THE IMPACT OF PSILOCYBIN AND EUGENOL ON BRAIN INFLAMMATION IN MURINE MODELS: UNRAVELING CUMULATIVE AND INDIVIDUAL EFFECTS

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ABSTRACT

Neuroinflammation represents a unique immune response within the central nervous system, involving glial cells such as microglia and astrocytes. Unlike peripheral inflammation, neuroinflammation affects the blood-brain barrier, glia, and neurons. Various factors can induce neuroinflammation, including surgical procedures, infections, traumatic brain injuries, toxin exposure, and immune dysregulation, involving interactions between multiple cell types and signaling molecules.

Neuroinflammation is a critical factor in various acute and chronic brain diseases. Recent research has emphasized the potential anti-inflammatory properties of naturally occurring compounds from mushrooms and plants. This study aimed to investigate the effects of psilocybin and eugenol, individually and in combination, on neuroinflammation. We used two different models to study the effects of treatment on neuroinflammation.

First, we used lipopolysaccharide (LPS) model to examine if our treatments can prevent an increase in cytokine levels in the brains of mice injected with LPS.

Second, we utilized dextran sulfate sodium (DSS) model to assess the combined anti-inflammatory effects of psilocybin and eugenol. While both psilocybin and eugenol individually displayed anti-inflammatory effects, their combined treatment demonstrated an additive effect on the reduction in neuroinflammation. This study adds to the growing body of evidence supporting the therapeutic potential of psilocybin and eugenol in psychiatric and neurodegenerative inflammatory disorders, with further research needed to understand their underlying mechanisms and clinical efficacy.

CONTRIBUTION OF AUTHORS

- **Conceptualization**: The conceptual framework for this research was developed by Igor Kovalchuk and Timur Zanikov.
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- **Validation**: Ensuring the validity of the research findings was a collaborative effort involving Igor Kovalchuk, Greg Robinson, and Olga Kovalchuk.
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- **Resources**: The necessary resources for this study were provided by Igor Kovalchuk and Olga Kovalchuk.
- **Data Curation**: Timur Zanikov and Igor Kovalchuk were responsible for curating and managing the research data.
- **Writing—Original Draft Preparation**: The initial draft of the manuscript was prepared by Timur Zanikov.
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LIST OF ABBREVIATIONS

LPS: Lipopolysaccharide
DSS: Dextran Sodium Sulfate
PCR: polymerase chain reaction
qRT-PCR: quantitative real-time polymerase chain reaction

ELISA: Enzyme-Linked Immunoassay

RNA: ribonucleic acid
ROS: reactive oxygen species
CNS: Central neuros system
PTSD: Post traumatic stress disorder
BBB: Blood-brain barrier
5-HT1A and 5-HT2A: 5-hydroxytryptamine (serotonin) receptor 1A and 2A
TRPV1: transient receptor potential cation channel of the vanilloid subtype 1
TRPA1: transient receptor potential cation channel ankyril 1
TRPM8: transient receptor potential ion channel Melastatin 8
COX-2: Cyclooxygenase 2
IL-1β: Interleukin beta
IL-2: Interleukin 2
IL-4: Interleukin 4
IL-5: Interleukin 5
IL-6: Interleukin 6
IL-8: Interleukin 8
IL-10: Interleukin 10
IL-12p40: Interleukin 12 subunit p40
IL-12p70: Interleukin 12 subunit p70
IL-13: Interleukin 13
MCP-1: Monocyte Chemoattractant Protein 1
GM-CSF: Granulocyte-macrophage colony-stimulating factor
TNF-α: tumor necrosis factor alpha
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
IgE and IgG4: Immunoglobulin E and Immunoglobulin G4
Th1: T helper 1 cells
Th2: T helper 2 cells
AD: Alzheimer's Disease
TLR4: Toll-like receptor 4
NF-κB: Nuclear Factor kappa betta
CD80: Cluster of differentiation 80
TREM2: Triggering Receptor Expressed on Myeloid Cells 2
BDNF: Brain-derived neurotrophic factor
TAS: Total antioxidant status
Eug: Eugenol
Psi: Psilocybin
SEM: Standard error for the mean
ANOVA: a one-way analysis of variance

