice susceptible to a depressive-like syndrome. No such changes were evident in mice resilient to stress-induced anhedonia. We also found in the CMS study that chronic administration of selective COX-2 inhibitor celecoxib counteracted the development of the stress-induced depressive-like syndrome, lowered the percentage of

anhedonic mice in the cohort, and normalized floating and hippocampal-dependent contextual learning behaviors in the stressed group of animals. The effects were similar in magnitude to those induced by citalopram administration. Bolus pre-treatment with celecoxib decreased floating behavior in the Porsolt test, further confirming the antidepressant effect of celecoxib. Together, these studies suggest a crucial role for hippocampal COX-2 activation in the mechanisms leading to susceptibility to a depressive-like syndrome and demonstrate the antidepressant activity of its inhibition with celecoxib, which is comparable to the widely used SSRI citalopram.

Our findings indicate a relationship between the COX-2 over-expression in the hippocampus and individual susceptibility to the depressive-like syndrome. Generally, it further supports the view that "neuroinflammation" contributes to an individual's predisposition to MDD [**64**,**75**]. Our results are in keeping with previous studies of Song et al. [**61**] who, using the 5-week CMS and LPS challenge to model depression in Wistar rats, reported elevated production of COX-2 and PGE2 in dendritic spines [**35**,**101**], in the CA1 area and dentate gyrus of the hippocampus, which were associated with decreased dendritic plasticity, oxidative stress, and depressive-like behaviors [**61**]. The normalizing effects of antioxidant treatment with N-acetylcysteine on these outcomes, together with our earlier reports linking susceptibility to CMS-induced anhedonia with decreased brain activities of catalase and superoxide dismutase activity in mice [**90**], suggest that oxidative stress may mediate the effects of over-expressed COX-2 on anhedonia development. As such, the beneficial effects of celecoxib on depressive features and hippocampus-dependent memory in the fear conditioning paradigm are likely to be due to its

normalizing effects on oxidative stress and cellular remodeling in the CA1 zone and dentate gyrus of hippocampal formation. Altered COX-2 expression in the hippocampus was shown to modulate its plasticity and LTD mechanisms, agreeing with earlier electrophysiological studies [**102**].

Previous studies with CMS variants stratifying mice for their susceptibility to stress-induced anhedonia showed that it can be associated with expression changes of several molecular and cellular markers of inflammation that are not displayed by resilience to anhedonic animals [**22**,**95**,**103**,**104**,**105**]. For example, CMS-exposed susceptible-to anhedonia-mice revealed significant elevations of COX-1 and IDO expression in the midbrain raphe region, suggesting a possible interaction of neuroinflammation with altered 5-HT transmission-relation mechanisms [**95**]. Anhedonic, but not resilient mice, showed an over-expression of TNF mRNA in the prefrontal cortex and an elevated number of Iba-1-positive cells in this brain structure [**95**]. These studies found similar increases in corticosterone blood levels, an important indicator of hyperactivity of the HPA axis in depressed patients [**104**,**106**] that in the context of the results reported here may be interpreted as a sign of dysregulation of COX-2 expression by glucocorticoids in a susceptible cohort of mice. Our data reporting the over-expression of inflammatory mediators in a susceptible depressive syndrome cohort of animals are in keeping with clinical data from depressed patients [**107**,**108**,**109**].

The functional effects of IL-1ß in the CNS, which include sickness behavior, were also shown to be antagonized by treatment with a selective COX-2 inhibitor [**110**]. While the antidepressant effects of celecoxib were earlier shown in CMS mice and other depression models in rats, these

experiments did not compare the effects of its pharmacological inhibition against the effects of standard antidepressants [**59**,**61**,**63**,**111**].

The current study revealed similar antidepressant-like activity of selective COX-2 inhibitor celecoxib and that of broadly used SSRI citalopram, suggesting that selective COX-2 inhibitors might be exploited to treat MDD. In comparison with the inhibition or genetic deletion of COX-1, which also counteracts the development of the depressive syndrome [**112**,**113**] and is functionally related to COX-2 [**114**,**115**], COX-2 inhibitors may display better compliance, since the constitutively expressed COX-1 is responsible for the maintenance of peripheral physiological functions and its inhibition causes significant side effects [**110**]. To date, several COX-2-selective inhibitors (coxibs) that have been used for the treatment of arthritis, post-operative pain, headaches, and inflammatory diseases of the brain and peripheral tissues have been developed [**116**]. However, due to their cardiovascular safety profiles, selective COX-2 inhibitors rofecoxib and valdecoxib were withdrawn from the market in 2005, whereas celecoxib is not reported to exhibit cardiovascular side effects, thereby remaining an FDA-approved drug. In any case, high affinity and selective coxibs can serve as promising prototypes in the development of novel, safe, and effective compounds that can be potentially beneficial for MDD patients [**117**].

Depressed patients display increased serum levels of pro-inflammatory cytokines, including TNF-α [**108**,**118**], that can trigger the activation of COX-2 [**119**,**120**], underlying the beneficial effects of treatment with celecoxib in previously reported clinical trials. Conversely, several studies have shown that antidepressants exert immunomodulatory properties suppressing low-level inflammation that may affect the human

immune system and may partly contribute to their efficacy [**121**]. The inconsistences with clinical studies using celecoxib and the accumulating clinical evidence of heterogeneity among MDD patients in the manifestation of low-degree inflammation argue for the refinement of anti-inflammatory treatment strategies in depression. It has been suggested that inflammatory components may be used to characterize a specific subgroup of patients with MDD; e.g., high baseline levels of CRP have been linked to greater depressive symptom severity in general and specific symptoms, such as bad mood, little interest, little activity, suicidality, and poor cognitive performance [**122**]. PET markers of COX-2, which are currently under development, may also potentially be useful [**38**]. This approach may help to identify those subgroups of MDD patients who may benefit from a targeted, and thus more effective, treatment approach. Together, targeting inflammatory markers such as COX-2 would likely be a move towards more advanced personalized treatment of depression.

4. Materials and Methods

4.1. Animals

Studies were performed using 3-month-old male C57BL/6N mice. Three-month-old male CD1 mice were used as intruders for social defeat stress and 2.5 month-old Wistar rats were used for predator stress. All animals were from Janvier, Charles River, France. C57BL/6J mice were housed individually for 10–14 days before the start of experiments; CD1 male 3-month-old mice were housed five per cage during the study; rats were housed in groups of five before the experiment and then individually.

Animals were kept under a 12-h light–dark cycle (lights on: 19:00 h) with food and water ad libitum, using controllable laboratory conditions (22 ± 1 °C, 55% humidity). All experiments were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals 2010/63/EU upon approval by the Ethical Committee of C. Bernard University 08-2008-2011RC and MSMU #11-18-2018/2019 on animal care and welfare, and were compliant with ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines, 2 January 2022).

4.2. Chronic Stress Experiments

This study used a previously validated 4-week stress protocol [**90**] that was adapted from previously described method [**88**,**93**]. The stress regimen comprised of a nighttime rat exposure and the daytime application of three stressors—a social defeat, restraint stress, and tail suspension, a combination of which was applied in a semi-random manner (for details see **Supplementary Material**). Briefly, between the hours of 09:00 and 18:00, three stressors per day were employed in the following sequence: social defeat for 30 min, restraint stress for 2 h, and tail suspension for 40 min with an inter-session interval of at least 4 h.

With the drug-free stress protocol, 12 naive control mice were used, and 20 mice were subjected to stress. At the baseline, control and stress groups of mice were balanced upon their sucrose preference, body weight, and social behavior (non-aggressive or aggressive) as described elsewhere ([**88**,**90**,**123**,**124**]; see also below). The sucrose preference test was repeated on the 2nd and 4th weeks of stress exposure. After the termination of the stress procedure, the latter group of mice was assigned to resilient and anhedonic cohorts according to their sucrose preference

and studied in the forced swim test (see below). Sucrose preference two-bottle test was performed during dark phase of animals' cycle, between 09.00–17.00, as described elsewhere [**97**]. All mice were tested behaviorally one day after the termination of chronic stress, i.e., starting 24 h after the last rat exposure stress session and sacrificed 36 h after the termination of stress (**Figure 1**A; see below). The sacrificed subgroups of control, resilient, and anhedonic animals were used to study the hippocampal COX-2 gene expression (each group was comprised of 7 mice) or immunohistochemical staining of COX-2-positive cells (5 controls, 5 resilient, and 5 anhedonic mice were used); remaining animals were used in the pilot studies.

In a follow-on chronic stress study, 58 mice were assigned to a non-stressed control group. Among them, 13 mice constituted each control group that was not treated or received i.p. injection of DMSO-vehicle; 16 control mice per group were treated with daily i.p. injections of citalopram (15 mg/kg/day) or celecoxib (30 mg/kg/day). Among the animals subjected to stress, 26 of them were untreated, 22 received vehicle, 32 were treated with citalopram (15 mg/kg/day), and 25 had daily injections of celecoxib (30 mg/kg/day) during the 7 days prior the onset of stress and during entire stress procedure, as described elsewhere ([**93**]; see **Supplementary data**). Mice were assigned to these groups after baseline measurements and subjected to the stress procedure and behavioral tests, as in the preceding CMS study (**Figure 1**B; see below). The percentage of animals that were categorized as susceptible to stress-induced anhedonia was calculated. In addition, mice were studied for their hippocampus-dependent memory in the fear conditioning paradigm of contextual learning, as described elsewhere [**98**,**125**]. All groups of mice

were trained on the second day of a post-stress period in the fear conditioning chamber and tested for a recall approximately 24 h thereafter.

4.3. A Study with the Porsolt Test

Mice were subjected to two swimming sessions with an interval of 24 h, where the i.p. administration of vehicle or celecoxib (30 mg/kg/day) was carried out 30 min prior the first swim session (**Figure 1**C) or 2 h thereafter (**Figure 1**D; see below). Twelve control untreated and 12 vehicle-treated mice were used in each study; celecoxib-treated mice constituted 11 mice in each experiment.

4.4. Chronic Stress Procedure and Determination of Anhedonia

In this study, the chronic stress procedure was applied as described previously [**98**]. Shortly, the mice were subjected to 4 different stressors (rat exposure, restraint stress, social defeat, and tail suspension procedure) over 4 weeks as described elsewhere ([**90**,**95**,**126**]; see **Supplementary File**). To assess the hedonic state in mice, the sucrose preference test was performed one week before the experiment (baseline measurement), on the 2nd week of stress and 4 weeks after the beginning of the stress procedure, (see below). Stressed mice that after the 4th week of stress showed a decrease of sucrose preference below 65%, were assigned to the anhedonic group, accordingly to the previously proposed criterion of anhedonia [**88**]. The remaining animals were considered as non-anhedonic (resilient to stress-induced anhedonia). Applied criterion of anhedonia was based on our previous results, which demonstrated that mice with a sucrose preference ≤65% showed a depressive-like syndrome,

consisting in increased floating and decreased exploration, whereas stressed mice with a sucrose preference above this value did not display this behavioral phenotype [**22**,**92**,**127**].

4.5. Sucrose Preference Test

Mice were given eight hours of free choice between two bottles of either 1% sucrose or standard drinking water. At the beginning and end of the period, the bottles were weighed and consumption was calculated. The beginning of the test started with the onset of the dark (active) phase of animals' cycle, i.e., at 9.00). To prevent the possible effects of side-preference in drinking behavior, the position of the bottles in the cage was switched at 4 h, halfway through testing. No previous food or water deprivation was applied before the test. To minimize the spillage of liquids during sucrose test, bottles were filled in advance and kept in the up-side-down position for at least 12 h prior to testing. In order to balance the air temperature between the room and the drinking bottles, they were kept in the same room where the testing takes place. This measure prevents the physical effect of liquid leakage resulting from growing air temperature and pressure inside the bottles, when they are filled with liquids which are cooler than the room air. Preliminary tests showed that with this method the error of measurement does not exceed 0.1 mL. In order to decrease variability in sucrose consumption during the very first sucrose test (baseline measurement), a day before, animals were allowed to drink 2.5% sucrose solution in a one-bottle paradigm for 2 h.

Percentage preference for sucrose is calculated using the following formula: $Sucrose\ Preference = Volume\ (Sucrose\ solution)/(Volume\ (Sucrose\ solution) + Volume\ (Water)) \times 100$. No mice from control groups

ever exhibited a preference for sucrose of <65% and, accordingly, mice exhibiting a sucrose preference of <65% were defined as susceptible. Mice that had undergone stress but maintained a sucrose preference of >65% were defined as resilient. Other conditions of the test were applied as described elsewhere [**22**,**89**,**92**].

4.6. Forced Swim Test

Two days after the termination of stress procedure, mice were tested in the forced swim test. Mice were introduced to a transparent pool (20 cm × 35 cm × 15 cm) filled with warm water (30 °C, height 9.5 cm) lit by red light for 2 min. The duration of floating behavior, defined as absence of directed movements of animals' heads and bodies, was estimated as described elsewhere [**123**,**128**].

4.7. Fear Conditioning Paradigm

The apparatus (Technosmart, Rome, Italy) consisted of a transparent plastic cubicle (25 × 25 × 50 cm) with a stainless-steel grid floor (33 rods, 2 mm in diameter). A single alternating electric current (AC, 50 Hz; 0.7 mA, 1 s, Evolocus LLC, Tarrytown, NY, USA) was delivered after a 2-min acclimatization period. After delivery of the current, the mouse was immediately placed back in the home cage. Freezing behavior was scored by visual observation during a test of memory recall that was carried out 24 h later as described elsewhere [**96**,**98**]. The occurrence of freezing behavior was assessed every 10 s for 180 s; each 10-s score was assigned to a freezing or non-freezing period, and the percentage of time spent in freezing was calculated. Mice spent in freezing ≥40% of time were defined as "good learners" as described elsewhere [**129**].

4.8. A Two-Day Forced Swimming Porsolt Test and Drug Administration

All sessions were 6-min long and were performed by placing a mouse in a transparent cylinder (Ø 17 cm) filled with water (23 °C, water height 13 cm, height of cylinder 20 cm). On day 2, the duration of floating behavior that was defined by the absence of any directed movements of the animals' heads and bodies, was scored manually using criteria, which were previously validated by automated scoring with Noldus EthoVision XT 8.5 (Noldus Information Technology, Wageningen, The Netherlands) and CleverSys (CleverSys, Reston, VA, USA) as described elsewhere [**100**,**130**]. The latency before floating and time spent floating were recorded.

4.9. Administration of Drugs

Citalopram (Cipramil: Lundbeck, Copenhagen, Denmark) was dissolved in sterile water for injection. Celecoxib (Celebrex: Pfizer, St. Louis, MO, USA) was dissolved in a vehicle containing 34% Hydroxypropyl-ß-cyclodextrin (Sigma, Steinheim am Albuch, Germany) and 10% DMSO (Sigma, Steinheim am Albuch, Germany). Mice were intraperitoneally injected with either DMSO-vehicle, citalopram, or celecoxib. The dose of citalopram was based on previous studies showing the efficacy under employed settings [**93**,**123**]. The dose of celecoxib was defined by previous reports [**59**,**131**].

4.10. Culling and Brain Dissection

Mice were terminally anaesthetized with isoflurane inhalation and sacrificed by cervical dislocation for a subsequent material collection. For gene expression assay, mice were perfused with ice-cold saline via left

ventricle, brains were removed, hippocampi were dissected and stored at −80 °C until use as described elsewhere [**132**]. For immunohistochemical study, mice were perfused with 10 mL ice-cold saline followed by 4% paraformaldehyde via left ventricle, brains were removed, post-fixed in PFA for 12 h and cryoprotected in 30% sucrose for 12 h and then embedded in a mold filled with OCT compound and snap-frozen in dry ice-cooled isopentane. Samples were stored at −30 °C until used as described elsewhere [**133**,**134**].

4.11. RNA Extraction and RT-PCR

First strand cDNA synthesis was performed using random primers and Superscript III transcriptase (Invitrogen, Darmstadt, Germany); 1 µg total RNA was converted into cDNA. Quantitative PCR for COX-2 gene and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using the SYBR Green master mix (Bio-Rad Laboratories, Philadelphia, PA, USA) and the CFX96 Real-time System (Bio-Rad Laboratories, Philadelphia, PA, USA). Sequences of primers used are: COX-2 (5′-CCGTGCTGCTCTGTCTTAAC-3′ and 5′-TTGGGAACCCTTCTTTGTTC-3′), GAPDH (5′-CTGCACCACCAACTGCTTAG-3′ and 5′-GGGCCATCCACAGTCTTC-3′). Data were normalized to GAPDH mRNA expression and calculated as relative-fold changes compared to control mice as described elsewhere [**100**,**134**]. Results of RT-PCR measurement were expressed as Ct values, where Ct is defined as the threshold cycle of PCR at which amplified product was 0.05% of normalized maximal signal. We used the comparative Ct method and computed the difference between the expression of the gene of interest and GAPDH in each cDNA sample ($2^{-\Delta\Delta Ct}$ method). Data are

given as expression-folds compared to the mean expression values in control mice.

4.12. Immunohistochemical Analysis of COX-2-Positive Cells in the Brain

Immunostaining with COX-2, NeuN, Iba-1, and Ki67 antibodies and image analysis in the hippocampus were performed as described elsewhere [**135**]. Coronal 10 µm-thick sections were cut on a cryostat microtome (Leica Biosystems, Nussloch, Germany) and mounted on gelatin-coated slides. Hippocampal sections were taken from lateral 3.6 to lateral 0.4 mm along the medial lateral axis up to bregma (Paxinos and Franklin, 2001). Slides were washed in PBS and blocked for non-specific protein binding with 10% goat serum in PBS for 1 h. Then, sections were incubated with primary antibody (COX-2: 1:1000, ab178846, Abcam, Cambridge, UK; NeuN: 1:1000, MAB377, Millipore, Billlerica, MA, USA; Iba1: 1:800, ab5076, Abcam, Cambridge, UK; Ki67: 1:500, ab15580, Abcam, Cambridge, UK) in 1% normal goat serum at 4°C for 12 h. Visualization was performed using secondary antibodies: anti-rat-Alexa Fluor 594 (1:500, Abcam, Cambridge, MA, USA), anti-rabbit-Alexa Fluor 488 (1:500, Abcam, Cambridge, MA, USA), and anti-chicken-Alexa Fluor 647 (1:500, ThermoFisher, Abingdon, UK) in 1% serum in PBS (Vector Laboratories, Burlingame, CA, USA) for two hours at room temperature. To visualize the nuclei of the hippocampal cells, sections were co-stained with 4′,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunostaining was examined using a light microscope Leitz Dialux 20 (Leica, Wetzlar, Germany) and digital camera Basler ACE (Basler Group, Ahrensburg, Germany). The areas of CA1 and CA3 zones, hilus, and the subgranular zone were specifically delineated according to the Paxinos atlas. Cell counting

was carried out using ImageJ software. Three sections per each structure per animal were analyzed.

4.13. Statistical Analysis

Data were analyzed with a statistical software package (Statistica 10.01, Chicago, IL, USA). ANOVA test followed by post hoc Tukey test was used for data analysis. One-way and two-way ANOVA were applied where appropriate. Assuming equal variability of differences no Geisser-Greenhouse correction was applied. Qualitative data were analyzed by the two-tailed Fisher's exact test. The level of confidence was set to 95% ($p < 0.05$).

5. Conclusions

Our findings demonstrate that the up-regulation of COX-2 expression in the CA1 zone and dentate gyrus of the hippocampus is associated with individual susceptibility to stress-induced depressive syndrome. We also report similar efficacy of antidepressant action of the selective inhibitor of COX-2 celecoxib compared to the SSRI citalopram in the CMS mouse model. In light of the considerable side effects reported for SSRIs and other classic antidepressants, resulting in premature discontinuation of the medication in over 70% of individuals [**136**], the use of COX-2 inhibitors would likely be beneficial. This add-on therapy might become particularly valuable as soon as appropriate clinical guidance for the use of anti-inflammatory therapy and new potentially safe COX-2 inhibitors will be developed.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23042061/s1.

Author Contributions

T.S. and R.C. conceived the study and designed experiments, T.S., D.P. and A.T. performed in vivo and in vitro assays, carried out statistical analysis, A.P. and A.U. contributed with the methodology and data analysis; A.S., A.L. and K.-P.L. supported the project with funds and helped with data interpretation and presentation; D.C.A. and T.S. drafted the ms; R.C. reviewed and validated the work. All authors have read and agreed to the published version of the manuscript.

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Data are available upon request.

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Conflicts of Interest

The authors declare no conflict of interest.

APPENDIX A

Supplementary File

Chronic stress procedure

Rat exposure while in a small container

Mice were introduced into cylindrical containers (Open Science, Moscow, Russia), which were placed into a rat home cage during 15 h (over-night, from 18h00 to 9h00). Containers were made from customized transparent plastic, size 15 cm x Ø 8 cm, with holes in covers (Ø < 0.5 cm), which ensured protection of the mouse from the rat, but allowed visual and odor contact. During the weekends, mice were kept in their home cage, which were situated on top of the rat cages.

Restraint stress

Animals were placed inside a plastic tube (internal diameter 26 mm) for 2 h during the dark phase of the light cycle and kept in a dark experimental room.

Tail suspension stress

Mice were submitted to the tail suspension procedure by hanging them by their tails in a tail suspension system (Bioseb, France) for about 6 min daily. The procedure was done during the dark phase of the animals' light cycle.

Social defeat stress

Social defeat procedures took place during the dark phase; to enable a visual control over the resident-intruder confrontation, the test was carried out under red light. In a preliminary test, aggressive individuals of the CD1 mouse strain that were able to attack the counter-partners in less than 60 sec without injuring them were selected for this procedure; these animals were introduced in the home cages of mice from the stress group during social defeat sessions for 5 min. During social defeat stress, test

mice typically showed flight response, submissive posture and vocalization. Pairs of animals were carefully observed in order to exclude any physical harm. In rare cases of its incidence, aggressive individuals were immediately removed from the cage of resident mice. After a 5-min period of social defeat C57BL/6 mice were introduced into small containers and again inside the CD1 cage, where they stayed for a 3 h-period. Thereafter, a 5-min social defeat procedure was repeated again. In order to randomize the procedure, the same pairs of C57Bl6 and CD1 mice were never put together.