Chapter 1 General Introduction

1.1. Neuroinflammation

Neuroinflammation refers to the diverse immune reactions that occur within the central nervous system, distinguishing it from peripheral inflammation in a number of ways, primarily due to the involvement of glial cells, such as microglia and astrocytes. Peripheral inflammation triggers a neuroinflammatory response involving blood–brain barrier (BBB), glia and neurons. Inflammation represents the innate immune system's reaction, designed to safeguard, and protect the body[1]. Within the central nervous system (CNS), glial cells hold the distinction of being the most numerous and extensively dispersed cell type. They interact with neurons, immune cells, and even blood vessels. Some glial cells function as resident innate immune cells in the CNS[2]. A multitude of factors can induce neuroinflammation, for instance aseptic insult like sterile surgical procedures, which cause tissue damage leading to an inflammatory response, as well as non-aseptic triggers such as bacterial or viral invasions, traumatic brain injuries, exposure to toxins and toxic metabolites, and immune dysregulation[3]. Inflammatory response is a complex process, that involves interactions of multiple cell types and signaling molecules.

1.1.1. Role of white blood cells (leucocytes) and endothelial cells in inflammatory response.

1.1.1.1. Leucocytes and their role in inflammatory response.

Inflammation unfolds as a sequential recruitment of leukocytes, often commencing with neutrophils as the initial cellular influx, succeeded by macrophages and lymphocytes. Within this process, pivotal microcirculatory events transpire, encompassing alterations in

vascular permeability, the mobilization and accumulation of leukocytes, and the release of inflammatory mediators[4,5].

Neutrophils, while circulating in the bloodstream, need to traverse the endothelial barrier to reach inflamed tissues rapidly. This swift migration is crucial for eliminating pathogens and facilitating tissue repair during acute inflammation. The initial phase of the inflammatory response involves a reconfiguration of the endothelial cell surface to capture drifting neutrophils. Inflammatory cytokines and peptides from bacteria stimulate the elevation of adhesive molecules on the luminal side of endothelial cells, fostering local, weak, and brief interactions between neutrophils and the endothelium, referred to as "rolling."

Chemokines deposited on the luminal side of endothelial cells subsequently activate leukocyte integrins, promoting firm adhesion and arrest through interactions with their ligands on the endothelial surface. Subsequently, activated neutrophils respond further to chemokines, undergo a significant change in cell shape from round to flat and highly polarized, defining a "front" and a "rear" or uropod. This polarized shape allows the cells to crawl on the luminal surface of the endothelium, seeking a nearby site to traverse the endothelial cell lining of blood vessels, a process known as diapedesis. Once they've crossed this endothelial barrier, the cells then navigate the pericyte layer within the venular basal membrane to access the inflamed interstitial tissues[6].

1.1.1.2. Endothelial cells and their role in inflammatory response

Endothelial cells at the BBB play a crucial role in regulating the entry of immune cells into the CNS. The BBB consists of a single layer of these specialized brain endothelial cells, known as cerebrovascular endothelial cells (BECs), and their unique properties are

responsible for maintaining cerebral homeostasis. Compared to peripheral endothelial cells, BECs possess distinctive features. These include a specific and selective transport barrier, a metabolic barrier, and a physical barrier characterized by a dense network of tight and adherens junctions. Together, these properties collectively provide a high level of resistance against the passage of molecules, polar solutes, and ions[7]. Endothelial cells also collaborate with immune cells to control both local and systemic inflammation. Both endothelial and immune cells can become activated in response to lipopolysaccharide (LPS) and tumor necrosis factor-alpha (TNFα), and this activation can result in dysfunction in both endothelial and immune cells. Endothelial dysfunction, triggered by both internal and external stimuli, can effectively initiate a state of systemic inflammation, and provoke various immune responses. This occurs through the upregulation of pro-inflammatory mediators, adhesion molecules, and an excessive adhesion and migration of immune cells[8].