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CHAPTER 5

General Discussion

Chapter 5. General Discussion

5.1. The 'double-hit' of stress-induced and environmental inflammation

The interplay of stress and inflammation in the development of depression has been examined in the scientific literature, both as separate etiological factors (Kim et al. 2022; Maydych 2019) and as a 'double-hit' model (Kim et al. 2020; Wijaya et al. 2022), highlighting the complexity and multifactorial nature of this disorder. We have shown that the 'double-hit' of low-dose LPS with CMS in mice augmented depressive-like behaviors such as a reduced sucrose preference, elevated immobility time in the tail suspension test and increased floating behavior in the forced swim test (**Chapter 2**). Both chronic stress alone and chronic stress combined with a low-grade systemic immune challenge induced anhedonic and depressive-like behaviors: animals that were exposed to both of these factors had a reduced sucrose preference, increased floating behaviors and decreased a latency of the first episode of floating in the forced swim test in comparison to control group and animals subjected to either factor separately. LPS alone did not induce such behavioral alterations.

Keeping with these findings the recent work performed on Wistar male rats that were submitted to LPS administrations followed by a CMS protocol demonstrated similar exacerbation of behavioral abnormalities and molecular dysregulation by a 'double-hit' challenge (Géa et al. 2019). The animals in that study received i.p. LPS injections for one week daily (0.25 mg/kg) and were submitted to a 6-week stress protocol that included

water and food deprivation, warm water at 30°C, cool water at 8°C, tail pinch, soiled bedding, and restraint stress. Rats that were exposed to stress alone in that study displayed anhedonic- and anxiety-like behavior but did not show elevated levels of blood IFNγ. Unlike rats that were exposed to both CMS and LPS challenges, LPS treated rats did not show this pro-inflammatory response either. The 'double-hit' of LPS and CMS increased IFNγ serum levels, that correlated with reduced sucrose consumption in LPS + CMS group (Géa et al. 2019), which is in consistency with our results showing exacerbated signs of anhedonia in the LPS + CMS group. Thus, similarly to our work, a combination of chronic stress and systemic inflammation has resulted in exacerbated depressive like behavior such as anhedonia. However, in this work the authors did not study central inflammation and peripheral inflammation in the organs.

Stress-induced HPA activation resulting in elevated peripheral glucocorticoid levels and suppressed cytokine activity play a pivotal role in emotional dysregulation in depression and is discussed by many researchers (Stanton et al. 2019; Strekalova et al. 2022; Sapolsky et al. 2000; O'Callaghan and Miller 2019; van Donkelaar et al. 2014). In a 'double-hit' concept, stress-induced neuroinflammatory priming, i.e. an exacerbation of the LPS-induced neuroinflammation by corticosterone, is considered as an etiological factor of neuropsychiatric disorders (Frank et al. 2016). Prior 1-week long exposure to corticosterone (200 mg/L in drinking water) was demonstrated to prime the neuroinflammatory response to LPS (2 mg/kg, s.c.) by increasing pro-inflammatory cytokine IL-1ß, IL-6, TNF production in cortex, hippocampus, striatum, hypothalamus, olfactory bulb, and cerebellum (Kelly et al. 2018). However, this 'double-hit' challenge did not cause astrogliosis (Kelly et al. 2018).

It should be noted that the phenomenon of priming is not limited to models of inflammation induced by LPS or PolyI:C administration (Kelly et al. 2018; Loram et al. 2011), but can also be observed in neuroinflammation caused by exposure to toxic substances. In mice, prolonged pretreatment with corticosterone significantly enhances the pro-inflammatory effects of acute exposure to organophosphate toxins such as chlorpyrifos oxon (CPO), diisopropyl fluorophosphate (DFP), pyridostigmine bromide (PB), or physostigmine (PHY), as measured by increased expression of IL-1β, IL-6, and TNF mRNA in the cortex and hippocampus (O'Callaghan et al. 2015; Locker et al. 2017). The role of corticosterone in inflammation-induced anhedonia is further supported by the observation that commonly used antidepressants in clinical settings, such as amitriptyline, tranylcypromine, and vortioxetine, as well as experimental substances with antidepressant properties, like Honokiol, a biphenolic neolignane, can restore hedonic sensitivity by decreasing corticosterone levels (Tomaz et al. 2020; Sulakhiya et al. 2014).

Although the depressive-like behaviors were more pronounced in LPS + CMS animals when compared with LPS or stress alone, such an effect was not additive in relation to the aggressive behaviors which were diminished by LPS exposure in stressed mice. While patients with agitated form of MDD are frequently characterized by elevated aggressiveness (Takahashi et al. 2018), decreased aggressiveness, such as submissive behavior, is known to be associated with higher predisposition to depressive episodes both in humans and animal models (Larrieu and Sandi 2018; Vollmayr and Henn 2001; Strekalova et al. 2004; Frank et al. 2019). We have observed that mice subjected to stress alone demonstrated increased measures of aggressive/dominant behavior and signs of impulsivity. Stress-induced

aggressiveness has been previously described using the CMS protocol employed here (Costa-Nunes et al. 2014). The opposite changes were found in stressed LPS-treated mice; therefore, CMS and CMS + LPS challenges gave generated opposing effects of aggressive behavior in mice, and a decrease in aggressiveness in mice exposed to a 'double-hit' challenge can be interpreted as a sign of higher predisposition to depressive features of animals.

Not all molecular and hormonal effects of LPS and CMS were additive either. We have shown that stressed, LPS-treated and stressed LPS-treated mice had similarly elevated level of corticosterone, while hepatic *Tnf* expression was elevated only in LPS-treated and *Il-1b* expression was increased only in stressed LPS-treated animals. Thus, glucocorticoid response alone cannot explain the behavioral differences between stressed and LPS-challenged animals, suggesting that stress-induced pro-inflammatory hepatic activation might not parallel changes in corticosterone. However, it is known that stress-induced corticosterone elevation is associated with hepatic inflammation in rats (Spiers et al. 2020), and a mechanism proposed behind this phenomenon implies a glucocorticoid-induced suppression of hepatic glucocorticoid receptor expression that decreases anti-inflammatory effects of glucocorticoids resulting in excessive liver and systemic inflammation (Jenniskens et al. 2018). Nevertheless, in our work pro-inflammatory hepatic activation was not necessarily associated with corticosterone elevation. Notably, the level of corticosterone was increased to the same extent by the two factors separately and by their combination in comparison with control. Other mechanisms of stress-induced inflammatory activation may play a role in the reported here phenomena such as epinephrin-mediated and vagus-

mediated inflammation (Ortega et al. 2021). Our data, showing the corticosterone-independent hepatic TNF and IL-1ß activation and behavioral differences, may also be explained by the possible anti-inflammatory activation due to stress exposure (Liu et al. 2017; Keller et al. 1983), as corticosterone is known to be an anti-inflammatory activator (Webster Marketon and Glaser 2008).

Thus, our data suggest that the combination of stress-/disease-driven inflammation and exogenously-induced systemic inflammation with LPS, results not in an additive response, but rather in a different response. We can hypothesize that this effect is due to distinct evolutionary-developed mechanisms to effectively deal with stress-induced inflammation vs exogenous infection-evoked inflammation separately. We can also summarize that chronic low-grade 'sterile' inflammation have a profound effect on stress-induced behaviors, and the mechanisms of these interactions are known to be of relevance in clinic as such combinations may provoke depressive episodes (Pahwa et al. 2022; Kim et al. 2022). The 'double-hit' experimental model of major depression in this context suggests that the combination of stress-induced inflammation and an environmental component, such as LPS exposure, would better mimic MDD pathophysiology and provide with a more translational approach (Géa et al. 2019).

5.2. Affective symptoms in ALS/FTLD: the role of inflammatory mechanisms

The results indicate that LPS treatment, similarly to the CMS model, exacerbated several behavioral abnormalities in FUS-tg mice (**Chapter 3**). Specifically, LPS-challenged FUS-tg mice showed a significant decrease in sucrose intake, reduced rearing behavior in the novel cage test, and a decreased number of displaced pellets in the marble test. In the forced swim test, LPS-challenged FUS-tg mice demonstrated a reduced latency to float and increased duration of floating. Together, these changes can be interpreted as depressive-like behaviors.

The observed behavioral abnormalities were associated with inflammatory changes in FUS-tg mice during the pre-symptomatic phase, which occurs prior to the onset of motor or cognitive manifestations of ALS/FTLD at the age of 2-2.5 months (**Chapter 3**). The results showed that the LPS injection caused a significant increase in the density of Iba-1-positive cells in all investigated CNS areas of FUS-tg mice but only in the dorsal horn of the SC and dentate gyrus of the Hip of the wild-type mice. In the PFC, LPS-challenged FUS-tg mice had increased mRNA expression of IL-1β, TNF, and COX-2 compared to saline-treated FUS-tg mice. In the Hip and SC, LPS-challenged FUS-tg mice had increased mRNA expression of TNF and COX-1 compared to saline-treated FUS-tg mice. The behavioral response of the FUS-tg mice to LPS injection was exacerbated compared to the wild-type controls.

These findings suggest that the initial abnormalities before the onset of motor symptoms may originate in the brain, rather than the spinal cord. These results correspond with previous study demonstrating that the pre-

symptomatic FUS[1-359]-tg mice demonstrate behavioral changes that are reminiscent of the FTLD-syndrome abnormalities, and they are attenuated by anti-inflammatory treatments such as celecoxib and Neuro-Cells (de Munter et al. 2020). In present study (**Chapter 3**), specifically, the PFC appeared to be particularly affected by the inflammatory challenge, as evidenced by increased microglial activation and augmented cytokine production, which was associated with the manifestation of depressive-like behaviors. The increased sensitivity of microglia to inflammatory stimuli and elevated cytokine production in FUS-tg mice suggest that microglial activation may be an independent mechanism in ALS/FTLD rather than a consequence of neurodegeneration (Lall and Baloh 2017).

The increased marker density observed in the stimulated PFC microglia in mutants was associated with depressive-like behaviors, which are primarily controlled by this brain region and can be triggered by cytokine administration to the PFC (Hayley and Litteljohn 2013). In addition, naive mutants exhibited deficits in the marble test and increased floating behavior. These abnormalities are likely linked to elevated baseline TNF and COX-1 expression in the PFC and Hip, which correspond to clinical and neuroanatomical features of the FTLD-FUS sub-type of this disease (Puppala et al. 2021).

Our study found that both genotypes showed a suppression of exploratory behavior in a novel cage after LPS challenge, which could be attributed to the short time interval between the inflammatory stimulus and the test. The absence of group differences in other behavioral tests rules out possible confounds in measuring LPS-induced responses. Notably, a single injection of a low dose of LPS did not alter the onset of the development of ALS-like paralysis in FUS-tg mice, which suggests that only

chronic increases in systemic inflammation of any origin, including environmental and infection factors, are likely to trigger the progression of ALS/FTLD caused by FUS dysfunction. Thus, anti-inflammatory prevention and treatment strategies that are based on chronic intervention may be beneficial in ALS/FTLD pathologies.

In conclusion, the present study provides evidence that pre-symptomatic FUS-tg mice exhibit an exacerbated immune response and behavioral abnormalities following a systemic inflammatory challenge with LPS. The effect is most prominent in the PFC, where pro-inflammatory cytokine expression is increased, and is accompanied by depressive-like behavior. Additionally, the deficits in hippocampus-dependent performance and increased floating behavior in naive mutants suggest a baseline pro-inflammatory state in the PFC and HIP. Our findings suggest that the brain of FUS-tg mice is more susceptible to systemic inflammatory challenges, and shows the need for timing therapeutic interventions with infections/injuries rather than as a generalized ongoing treatment regimen. Future studies may focus on the mechanisms underlying the observed immune and behavioral changes, as well as potential therapeutic targets. The FUS[1-359]-tg mouse line is a valuable model for investigating the role of microglia and inflammation in the mechanisms of ALS/FTLD syndrome.