1.2. Role of cytokines, chemokines, and enzymes in neuroinflammation.

Inflammatory processes play a significant role in the development of various chronic diseases, involving shared inflammatory mediators and regulatory pathways. When the body encounters inflammatory stimuli, it initiates intracellular signaling pathways that, in turn, lead to the production of inflammatory mediators. These primary inflammatory stimuli encompass microbial products and cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). They induce inflammation by interacting with specific receptors such as Toll-like receptors (TLRs), the interleukin-1 receptor (IL-1R), the interleukin-6 receptor (IL-6R), and the tumor necrosis factor receptor (TNFR). Activation of these receptors initiates crucial intracellular signaling pathways, including the mitogen-

activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways[5].

1.2.1. Interleukin 1 beta (IL-1β).

IL-1β, a member of the IL-1 cytokine family, serves various crucial roles, including its involvement in maintaining normal bodily functions such as regulating appetite, sleep, and body temperature[9]. The IL-1 cytokine family plays a role in regulating the production of other cytokines, activating phagocytosis, and inducing programmed cell death[10]. IL-1β plays a key role in neuroinflammatory processes. This includes its involvement in the central nervous system's response to peripheral challenges like LPS, as well as its signal transduction mechanism, which requires the binding of IL-1β to its cell surface receptor, IL-1R1. Subsequently, its coreceptor, IL-1RAcP, is recruited to form an activated IL-1R complex. This activated receptor complex sets off the canonical IL-1 signaling pathway, involving various components such as adaptor proteins kinases (IRAKs, TAK1), and enzymes (TRAF-6). Ultimately, this cascade leads to the activation of NF-κB, p38 MAPK, and c-Jun N-terminal kinase (JNK) pathways. Notably, the components of the IL-1/IL-1R system are also expressed in neurons, implying that IL-1 signaling can induce alterations in neuronal activity[11]. IL-1β also pivotal in the advancement of both chronic neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson's disease (PD), as well as acute neuroinflammatory situations such as stroke, ischemia, and brain injuries[12].

Nonetheless, excessive production of IL-1β is linked to the pathological alterations observed in various disease conditions, including but not limited to rheumatoid arthritis, neuropathic pain, inflammatory bowel disease, osteoarthritis, vascular disease, multiple sclerosis, and AD[9]. The sentinel cells of the innate immune system, such as macrophages and monocytes, serve as a significant source of IL-1α and IL-1β. However, numerous other cell

types, including epithelial cells, endothelial cells, and fibroblasts, are also capable of producing these cytokines. IL-1α predominantly remains anchored to the cell membrane, signaling through autocrine or juxtacrine mechanisms. In contrast, IL-1β follows an unconventional protein secretion pathway and is released for potential paracrine or systemic actions[13].

1.2.2. Tumor necrosis factor alfa (TNF-α)

TNF-α, a member of the TNF protein family, is recognized for its potent proinflammatory properties and plays a pivotal role in immune response regulation, while also serving as a crucial mediator of inflammation and participating in vital physiological functions such as maintaining homeostasis, supporting overall health, and enhancing antimicrobial immunity[14].

TNF-α is synthesized by various immune cells, such as macrophages and lymphocytes, initially as transmembrane TNF-α (tmTNF-α). An enzyme called TNF-α converting enzyme (TACE or ADAM17) cleaves tmTNF-α into its soluble form, known as soluble TNF-α (sTNF-α). TNF-α exerts its functions by binding to two distinct receptors: TNF receptor 1 (TNFR1, p55) and TNFR2 (P75). Interestingly, tmTNF-α can also act as a receptor by itself. Thus, after binding to TNFRs, it can transmit signals back into the cell.

TNF-α initiates various signaling pathways upon attaching to TNFR1 and TNFR2 receptors. The binding of TNF-α to TNFR1 triggers the trimerization of TNFR1, activating its intracellular death domain, which in turn recruits TNFR1-associated death domain (TRADD). This sequence of events sets in motion intracellular TNFR1 signaling. In contrast, TNFR2, when bound by TNF-α, also trimerizes, but it lacks a death domain. Instead, TNFR2 directly stimulates TNF receptor-associated factor (TRAF), initiating intracellular TNFR2 signaling. It's worth noting that tmTNF-α predominantly activates TNFR2. These two distinct

pathways have different outcomes. TNFR1 activation typically leads to proinflammatory responses and apoptosis (cell death), whereas TNFR2 stimulation results in cell migration and proliferation. Furthermore, soluble TNFR can bind to TNF-α with relatively high affinity. This interaction reduces the amount of available TNF-α and modulates TNF-α signaling[15].

TNF-α, a prominent proinflammatory molecule and an early initiator of neuroinflammation, engages with two receptors, R1 and R2. Through R1, it orchestrates extrinsic apoptotic signals via the Fas-associated death domain (FADD), while R2 triggers inflammation via the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). NF-κB stands as a pivotal regulatory transcription factor that plays a significant role in the activation of genes associated with inflammation.

Normally, NF-κB resides in a dormant state within the cytosol, bound to inhibitory proteins known as inhibitors of NF-κB (IκB), predominantly the alpha inhibitor of NF-κB (IκBα). In this state, NF-κB cannot translocate into the nucleus. However, various brain pathological processes, such as cerebral ischemia, trigger inflammatory stimuli, which lead to the degradation of these inhibitors, accomplished through their phosphorylation by the IκB kinase (IKK). This degradation allows NFκB to migrate into the nucleus, where it binds to DNA and initiates the transcription of numerous proinflammatory genes. This includes the upregulation of genes responsible for proinflammatory cytokines, chemokines, enzymes that produce inflammation mediators, and adhesion molecules. As a result, TNF-α not only activates NF-κB but is also activated by it, creating a positive regulatory loop that amplifies and sustains local inflammation. Consequently, these pathways clarify how TNF-α induces other inflammatory cytokines like IL-6 and IL-8 and synergizes with interferons[14].

1.2.3. Cyclooxygenase 2 (COX-2)

Cyclooxygenases (Cox) represent a family of heme-containing enzymes; they facilitate a crucial and rate-limiting process: the conversion of arachidonic acid (AA) into predominantly biologically active prostaglandins (PGs) by incorporating molecular oxygen. Notably, COX-2 is known to be upregulated in various brain cells following seizure induction, leading to an increased production of proinflammatory mediators, specifically PGs, which exacerbate the severity of seizures[16,17]. COX-2 can assume either a neurotoxic or an anti-inflammatory role, and this is contingent on the specific stimulus and the type of cells affected by the insult. Notably, COX-2 expression is predominantly detected in neurons, where it plays a role in synaptic function and memory formation. Elevated COX-2 levels are typically observed in cases of direct neuronal damage and have been identified in the pathways associated with neurodegenerative diseases[12]. COX-2 has been identified as a factor that promotes the generation of proinflammatory molecules, including IL-1β and TNFα. This activity can play a role in the initiation and progression of neuroinflammation, ultimately leading to additional damage to neurons[18].

1.2.4. Interleukin 6 (IL-6)

IL-6, a versatile cytokine, functions as a potent mediator of cellular communication and plays a critical role in regulating both innate and adaptive inflammatory responses. Its impact extends beyond immune system cells, also affecting cardiomyocytes in response to injuries[19,20]. IL-6 exerts its influence through either its membrane-bound receptor, IL-6R alpha (α) subunit (known as classical signaling), or the soluble receptor (sIL-6R), which, in association with the signal transduction protein glycoprotein 130 (gp130), triggers the activation of Janus kinase (JAK)-related signaling pathways[19].