5.3. Differential roles of pro-inflammatory cytokines and associated mechanisms in the CNS pathology

Both the CMS model and ALS share commonalities in terms of pro-inflammatory changes. In ALS, pro-inflammatory cytokines such as IL-1β, TNF, and IL-6 are elevated in the brain and spinal cord, and are thought to contribute to the degeneration of motor neurons. Similarly, in the CMS model, chronic stress leads to an increase in pro-inflammatory cytokines in the brain (**Chapter 2**). This chronic inflammatory response has been implicated in the development of emotional disturbances such as depression and anxiety, which are also common in ALS (Roos et al. 2016). The commonalities in pro-inflammatory changes observed in both models suggest that targeting these mechanisms may offer new therapeutic avenues for the treatment of both emotional disturbances and neurodegenerative diseases.

In our study, the 'double-hit' combination had no effect on LPS-induced TNF hepatic mRNA expression but did suppress LPS-induced IL-1β hepatic mRNA expression. Here, hepatic TNFα and IL-1β mRNA levels differed between stressed mice injected with LPS compared to LPS alone in a surprising manner revealing a dissociation between the regulation of TNFα and IL-1β mRNA expression. Moreover, these changes in hepatic cytokine expression appeared to be independent of corticosterone induction (**Chapter 2**).

Based on the results from **Chapter 2**, it appears that inflammatory mechanisms may play a crucial role in determining the behavioral outcomes of the 'double-hit' model. CMS can induce low-grade 'sterile' inflammation by producing DAMPs. In stressed animals, an increased level

of proinflammatory cytokine mRNA expression in the brain may indicate the activation of COX-2 synthesis as a result of CMS. Additionally, the elevated expression of SERT in the hippocampus of stressed mice may also be caused by COX activation (Perrone et al. 2020). Although the level of COX was not examined in our study (**Chapter 2**), it is well-known that chronic stress increases its production. For example, in an experiment on Wistar rats, 6-hour restraint stress increased COX-2 and PGE2 levels in the brain for 2-6 hours (Madrigal et al. 2003). Therefore, it is possible that the activation of COX-2 and subsequent inflammation may be key factors in the behavioral changes observed in the 'double-hit' model.

ALS/FTLD is also associated with chronic low-grade 'sterile' inflammation, which leads to neuroinflammation during the progression of the disease (Béland et al. 2020). Similarly to the CMS model investigated in **Chapter 2**, the results of the present study (**Chapter 3**) suggest an exacerbated response of young pre-symptomatic FUS-tg mice to systemic inflammation following a 24-hour post-challenge, which predominantly affects the PFC and emotional behavior of the mutant animals. However, at 48 hours post-LPS injection, no genotype differences in gene expression between LPS-treated groups were observed, suggesting a rapid decay of the increased pro-inflammatory response in the mutants. This may explain the unchanged onset of the ALS-like motor syndrome in FUS-tg mice subjected to a single LPS challenge found in our work.

Our study (**Chapter 3**) also demonstrated that FUS-tg mice exhibit signs of neuroinflammation during the pre-symptomatic stage, before the onset of motor symptoms, such as increased hippocampal COX-1 mRNA expression. Given these findings, it is possible that cyclooxygenases may play a crucial role in the transfer of chronic 'sterile' inflammation into

'sterile' neuroinflammation, which may affect the brain molecular mechanisms in such a way that pathogen-induced inflammation, acting as a second hit, may lead to depressive symptoms. This question was addressed in **Chapter 4** of the present study.

5.4. The role of COX-2 in the mechanisms of the depressive-like behavior and individual vulnerability to stress-induced syndrome

In the study with double hit depression model, we found that COX-2 expression is related to an individual susceptibility to stress induced depressive syndrome. We have shown that anhedonic (susceptible), but not non-anhedonic (resilient) mice displayed over-expression of COX-2 in the dentate gyrus and CA1 area of the hippocampus (**Chapter 4**). These data support a recently proposed concept that COX-2 plays role as one of key molecular factors of depression (He et al. 2022). COX-2 expression in the brain can be triggered by inflammation (Font-Nieves et al. 2012) or stress (Madrigal et al. 2003) and influence the neuronal functioning through several pathways in the brain including induction of neuroinflammation (Choi et al. 2009), dysregulation of glutamatergic, serotoninergic and dopaminergic neuronal transmission (López and Ballaz 2020), HPA axis hyperactivation (Ma et al. 2013; Adzic et al. 2015), mitochondrial dysfunction (Yan et al. 2021), a common factor for CNS disorders (Reichart et al. 2018), and hippocampal neuronal damage (Wang et al. 2015). There is an increasing number of literature acknowledging the therapeutic value

of COX-2 inhibitors, such as coxibs, for the treatment of depression (Song et al. 2019; El-Malah et al. 2022).

Previously, 5-week CMS or a 1-week LPS challenge (i.p. 0.5 mg/kg/day) to model depression in Wistar rats were shown to increase the production of COX-2 and PGE_2 in dendritic spines in the CA1 area and dentate gyrus of the hippocampus which was associated with depressive-like behaviors such as increased floating behavior and decreased sucrose preference (Song et al. 2019). These findings, similarly to our work, show the role of COX-2 over-expression precisely in the hippocampal CA1 area in the manifestation of depressive behaviors. However, the combined effects of the two factors in a 'double-hit' paradigm were not studied in this work, and the relationship of COX-2 with individual predisposition was not studied either (Song et al. 2019).

Other mechanisms involved in the stress-susceptibility of stress-exposed animals with connection to COX dysregulation comprise altered serotonin transmission. Susceptible to anhedonia mice due to CMS exposure demonstrate elevations of COX-1 expression in the raphe nucleus, one of the main serotoninergic areas (Couch et al. 2013). Moreover, as COX-2 over-expression in the brain leads to an excessive production of prostaglandins, such as PGE_2, inducing neuroinflammatory response in rodent models of depression (Sethi et al. 2019; Chen et al. 2017), this may elevate the activity of SERT via MAPK pathway in neurons of various brain areas including prefrontal cortex, midbrain and striatum (Zhu et al. 2006; Couch et al. 2013).

Inflammatory processes in the brain, particularly in monoamine nuclei locus coeruleus and dorsal raphe are associated with susceptibility to stress in adult male Sprague Dawley rats determined as a passive coping

behavior (or submissive behavior) in the resident-intruder test, characterized by immobility and withdrawal in response to stressors (Wood et al. 2015). This generally is in line with the hypothesis on the evolutionary function of SERT, that was proposed to be a regulator of social interaction and social cognition on the population level across species (Canli and Lesch 2007; Lesch 2007).

Individual vulnerability to stress is also linked to the orexin system, which is known to regulate feeding behavior, sleep, cardiovascular function, and reward-seeking behavior, as well as higher cognitive functions such as attention, cognition, and mood. (Katzman and Katzman 2022). While social defeat in rodents is a valid paradigm for studying behaviors resembling affective disorders (Hammels et al. 2015), inhibition of orexin-expressing neurons in vulnerable to social defeat stress adult male Sprague Dawley rats increased their social interaction behavior and decreased floating behavior in the forced swim test (Grafe et al. 2018). In a clinical study using a selective serotonin reuptake inhibitor sertraline which specifically blocks SERT, orexin levels in the cerebrospinal fluid were reduced (Salomon et al. 2003), suggesting that orexins may act as an inflammation-induced downstream regulator of social behavior in active / passive coping dichotomy that, in turn, determines whether an individual becomes susceptible or resilient to stress. In our study, reduced signs of neuroinflammation were also associated with stress-resilience in mice, that also had higher level of neurogenesis in the hippocampal DG (**Chapter 4**).

In our study, prolonged treatment with the selective COX-2 inhibitor celecoxib reduced signs of neuroinflammation and depressive-like behaviors in the forced swim test and fear conditioning paradigm (**Chapter**

4). Interestingly, endogenous opioids enkephalin and dynorphin, that possess anti-inflammatory properties, are increased within specific nuclei of the basal forebrain of stress-resilient Sprague-Dawley rats exposed to a resident-intruder model of defeat for 7 days (Bérubé et al. 2013). All in all, our results along with the literature suggests the contribution of neuroinflammatory response in susceptibility to stress-induced depressive-like behaviors, while reduction of pro-inflammatory cytokines by COX-2 inhibitor celecoxib (**Chapter 4**) or a similar dose of resveratrol (30 mg/kg/day) that also exerts a COX-2-inhibiting activity demonstrates protective properties against stress-induced depressive-like behavior (Zykova et al. 2008; Finnell et al. 2017).

COX-2 may be involved in the mechanisms of depression through vascular dysfunction, as shown by the increased COX-2-dependent relaxation of small resistance arteries in CMS-induced anhedonic 6 w.o. Wistar male rats upon inhibition of vascular COX-2 activity with NS398 (10 μM). This may affect peripheral resistance and organ perfusion in major depression (Bouzinova et al. 2014). The anhedonic animals were identified through the sucrose preference test, and the CMS protocol included various stressors that lasted for 8 weeks. While COX-2 activity or level was not assessed in any tissue, only the anhedonic subgroup of rats responded to COX-2 but not COX-1 inhibition by enhanced acetylcholine-stimulated vascular relaxation (Bouzinova et al. 2014).

The relevance of CMS models in studying the mechanisms of individual stress-vulnerability and higher predisposition to depressive symptoms is based on the observation that only a subset of animals subjected to CMS exhibit depressive-like behaviors, characterized by passive coping strategies in social interaction tests, increased floating

behaviors, and decreased sucrose preference, i.e., anhedonia (Strekalova et al. 2004). Susceptible animals also display signs of neuroinflammation in the brain areas responsible for emotional regulation (locus coeruleus) and cognitive functions (prefrontal cortex, hippocampus) and have a higher risk of CVD due to autonomic imbalance and altered endothelial function (Carnevali et al. 2018; Couch et al. 2013). Based on the findings from (Bouzinova et al. 2014) and our own research, COX-2 may be a key mechanism in the predisposition to stress-induced depression and comorbid CVD.

We studied the involvement of COX-2 in depressive syndrome in a 'double-hit' paradigm using FUS-tg model of ALS/FTLD and LPS treatment (**Chapter 3**). Exogenous inflammatory stimulation increased central expression of COX-2 mRNA only in FUS-tg, but not in wild type mice. This elevation was associated with manifestations of depressive-like behaviors of LPS-challenged FUS-tg animals, such decreased sucrose preference and elevated floating behavior. It was previously shown that FUS-tg mice have elevated TNF and COX-1 mRNA in the hippocampus when compared with wild type animals, which is associated with impaired social behavior, increased anhedonia in sucrose preference test and elevated floating behavior in forced swim test (de Munter et al. 2020). These abnormalities were attenuated by Celecoxib treatment, which further supports the role of COX-2 in depressive symptoms not only in stress models (Song et al. 2018), but in other conditions, such as ALS/FTLD.