The multifaceted nature of IL-6, as influenced by its regulation of numerous genes, is thought to explain its diverse effects. Consequently, the consequences of IL-6 production encompass both pro-inflammatory and anti-inflammatory outcomes, underscoring IL-6 central role in initiating and controlling immune responses. These effects involve controlling the differentiation of monocytes into macrophages, enhancing B-cell IgG production, suppressing dendritic cell maturation through STAT3 signaling, promoting the Th2 response while inhibiting Th1 polarization. Two distinct mechanisms have been described for IL-6 inhibition of Th1 polarization: it stimulates CD4 T cells to secrete IL-4, steering the response toward Th2, and it influences the secretion of IFNγ by CD4 T cells, an essential interferon for promoting Th1 polarization[21,22].

1.2.5. Interleukin 2 (IL-2)

IL-2 is crucial for the differentiation of CD4+ T cells into Th1 and Th2 effector subsets, while inhibiting Th17 differentiation. It is also essential for the development of memory T cells. The IL-2 receptor (IL-2R) is composed of three membrane-bound subunits: α (CD25), β (CD122), and common γ (γc) (CD132) chains. When IL-2 binds to the IL-2R on T cells, it triggers signaling pathways such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, mitogen-activated protein kinase, and phosphoinositide 3-kinase. These pathways lead to the transcription of genes related to proinflammatory cytokines, cell survival, and cell cycle regulation. IL-2 also upregulates the expression of CD25 and IL-2Rβ and influences genes involved in cell cycle regulation, promoting T-cell survival and differentiation into effector and memory cells. In summary, IL-2 is pivotal for immune response activation and control. It is produced primarily by CD4+ helper T cells and plays a crucial role in differentiating T cells into effector subsets, inhibiting

Th17 differentiation and fostering memory T cell development. IL-2 achieves these functions through the IL-2 receptor and its associated signaling pathways[23,24].

1.2.6. Interleukin 4 (IL-4) and Interleukin 13 (IL-13)

IL-4 and IL-13 belong to the Th2-type cytokine family, which also includes IL-5. These cytokines share a considerable homology of about 20–25% at the amino acid level and are closely located on chromosome 5q31. These Th2-type cytokines, IL-4 and IL-13, play a pivotal role in orchestrating type II inflammatory responses associated with allergies or parasite infections. They stimulate the proliferation of B cells and activate eosinophils, basophils, and mast cells. Additionally, IL-4 and IL-13 are involved in promoting fibrosis in both skin and internal organs. Furthermore, these cytokines have the capacity to induce a switch in the immunoglobulin (Ig) class, influencing the production of IgE and IgG4.[25,26]

1.2.7. Interleukin 5 (IL-5)

IL-5 is a potent pro-inflammatory cytokine responsible for orchestrating the maturation, proliferation, activation, and migration of eosinophils. This cytokine plays pivotal roles in the pathogenesis of eosinophilic asthma, a condition often responsive to corticosteroid therapy due to its potent capacity to induce eosinophil apoptosis. Its primary cellular sources encompass T helper-2 (Th2) lymphocytes and group 2 innate lymphoid cells (ILC2). Th2 cells undergo a complex activation process triggered by inhaled allergens and guided by dendritic cells, eventually leading to the production and secretion of IL-5. This intricate relationship between IL-5 and eosinophilic inflammation has been convincingly established through both animal and human experimental models of asthma[27].

1.2.8. Interleukin 8 (IL-8)

Interleukin 8 (IL-8), also known as chemokine (C-X-C motif) ligand 8 (CXCL8), is a proinflammatory cytokine with production originating from various cell types, including macrophages and microglia. It exerts its effects by activating multiple intracellular signaling pathways through two cell-surface receptors, namely CXCR1 and CXCR2. In the brain, IL-8 is predominantly synthesized and released by microglia and astrocytes. Unlike most other inflammatory cytokines, IL-8 has a unique profile, potentially persisting for extended periods, spanning days or even weeks. This characteristic suggests its specificity for chronic inflammatory changes associated with neurodegenerative and neuropsychological alterations within the brain. Clinically, IL-8 plays a pivotal role in numerous inflammatory and autoimmune diseases. Elevated levels of peripheral IL-8 have been observed in patients with various inflammatory conditions, autoimmune diseases, infections, and cancers[28,29].