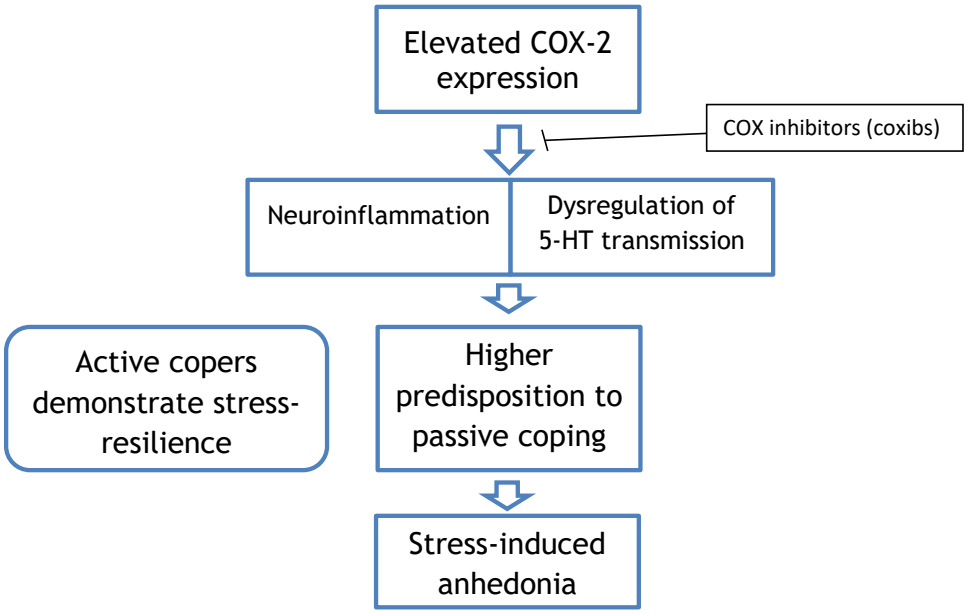

Fig. 5.1. Proposed mechanisms of the role of COX-2 in individual susceptibility to stress-induced syndrome

To sum up, elevated COX-2 expression and decreased Ki67 expression, signs of neuroinflammation, microglial activation and suppressed neurogenesis in the hippocampus accompanied a depressive-like syndrome and are observed in stress-susceptible anhedonic and LPS-treated pre-symptomatic FUS-tg mice. We can hypothesize that the role of COX-2 in susceptibility to stress and development of depression may imply several mechanisms, including the induction of pro-inflammatory cytokines that are involved in anhedonia themselves (Pan et al. 2017) and dysregulated 5-HT transmission leading to passive coping in stressful condition and subsequent development of depressive-like behaviors

(**Figure 5.1**). The results of the study and other literature suggest that reducing signs of neuroinflammation, and specifically COX-2, may be an effective therapeutic strategy for treating depression.

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SUMMARY

In my work, I aimed to study the role of neuroinflammation in mechanisms of emotional abnormalities and pathology hallmarks in two mouse models of neuropsychiatric disorders. I particularly focused on clinically relevant situation of the interaction of disease-driven and environmentally induced inflammation in disease manifestations. Specifically, I used a mouse chronic stress depression model (**Chapter II**) and a transgene mouse model of amyotrophic lateral sclerosis (ALS)/ frontotemporal lobar degeneration (FTLD) with truncated highly aggregate-prone form of human Fused in Sarcoma (FUS) protein, FUS[1-359] (**Chapter III**), in which the role of disease-associated inflammation in manifestation of emotional abnormalities has been previously established. Using these paradigms, I induced systemic inflammation by the administration of a low-dose lipopolysaccharide injection (**Chapters II and III**).

Chronic stress and systemically induced inflammation are both paradigms established to model major depressive disorder, however their combined 'double-hit' effects remain largely unexplored. At the same time, a combination of environmentally induced inflammation and disease-driven HPA activation is a common clinical situation. I aimed to investigate behavioral and molecular consequences of stress-induced and environmental inflammation leading to manifestations of depressive-like behaviors and molecular hallmarks of MDD, i.e., levels of corticosterone

and inflammatory cytokine expression. I have shown that the administration of a low dose of LPS, when combined with a CMS, exacerbated depressive-like behavior in terms of behavioral signs of helplessness and anhedonia, a decreased sensitivity to a reward, while behavioral scores of aggression and impulsivity were found to be decreased after LPS treatment in stressed mice (**Chapter II**). LPS treatment alone did not cause overt behavioral changes in my study. Blood corticosterone level was similarly elevated in response to LPS or CMS alone at the same extent as to the combination of both factors. Pro-inflammatory cytokines were increased only in stressed LPS challenged mice. No exacerbation of CNS gene expression of pro-inflammatory cytokine *Il-1β*, *Tnf* and serotoninergic *5-Ht$_{2a}$*, *Sert* was observed after LPS treatment of stressed mice. This suggests dissociated changes in depressive-like and aggressive behaviors, as well as in brain and hepatic expression of pro-inflammatory cytokine genes.

Thus, my suggestion is that various adaptive mechanisms has evolved to allow the organism to respond to stress and systemic inflammation separately, whereas a low degree inflammation likely to lead to significant behavioral abnormalities in response to stress.

Neurodegenerative disorders are well known to be accompanied by pro-inflammatory changes that are thought to underlie emotional abnormalities in patients. In this context I ran the study, in which I aimed to investigate whether a systemic inflammatory challenge stress may interfere with emotional abnormalities and pathology hallmarks of FUS[1-359] transgenic mice of FUS-tg mice a model of ALS/FTLD (**Chapter III**). Induction of a systemic inflammatory response with low-dose LPS injection

during the pre-symptomatic phase of the disease exacerbates emotional abnormalities and molecular hallmarks in these mice. Thus, regardless the etiology, double–hit disease-driven and environmental inflammation results in aggravated emotional abnormalities and pathology hallmarks in mouse models of depression and ALS/FTLD.

To investigate the role inflammatory mechanisms in MDD further I studied the role of one of the main pro-inflammatory enzymes COX-2 which regulates the arachidonic and cytokines (**Chapter IV**). I used a variant of the CMS model that is based on the induction of anhedonia, a decreased sensitivity to reward, as the core depressive symptom. The anhedonic state in stressed mice in this model is defined by a decrease in sucrose preference and thus were defined as susceptible, while non-stressed control animals do not exhibit such behavior and defined as resilient and can be regarded as an internal control for the effects of stress that are not related to depressive-like changes. In this study, I stratified all the stressed animals in hedonic and anhedonic cohorts to measure the level of COX-2 expression and microglial activation, as well as the intensity of adult neurogenesis. This study suggests the role of COX-2 in individual predisposition to depression. Hippocampal COX-2 is known to play a key role in the hedonia/anhedonia dichotomya. In my study, anhedonic (i.e., depressive) mice exhibited higher number of Iba-1- and COX-2-positive and lower number of Ki67-positive cells accompanied by elevated COX-2 gene expression in the dentate gyrus and the CA1 zone of the hippocampus of compared to non-anhedonic animals. These results further support the view that neuroinflammation contributes to predisposition to depressive syndrome.

My study contains the comparison of celecoxib (a selective inhibitor of COX-2) with a classical antidepressant citalopram (a selective serotonin re-uptake inhibitor) in one animal study as a possible therapy for managing depressive outcomes (**Chapter IV**). This demonstrates similar antidepressant-like properties of celecoxib and that of citalopram suggesting that coxibs can be exploited to treat depressive disorders.

Thus, preventive measures of inflammatory factors and anti-inflammatory therapy that includes as for instance coxibs, might be useful in the prevention and treatment of depressive-like and neurodegenerative conditions. The approach of mimicking an interplay between known etiological and environmental factors in experimental animals is likely to help a better translational research with neuropsychiatric disorders. The two types of inflammation when occur simultaneously do not sum up in an additive way but result in different responses.

SAMENVATTING

In mijn werk richtte ik me op het bestuderen van de rol van neuro-inflammatie in de mechanismen van emotionele afwijkingen en pathologische kenmerken bij twee muismodellen van neuropsychiatrische stoornissen. Ik richtte me met name op een klinisch relevante situatie van de interactie tussen ziektegerelateerde en door de omgeving veroorzaakte ontsteking bij ziekteverschijnselen. Specifiek gebruikte ik een muismodel van chronische stressdepressie (**Hoofdstuk II**) en een transgeen muismodel van amyotrofische laterale sclerose (ALS)/ frontotemporale lobaire degeneratie (FTLD) met een afgeknotte, sterk aggregatiegevoelige vorm van menselijk Fused in Sarcoma (FUS) — eiwit, FUS [1-359] (**Hoofdstuk III**), waarin de rol van ziektegerelateerde ontsteking bij het ontstaan van emotionele afwijkingen eerder is vastgesteld. Met behulp van deze paradigma's veroorzaakte ik systemische ontsteking door toediening van een lage dosis lipopolysaccharide-injectie (**Hoofdstuk II en III**).

Chronische stress en systemisch opgewekte ontsteking zijn beide gevestigde paradigma's om een grote depressieve stoornis te modelleren, maar hun gecombineerde effecten blijven grotendeels onontdekt. Tegelijkertijd is een combinatie van door de omgeving veroorzaakte ontsteking en ziektegerelateerde HPA-activatie een veelvoorkomende klinische situatie. Ik richtte me op het onderzoeken van gedrags- en moleculaire consequenties van stress-geïnduceerde en omgevingsontsteking die leiden tot manifestaties van depressieve gedragingen en moleculaire kenmerken van MDD, d.w.z. niveaus van corticosteron en inflammatoire cytokine-expressie. Ik heb aangetoond dat toediening van een lage dosis LPS, in combinatie met CMS, depressief

gedrag verergert in termen van gedragsmatige tekenen van hulpeloosheid en anhedonie, een verminderde gevoeligheid voor beloning, terwijl gedragsmatige scores van agressie en impulsiviteit werden gevonden om te worden verminderd na LPS-behandeling bij gestreste muizen (**Hoofdstuk II**). LPS-behandeling alleen veroorzaakte geen duidelijke gedragsveranderingen in mijn onderzoek. Het niveau van bloedcorticosteron werd evenveel verhoogd bij reactie op LPS of CMS alleen als bij de combinatie van beide factoren. Pro-inflammatoire cytokines werden alleen verhoogd bij gestreste LPS-uitgedaagde muizen. Er werd geen verergering van CNS-genexpressie van pro-inflammatoire cytokine *Il-1β*, *Tnf* en serotonerge *5-Ht2a*, *Sert* waargenomen na LPS-behandeling van gestreste muizen. Dit suggereert gedissocieerde veranderingen in depressieve en agressieve gedragingen, evenals in de expressie van pro-inflammatoire cytokinegenen in de hersenen en lever.

Dus, mijn suggestie is dat verschillende aanpassingsmechanismen zijn geëvolueerd om het organisme in staat te stellen afzonderlijk te reageren op stress en systemische ontsteking, terwijl een lichte ontsteking waarschijnlijk leidt tot significante gedragsstoornissen als reactie op stress.

Neurodegeneratieve aandoeningen gaan gepaard met pro-inflammatoire veranderingen die verondersteld worden de emotionele afwijkingen bij patiënten te veroorzaken. In deze context heb ik het onderzoek uitgevoerd, waarin ik heb geprobeerd te onderzoeken of een systemische inflammatoire uitdaging stress kan interfereren met emotionele stoornissen en pathologiekenmerken van FUS[1-359] transgene muizen van FUS-tg muizen, een model van ALS/FTLD (**Hoofdstuk III**).

Inductie van een systemische inflammatoire respons met een lage dosis LPS-injectie tijdens de pre-symptomatische fase van de ziekte verergert emotionele stoornissen en moleculaire kenmerken bij deze muizen. Dus, ongeacht de oorzaak, resulteert een dubbel-effect van door ziekte gedreven en milieu-ontsteking in verergerde emotionele stoornissen en pathologiekenmerken bij muismodellen van depressie en ALS/FTLD.

Om de rol van ontstekingsmechanismen bij MDD verder te onderzoeken, heb ik de rol bestudeerd van een van de belangrijkste pro-inflammatoire enzymen COX-2, dat de arachidonzuur- en cytokines reguleert (**Hoofdstuk IV**). Ik heb een variant van het CMS-model gebruikt dat is gebaseerd op de inductie van anhedonie, een verminderde gevoeligheid voor beloning, als het kernsymptoom van depressie. De anhedonische toestand bij gestreste muizen in dit model wordt gedefinieerd door een afname van de sucrosevoorkeur en wordt dus als vatbaar gedefinieerd, terwijl niet-gestreste controledieren dergelijk gedrag niet vertonen en veerkrachtig worden genoemd en als interne controle kunnen dienen voor de effecten van stress die niet gerelateerd zijn aan depressieve veranderingen. In deze studie heb ik alle gestreste dieren onderverdeeld in hedonische en anhedonische cohorten om het niveau van COX-2-expressie en microglia-activatie te meten, evenals de intensiteit van volwassen neurogenese. Deze studie suggereert de rol van COX-2 bij individuele predispositie voor depressie. Hippocampale COX-2 speelt een sleutelrol bij de hedonie/anhedonie-dichotomie. In mijn studie vertoonden anhedonische (dwz depressieve) muizen een hoger aantal Iba-1- en COX-2-positieve en een lager aantal Ki67-positieve cellen vergezeld van verhoogde COX-2-genexpressie in de dentate gyrus en de CA1-zone van

de hippocampus in vergelijking met niet-anhedonische dieren. Deze resultaten ondersteunen verder het idee dat neuro-inflammatie bijdraagt aan de predispositie voor het depressieve syndroom.

Mijn onderzoek bevat de vergelijking van celecoxib (een selectieve remmer van COX-2) met een klassiek antidepressivum citalopram (een selectieve serotonineheropnameremmer) in één dierstudie als mogelijke therapie voor het beheersen van depressieve uitkomsten (**Hoofdstuk IV**). Dit toont vergelijkbare antidepressieve eigenschappen van celecoxib en citalopram, wat suggereert dat coxibs kunnen worden gebruikt om depressieve stoornissen te behandelen.

Preventieve maatregelen van inflammatoire factoren en anti-inflammatoire therapie, inclusief coxibs, kunnen dus nuttig zijn bij de preventie en behandeling van depressie-achtige en neurodegeneratieve aandoeningen. De benadering van het nabootsen van een interactie tussen bekende etiologische en omgevingsfactoren bij experimentele dieren zal waarschijnlijk bijdragen aan beter vertaalbaar onderzoek naar neuropsychiatrische aandoeningen. De twee soorten ontsteking die tegelijkertijd optreden, worden niet opgeteld op een additieve manier, maar leiden tot verschillende reacties.

Valorization

Relevance for society

Major depressive disorder (MDD) remains to be one of the most common neuropsychiatric disorders recognized among the top causes of health loss worldwide. The etiology of MDD is multifactorial and comprises both hereditary and environmental factors, including stressful and pro-inflammatory triggers. A combination of disease-driven 'sterile' inflammation due to emotional stress or genetically-determined neurodegeneration and pathogen-induced inflammation is a common clinical situation. An interaction of various factors when combined may lead to an altered progression of pathological processes in the CNS, including possible exacerbation of neuropsychiatric outcomes. Investigation of molecular mechanisms of such interactions is necessary for better understanding of the factors influencing the onset and progression of MDD, which will be used for the development of therapeutic approaches. Additionally, the FUS[1-359]-tg mouse line used in this study can serve as a useful model to further explore the role of microglia and inflammation in the mechanisms of ALS/FTLD syndrome.

Target groups

Patients with specific etiological factors of neuropsychiatric disorders, such as abnormal HPA activity due to chronic stress or neurodegenerative processes, and systemic inflammation. The combination of both factors may exacerbate neuropsychiatric symptoms.

Activity / Products

My work has demonstrated that the use of anti-inflammatory treatments can be beneficial in the management of neuropsychiatric and neurodegenerative disorders. The exploitation of coxibs in the management of depressive-like syndrome can be such example, suggesting that this therapeutic approach needs further investigation and development.

Innovation

My work is innovative in terms of exploitation of animal models that mimic an interplay between etiological factors of neuropsychiatric disorders rather than use a single challenge. This approach is likely to result in more valid translational research, as a combination of various etiological factors for neuropsychiatric disorders with a systemic inflammation represent a common clinical situation, and an interaction between systemic inflammation and specific etiological factors of neuropsychiatric disorders can exacerbate their symptoms.

Implementation

The results of my research have been published in peer-reviewed international journals, presented at international conferences, and contributed to our understanding of the overlapping molecular mechanisms underlying depressive syndrome induced by emotional stress, neurodegeneration, and systemic inflammation.

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I am filled with the utmost gratitude for all of **my school teachers and university professors** who played an integral role in shaping my education and inspiring my passion for the life sciences. However, I would like to extend a particularly heartfelt thank you to **my first school teacher of Biology, Vladimir Koshman**. His dedication to teaching ignited my love for the field of biology and set me on the path that led me to where I am today. His influence has had a profound impact on my life and career, and for that, I am forever grateful.

I would like to express my heartfelt gratitude to **my parents and relatives** for their unwavering love and support throughout my academic journey. Their belief in my potential and their encouragement during times of self-doubt and lack of motivation have been invaluable. Their constant presence in my life has given me the strength and determination to pursue my dreams, and I am forever grateful for their guidance and love. To my family and to my beloved fiancée, I offer the warmest thanks for being my rock and my constant source of inspiration.

About the author

Alexander Trofimov was born on February 24th, 1987, in Solovetskoye village, Kostroma region, USSR (now Russia). He studied genetics and biochemistry at Saint Petersburg State University from 2005 to 2011, conducting research on molecular mechanisms of cognitive dysfunctions caused by early-life immune challenges in rats under the guidance of Dr. Olga Zubareva and Prof. Victor Klimenko at the Laboratory of Neurobiology of Brain Integrative Functions, Institute of Experimental Medicine.

After earning his MSc in biochemistry in 2011, Alexander continued his studies in the field of behavioral neurophysiology. He completed a research project in September 2014 and was then invited by Prof. Tatyana Strekalova and Prof. Klaus-Peter Lesch to perform collaborative experiments at the Division of Molecular Psychiatry, Department of Psychiatry and Psychotherapy, University Hospital of Würzburg, Germany. Alexander traveled to Germany multiple times between 2014 and 2016 to learn research methods, including immunohistochemical assays, cell and slice electrophysiology, mammalian cell cultures, and RT-qPCR.

In 2015, Alexander was awarded a scholarship from the German Academic Exchange Service (DAAD) to conduct one year of research at Prof. K.-P. Lesch's laboratory, and he was employed there as a research fellow for one year in 2016. During this time, he also undertook short research visits to the National Institute of Hygiene and Tropical Medicine in Lisbon, Portugal (Dr. João Costa-Nunes, November 2014), the University of Maastricht in the Netherlands (Prof. Harry Steinbusch and Prof. Tatyana

Strekalova, March 2015), and the University of Lyon in France (Prof. Raymond Cespuglio, November 2016).

From October 2017 to December 2018, Alexander worked as a research fellow at the Laboratory of Psychiatric Neurobiology at I.M. Sechenov First Moscow State Medical University. His research focused on mouse models that combine inflammatory (LPS), stressful (chronic mild stress), and neurodegenerative (FUS model of ALS/FTLD) factors of pathogenesis of neuropsychiatric disorders, under the guidance of Prof. Tatyana Strekalova and general supervision of Prof. Klaus-Peter Lesch.

Alexander has presented his research at several international conferences, including the Göttingen Meeting of the German Neuroscience Society, the Annual MHeNs Research Day in The Netherlands, the Sechenov International Biomedical Summit in Russia, and the International Neuroscience and Biological Psychiatry Conference "Stress and Behavior" in Russia. He has given one oral presentation and presented three posters. Alexander has co-authored 16 papers in the field of neurobiology, five of which were written with Prof. K.-P. Lesch and Prof. T. Strekalova. These publications have been published in international peer-reviewed journals, including the Journal of Neuroinflammation, Neurotoxicity Research, Molecular Neurobiology, and the International Journal of Molecular Sciences, as well as one paper in a Russian journal. These publications have a cumulative impact factor of 20. Alexander's h-index is currently 5.

In 2022, Alexander Trofimov was appointed as an external Ph.D. student at the Department of Neuroscience, School for Mental Health and Neuroscience, Maastricht University, The Netherlands.

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Employment

- Institute of Experimental Medicine (FSBSI "IEM"), Saint Petersburg, Russia; Senior Researcher at the Laboratory of Neurobiology of the Brain Integrative Functions, I.P. Pavlov Department of Physiology, Nov 2008 — Present.
- St. Petersburg Chemical and Pharmaceutical University (SPCPU), Saint Petersburg, Russia; Associate Professor at the Department of Physiology and Pathology, Sep 2020 — Present.
- Nazarbayev University, Astana, Republic of Kazakhstan; Research Assistant at the School of Sciences and Humanities, Aug 2023 — Present.

Training experiences in other research Institutes

- Institute of Physiologically Active Compounds, Russian Academy of Sciences (Chernogolovka, Russia), Laboratory of Biomolecular Screening (Prof. Tatyana Strekalova); Research Fellow, Feb 2014 — Dec 2016.
- University Hospital of Würzburg (Germany), Division of Molecular Psychiatry, Department of Psychiatry, Psychosomatics and Psychotherapy (Prof. Klaus-Peter Lesch); Research Fellow, DAAD Scholar, Sep 2014 — Dec 2016.
- Portugal's National Institute of Hygiene and Tropical Medicine (Dr. João Pedro Costa-Nunes); Visiting Researcher, Oct 2014 — Nov 2014.
- Claude Bernard University Lyon 1 (France), Faculty of Medicine (Prof. Raymond Cespuglio); Visiting Researcher, Nov 2016.

- I.M. Sechenov First Moscow State Medical University, Moscow, Russia (Prof. Klaus-Peter Lesch, Prof. Tatyana Strekalova); Researcher at the Laboratory of Psychiatric Neurobiology, Oct 2017 — Dec 2018.
- University of Coimbra, Center for Neuroscience and Cell Biology (CNC), Coimbra, Portugal (Prof. Ana Luisa Carvalho); Visiting Researcher at the Synapse Biology group, Jan 2023 — Jul 2023.

Conferences attended, given presentations and seminars

(*relevant to this Thesis are given in italics*)

Oral presentations

1. **Trofimov AN**, Zubareva OE, Simbirtsev AS, Klimenko VM. Spatial memory derangements are caused by increased level of interleukin-1ß in early postnatal ontogenesis // 14th Multidisciplinary International Conference on Neuroscience and Biological Psychiatry "Stress and Behavior", St. Petersburg, Russia (May, 2010)
2. **Trofimov AN**, Schwarz AP. Spatial memory impairments caused by interleukin-1ß administration in early postnatal ontogenesis // All-Russian Scientific Conference for Young Researchers "The issues of biomedical science in the 3rd millennium", St. Petersburg, Russia (December, 2010)
3. **Trofimov AN**, Sechina MS. Lipopolysaccharide injections during early postnatal development impair the formation of exploratory behavior and conditioned learning of adult rats // 18th International Pushchino

School-Conference for Young Scientists “Biology is the science of the XXI century”, Pushchino, Russia (April, 2014)

4. **Trofimov AN**, Schwarz AP, Sechina MS, Veniaminova EA, Fomalont KJ, Zubareva OE, Klimenko VM. Altered exploratory behavior and cognitive dysfunction in mature rats caused by neonatal administration of pro-inflammatory factors are associated with impairment in the expression of neuroplasticity genes // 21st Annual International "Stress and Behavior" Neuroscience and Biopsychiatry Conference, St. Petersburg, Russia (May, 2014)
5. **Trofimov AN**, Schwarz AP, Sechina MS, Schukina VA, Veniaminova EA, Markova NA, Fomalont KJ. Formation of cognitive deficiency due to perinatal pathology: possible role of extracellular proteolytic system Mmp9/Timp1 // XX International Conference “Fundamental Science and Clinical Medicine — Man and Health” For Young Researchers, St. Petersburg, Russia (April, 2017)
6. **Trofimov AN**, Schwarz AP, Fomalont K, Schukina VA, Veniaminova EA, Markova NA, Zubareva OE, Klimenko VM. Effects of neonatal lipopolysaccharide treatment on *Mmp9* and *Timp1* mRNA expression in the rat brain // 24th Multidisciplinary International Neuroscience and Biological Psychiatry Conference “Stress and Behavior”, St. Petersburg, Russia (May, 2017)
7. ***Trofimov AN**, de Munter J, Lysikova EA, Veniaminova EA, Gorlova AV, Wolters E, Klimenko VM, Lesch K-P, Strekalova TV. Behavioral alterations and response to systemic inflammation in mice with the FUS gene mutation, a new model of amyotrophic lateral sclerosis // 25th Multidisciplinary International Neuroscience and Biological*

Psychiatry Conference "Stress and Behavior", St. Petersburg, Russia (May, 2018)

8. **Trofimov AN**, Krytskaya DU, Schwarz AP, Shcherbakova KP. Medium Chain Triglyceride (C8–C10) supplementation improves memory in rat // Fundamental Science and Clinical Medicine — Homo and Health: 22nd International Medical Biological Conference of Young Researchers, St. Petersburg, Russia (April, 2019)
9. **Trofimov AN**, Krytskaya DU, Schwarz AP, Shcherbakova KP, Klimenko VM. Mild ketogenic diet with medium-chain (C8-C10) triglyceride supplementation improves the memory in rats // 26th Multidisciplinary International Neuroscience and Biological Psychiatry Conference "Stress and Behavior", St. Petersburg, Russia (May, 2019)
10. **Trofimov AN**, Zubareva OE, Schwarz AP, Veniaminova EA, Fomalont K, Klimenko VM. Delayed cognitive deficit as a result of neonatal lipopolysaccharide exposure: a presumable implication of long-lasting changes of neuroplastic gene expression // VII International Symposium "Interaction of the Nervous and Immune Systems in Health and Disease", St. Petersburg, Russia (May, 2019)
11. **Trofimov AN**, Zubareva OE, Schwarz AP, Veniaminova EA, Fomalont K, Klimenko VM. Long-lasting impairment of neuroplastic gene expression as a mechanism of cognitive deficit caused by neonatal LPS exposure // 23rd ESN Biennial Meeting – 7th Conference on Molecular Mechanisms of Regulation in the Nervous System, Milan, Italy (September, 2019)
12. **Trofimov AN**, Nikitina VA, Zakharova MV, Kovalenko AA, Tsikunov SG, Beznin GV, Krytskaya DU, Schwarz AP, Zubareva OE. Stress-induced

changes of NMDA and AMPA receptor expression in the rat brain are aggravated by neonatal bacterial endotoxin exposure // CNS Diseases: Advanced Diagnostics and Treatment Conference (CNS Diseases-2019), Melbourne, Australia (September, 2019)

13. Zubareva OE, Nikitina VA, **Trofimov AN**, Zakharova MV, Kovalenko AA, Beznin GV, Krytskaya DU, Schwarz AP, Tsikunov SG. Early life lipopolysaccharide exposure alters stress-induced changes of NMDA and AMPA receptor expression in the rat brain // 27th Multidisciplinary International Neuroscience and Biological Psychiatry Conference "Stress and Behavior", St. Petersburg, Russia (September, 2020)

14. **Trofimov AN**, Ivleva IS, Schwarz AP, Krytskaya DU, Nikitina VA, Apryatin SA, Karpenko MN, Shcherbakova KP. Medium-chain triglyceride feeding promotes intermittent ketosis and improves working memory with no adverse effects on metabolic markers in rats // 1st ESN Virtual Conference "Future Perspectives for European Neurochemistry – a Young Scientist's Conference" (May, 2021)

15. ***Trofimov A**, Pavlov D, Goswami A, Gorlova A, Chaprov K, Umriukhin A, Kalueff A, Deykin D, Lesch K-P, Anthony D, Strekalova T. Lipopolysaccharide triggers exacerbated neuroinflammation and behavioural disturbances in mice with truncated Fused-in-Sarcoma Protein (FUS) // International Symposium and Research Workshop Series "Modern approaches in translational research on neurodegeneration and neuropathology: from mechanisms to new therapies", Astana, Kazakhstan (April, 2023; online presentation)*

Posters

1. **Trofimov AN.** Expression of *Creb1* gene in the rat brain during the locomotor conditioning // III Youth International Medical Congress "St. Petersburg scientific readings", St. Petersburg, Russia (December, 2009)
2. **Trofimov AN**, Zubareva OE. Expression of the *Creb1* gene in the hippocampus and frontal cortex of rats during active avoidance conditioning // 14th International Pushchino School-Conference for Young Scientists "Biology is the science of the XXI century", Pushchino, Russia (April, 2010)
3. **Trofimov AN.** The influence of the neonatal interleukin-1β administrations on the rats' spatial memory formation // XVI Intercity Conference for Young Scientists "The current issues of pathophysiology", St. Petersburg, Russia (April, 2010)
4. **Trofimov AN**, Simbirtsev AS, Klimenko VM, Zubareva OE, Karpenko MN, Schwarz AP. The role of immune factors in the formation of susceptibility to schizophrenia // 1st International Internet-Conference "The Molecular Mechanisms of Schizophrenia" (March, 2011)
5. **Trofimov AN**, Zubareva OE, Klimenko VM. Neonatal interleukin-1β increase causes the spatial memory impairment in adult rats // All-Russian Conference-School for Young Scientists "The neurobiology of the brain integrative functions", St. Petersburg, Russia (November, 2011)
6. **Trofimov AN**, Schwarz AP, Zubareva OE, Klimenko VM. Cellular and molecular mechanisms of the formation of delayed cognitive deficit

caused by increased level of interleukin-1β in neonatal period // 17th Annual International "Stress and Behavior" Neuroscience and Biopsychiatry Conference, St. Petersburg, Russia (May, 2012)

7. **Trofimov AN**, Schwarz AP, Veniaminova EA, Zubareva OE, Markova NA, Steinbusch HMW, Strekalova TV, Klimenko VM // Neonatal Elevation of Interleukin-1β, Cognitive Functioning of Adult Brain and *Fgf2* Expression in Brain Structures in a Rat // 11th Göttingen Meeting of the German Neuroscience Society, Göttingen, Germany (March , 2015)

8. ***Trofimov A**, Couch Y, Markova N, Steinbusch HW, Lesch KP, Anthony DC, Strekalova T. Synergistic and opposing effects of chronic stress alone or combined with lipopolysaccharide injection on aggressive and depressive-like behaviors: a possible role of SERT // 8th Annual MHeNs Research Day 2015, Maastricht, Netherlands (October, 2015)*

9. **Trofimov AN**, Schwarz AP, Sechina MS, Schukina VA, Veniaminova EA, Markova NA, Fomalont KJ. Cognitive malfunctions caused by neonatal inflammatory process: a possible role of Mmp9/Timp1 extracellular proteolytic system // St. Petersburg Scientific forum devoted to 100 anniversary of the Russian Physiological Society by I.P. Pavlov, St. Petersburg, Russia (April, 2017)

10. **Trofimov A,** Schwarz A, Fomalont K, Zubareva O, Klimenko V. Immediate and long-lasting effects of early life LPS treatment on *Mmp9* and *Timp1* mRNA expression in the rat brain: rescue by learning // VI International Symposium “Interaction of the Nervous and Immune Systems in Health and Disease”, St. Petersburg, Russia (June, 2017)

11. **Trofimov A**, Schwarz A, Fomalont K. Immediate and delayed effects of neonatal inflammatory process on *Mmp9* and *Timp1* mRNA expression in the rat brain: rescue by repeated training // FENS Regional Meeting, Pécs, Hungary (September, 2017)
12. **Trofimov A**, Schwarz A, Krytskaya D, Shavva V, Veniaminova E, Sizov V, Scherbakova K. Improved memory of rats fed with medium chain (C8-C10) triglyceride enriched diet // III All-Russian Youth Conference with International Participation "Neurobiology of Integrative Functions of the Brain", St. Petersburg, Russia (October, 2017)
13. ***Trofimov A**, de Munter J, Gorlova A, Lysikova E, Ovchinnikov R, Serebryakova O, Pavlov D, Lesch K-P, Anthony D, Strekalova T. Pro-inflammatory CNS changes and altered emotionality as prodromal phase of the amyotrophic lateral sclerosis syndrome: a study on FUS transgenic mouse model // Sechenov International Biomedical Summit SIBS-2018, Moscow, Russia (May, 2018)*
14. **Trofimov AN**, Schwarz AP, Krytskaya DU, Shavva VS, Veniaminova EA, Sizoff VV, Shcherbakova KP. Medium-chain (C8-C10) triglyceride-enriched diet improves the memory in rats // 11th FENS Forum of Neuroscience, Berlin, Germany (June, 2018)
15. **Trofimov AN**, Nikitina VA, Krytskaya DU, Schwarz AP, Tsikunov SG, Zubareva OE. Life-threatening stress reverses caused by neonatal LPS exposure alterations of NMDA and AMPA receptor subunit expression in the rat brain // FENS 2020 Virtual Meeting (July, 2020).
16. **Trofimov AN**, Schwarz AP, Krytskaya DU, Shavva VS, Veniaminova EA, Apryatin SA, Karpenko MN, Shcherbakova KP. Prolonged treatment with

medium chain triglycerides (C8, C10) induces positive effect on cognitive abilities of intact rats // Virtual Mini-Conference “Molecular Mechanisms of Cognitive Impairment and Intellectual Disability” organized by European Society for Neurochemistry (ESN) in collaboration with Federation of European Neuroscience Societies (FENS) (July, 2020).

17. ***Trofimov A**, Strekalova T, Pavlov D, Anthony DC, Gorlova A, Efimochkina S, Cespuglio R, Lesch K-P. The role of hippocampal cyclooxygenase-2 (COX-2) over-expression in the susceptibility and resilience to stress-induced anhedonia: a study in mice // EURON PhD Days (February, 2022)*

18. **Trofimov AN**, Nikitina VA, Ivleva IS, Schwarz AP, Krytskaya DU, Apryatin SA, Karpenko MN, Shcherbakova KP. Dose-dependent differential effects of intermittent ketosis established by medium-chain triglyceride supplementation on cognitive parameters in rats // FENS Forum 2022, Paris, France (July, 2022)

19. **Trofimov A**, Schwarz A, Ivleva I, Nikitina V, Krytskaya D, Apryatin S, Karpenko M, Shcherbakova K. Intermittent ketosis established by medium-chain triglyceride (MCT) supplementation: dose-dependent differential effects on cognitive and metabolic parameters in rats // ISN-APSN 2022 Biennial Meeting, Honolulu, USA (August, 2022)

20. **Trofimov A**, Schwarz A, Ivleva I, Nikitina V, Krytskaya D, Apryatin S, Karpenko M, Shcherbakova K. Dose-dependent effects of MCT supplementation on cognitive and metabolic parameters in rats // FENS Regional Meeting 2023, Algarve, Portugal (May, 2023)

List of publications

(*relevant to this Thesis are given in italics*)

1. **Trofimov AN**, Zubareva OE, Simbirtsev AS, Klimenko VM. Effects of neonatal increases in interleukin-1ß levels on the formation of spatial memory in adult rats. Neuroscience and Behavioral Physiology. 2014;44(3):359-364. doi: 10.1007/s11055-014-9918-1

2. **Trofimov AN**, Zubareva OE, Shvarts AP, Ishchenko AM, Klimenko VM. Expression of the *Fgf2* and *Timp1* Genes in the Adult Rat Brain after Administration of Interleukin-1ß during Early Postnatal Ontogeny. Neuroscience and Behavioral Physiology. 2016;46(4):413-420. doi: 10.1007/s11055-016-0252-7

3. Schwarz AP, Rotov AYu, Chuprina OI, **Trofimov AN**, Ischenko AM, Zubareva OE, Klimenko VM. Chronic elevation of Interleukin-1beta level during early postnatal period causes delayed impairments of working memory in rats. Medical Academic Journal. 2016;16(4):39-40.

4. *Couch Y*, **Trofimov A***, Markova N*, Nikolenko V, Steinbusch HW, Chekhonin V, Schroeter C, Lesch KP, Anthony DC, Strekalova T. Low-dose lipopolysaccharide (LPS) inhibits aggressive and augments depressive behaviours in a chronic mild stress model in mice. J Neuroinflammation. 2016;13(1):108. PMID: 27184538 doi: 10.1186/s12974-016-0572-0.*

5. **Trofimov A**, Strekalova T, Mortimer N, Zubareva O, Schwarz A, Svirin E, Umriukhin A, Lesch K-P, Klimenko V. Postnatal LPS challenge impacts escape learning and expression of plasticity factors Mmp9 and Timp1 in

rats: effects of repeated training. Neurotox Res. 2017;32(2):175-186. PMID: 28421528 doi: 10.1007/s12640-017-9720-2.

6. Schwarz A., **Trofimov A.**, Zubareva O., Lioudyno V., Kosheverova V., Ischenko A., Klimenko V. Prefrontal mRNA expression of Long and Short isoforms of D2 dopamine receptor: possible role in delayed learning deficit caused by early life interleukin-1ß treatment. Behav Brain Res. 2017;333:118-122. PMID: 28673768 doi: 10.1016/j.bbr.2017.06.052.
7. Strekalova T., Bazhenova N., **Trofimov A.**, Schmitt-Böhrer A.G., Markova N., Grigoriev V., Zamoyski V., Serkova T., Redkozubova O., Vinogradova D., Umriukhin A., Fisenko V., Lillesaar C., Shevtsova E., Sokolov V., Aksinenko A., Lesch K.P., Bachurin S. Pro-neurogenic, memory-enhancing and anti-stress effects of DF302, a novel fluorine gamma-carboline derivative with multi-target mechanism of action. Mol Neurobiol. 2018;55(1):335-349. PMID: 28856531 doi: 10.1007/s12035-017-0745-6
8. Strekalova TV, Lysko AI, **Trofimov AN**, Proshin AT, Pomytkin IA, Umriukhin AE. Citalopram counteracts the development of cognitive impairment in the model of stress-induced anhedonia in mice. Modern problems of science and education. 2018;6:54.
9. Schwarz AP, Rotov AY, Chuprina OI, Krytskaya DU, **Trofimov AN**, Kosheverova VV, Ischenko AM, Zubareva OE. Developmental prefrontal mRNA expression of D2 dopamine receptor splice variants and working memory impairments in rats after early life Interleukin-1ß elevation. Neurobiol Learn Mem. 2018;155:231–238. PMID: 30092312 doi: 10.1016/j.nlm.2018.08.008

10. Schwarz AP, Malygina DA, Kovalenko AA, **Trofimov AN**, Zaitsev AV. Multiplex qPCR assay for assessment of reference gene expression stability in rat tissues/samples. Mol Cell Probes. 2020;53:101611. PMID: 32485234 doi: 10.1016/j.mcp.2020.101611

11. **Trofimov AN**, Rotov AYu, Veniaminova EA, Fomalont K, Schwarz AP, Zubareva OE. Changes in Behavior and the Expression of Ionotropic Glutamate Receptor Genes in the Brains of Adult Rats after Neonatal Administration of Bacterial Lipopolysaccharide. Neuroscience and Behavioral Physiology. 2020;50(9):1239–1248. doi: 10.1007/s11055-020-01025-7

12. Nikitina VA, Zakharova MV, **Trofimov AN**, Schwarz AP, Beznin GV, Tsikunov SG, Zubareva OE. Neonatal exposure to bacterial lipopolysaccharide affects behavior and expression of ionotropic glutamate receptors in the hippocampus of adult rats after psychogenic trauma. Biochemistry (Moscow). 2021;86:761–772. PMID: 34225597 doi: 10.1134/S0006297921060134

13. *Strekalova T*, Pavlov D*, **Trofimov A***, Anthony DC, Svistunov A, Proshin A, Umriukhin A, Lyundup A, Lesch K-P, Cespuglio R. Hippocampal Over-Expression of Cyclooxygenase-2 (COX-2) Is Associated with Susceptibility to Stress-Induced Anhedonia in Mice. International Journal of Molecular Sciences. 2022;23(4):2061. PMID: 35216176 doi: 10.3390/ijms23042061*

14. Shcherbakova K, Schwarz A, Apryatin S, Karpenko M, **Trofimov A**. Supplementation of Regular Diet With Medium-Chain Triglycerides for Procognitive Effects: A Narrative Review. Frontiers in Nutrition. 2022;9:934497. PMID: 35911092 doi: 10.3389/fnut.2022.934497

15. Shcherbakova KP, Schwarz AP, Ivleva IS, Nikitina VA, Krytskaya DU, Apryatin SA, Karpenko MN, **Trofimov AN**. Short- and long-term cognitive and metabolic effects of medium-chain triglyceride supplementation in rats. Heliyon. 2023; 9(2):e13446. PMID: 36825166 doi: 10.1016/j.heliyon.2023.e13446

16. Schwarz AP, Nikitina VA, Krytskaya DU, Shcherbakova KP, **Trofimov AN**. Reference gene expression stability within the rat brain under mild intermittent ketosis induced by supplementation with medium-chain triglycerides. PLoS One. 2023 Feb 9;18(2):e0273224. PMID: 36757952; doi: 10.1371/journal.pone.0273224

17. Apryatin S, Traktirov D, Karpenko M, Ivleva I, Pestereva N, **Trofimov A**, Fesenko Z, Bolshakova M, Klimenko V. Antioxidant system alterations and physiological characteristics of juvenile dopamine transporter knockout rats. Journal of Neuroscience Research. 2023 Oct;101(10):1651-1661. PMID: 37394966. doi: 10.1002/jnr.25228

18. ***Trofimov A*,*** *Pavlov D*, Goswami A, Gorlova A, Chaprov K, Umriukhin A, Kalueff A, Deykin A, Lesch K-P, Anthony D, Strekalova T. Lipopolysaccharide triggers exacerbated microglial activation, excessive cytokine release and behavioural disturbances in mice with truncated Fused-in-Sarcoma Protein (FUS). Brain, Behavior, and Immunity — Health. 2023 Sep 15;33:100686. PMID: 37767237. doi: 10.1016/j.bbih.2023.100686*

Papers under preparation

1. Abdurasulova I, Tarasova E, Matsulevich A, Grefner N, Serebryakova M, Kudryavtsev I, **Trofimov A**, Ermolenko E. *Enterococcus faecium* L-3 Alleviates Severity of Experimental Allergic Encephalomyelitis (EAE) in Rats by Modulating Gut Microbiota, Enterocytes, and Immune Functions.
2. Rodrigues B, Leitão R, Santos M, **Trofimov A**, Silva M, Inácio Â, Abreu M, Nobre R, Costa J, Cardoso AL, Milosevic I, Peça J, Pereira de Almeida L, Pinheiro PS, Carvalho AL. MiR-186-5p inhibition restores synaptic transmission and neuronal network activity in a model of chronic stress.
3. Suleimen Y, **Trofimov A**, Mamytbekova G, Suleimen R, Ashimbayeva M, Akbay B, Tokay T. Sustainable Squalene Recovery from Uzen Oil Field Wastewater: Harnessing a New Non-Biological Resource.
4. **Trofimov A**, Shcherbakova K, Schwarz A, Akbay B, Tokay T. The Therapeutic Potential of Anti-Inflammatory Approaches in Epilepsy: Exploring the Link between Epilepsy and Neuroinflammation.

Grants and awards

1. Research grant for PhD students from the Science and Higher Education Committee of the city of St. Petersburg, Dec 2014
2. DAAD (Deutscher Akademischer Austauschdienst, German Academic Exchange Service) Research Scholarship, Aug 2015 — July 2016

3. RFBR (Russian Foundation for Basic Research) Grant for Young Scientists “My First Grant” 16-34-00316, Jan 2016 — Dec 2017
4. Federation of European Neuroscience Societies Regional Meeting (FENS RM 2017, Pécs, Hungary), Travel Grant, Sep 2017
5. European Society for Neurochemistry (23rd ESN Biennial Meeting — 7th Conference on Molecular Mechanisms of Regulation in the Nervous System, Milan Italy), Travel Grant, Sep 2019
6. Russian Science Foundation RSF, funding of projects conducted by scientific groups led by young scientists, Project 19-75-10076, Aug 2019 — Jul 2022
7. ESN–FENS Fellowship Award (FENS Forum 2022, Paris, France), Travel Grant, Jul 2022
8. ISN (International Society for Neurochemistry, ISN-APSN 2022 Biennial Meeting, Honolulu, USA) Travel Award, Aug 2022
9. ESN (European Society for Neurochemistry) Scholarship for ESN members Students/Postdoctoral Fellows (Center for Neuroscience and Cell Biology CNC, University of Coimbra, Portugal), Research Scholarship, Jan 2023 — Jul 2023
10. Federation of European Neuroscience Societies Regional Meeting (FENS RM 2023, Algarve, Portugal), Travel Grant, May 2023