1.2.9. Interleukin 10 (IL-10)

Interleukin-10 (IL-10) is widely recognized as a prominent anti-inflammatory cytokine, exerting multifaceted immunomodulatory functions during inflammatory responses, particularly in the resolution phase. In the context of CNS pathology, IL-10 expression increases, promoting the survival of both neuronal and glial cells. It achieves this by dampening inflammatory responses through several signaling pathways. Originally identified as a cytokine synthesis inhibitory factor, IL-10 not only attenuates the production of proinflammatory cytokines but also curbs inflammation by reducing the expression of cytokine receptors and inhibiting receptor activation. Furthermore, IL-10 exerts potent and diverse effects on various hematopoietic cells that infiltrate the brain following injury. For

instance, it diminishes the activation and effector functions of T cells, monocytes, and macrophages, effectively bringing an end to the inflammatory response in the aftermath of injury[30].

IL-10 holds a crucial position as one of the most significant anti-inflammatory cytokines in humans. It is secreted by a variety of cells, including monocytes, macrophages, dendritic cells, T cells, B cells, granulocytes, epithelial cells, keratinocytes, and mast cells. IL-10's functions encompass limiting the secretion of pro-inflammatory cytokines like TNF-α, IL-1, IL-6, and IL-12, deactivating macrophages, inhibiting the release of Th1 cytokines such as IL-2 and IFN-γ, and regulating the differentiation and proliferation of macrophages, T cells, and B cells. By effectively controlling pro-inflammatory events, IL-10 acts as a safeguard against excessive immune responses and tissue damage. Its pivotal role in maintaining immune system balance is underscored by the fact that mutations in IL-10 or components of it signaling pathway, which reduce or eliminate its anti-inflammatory properties, have been implicated in the pathogenesis of hyperinflammatory disorders like rheumatoid arthritis or inflammatory bowel disease (IBD)[31].

1.2.10. Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Originally classified as a hematopoietic growth factor capable of inducing the proliferation and differentiation of bone marrow progenitor cells, GM-CSF (CSF2) later revealed its influence on mature myeloid cells like macrophages and neutrophils, functioning as a prosurvival and activating factor with implications in inflammation[32]. The GM-CSF receptor (GM-CSFR), a type I cytokine receptor, forms a multimeric complex comprising a binding (α) subunit and a signaling (β) subunit, the latter being shared with receptors for IL-3 and IL-5[33]. GM-CSF is produced by a diverse range of cell types, including activated T and B

cells, monocytes/macrophages, endothelial cells, fibroblasts, and others like neutrophils, eosinophils, epithelial cells, mesothelial cells, chondrocytes, Paneth cells, and tumor cells. Various factors stimulate GM-CSF production, such as IL-1β, IL-23, IL-1β, IL-12, prostaglandin E2, TNF-α, IL-1, and toll-like receptor (TLR) activation, depending on the cell type. In lymphocytes, the transcription factor nuclear factor of activated T cells (NFAT) plays a role in GM-CSF production. Conversely, GM-CSF production can be inhibited by factors like IFN-γ, IL-4, IL-10, and pharmacological agents like cyclosporine A or glucocorticoids[34].

1.2.11. Monocyte Chemoattractant Protein 1 (MCP-1)

MCP-1, also referred to as chemokine (CC motif) ligand 2 (CCL2), is a pivotal chemokine involved in the regulation of neuroinflammation. In both mouse and human brains, MCP-1 and its receptor, C-C chemokine receptor type 2 (CCR2), are primarily observed in microglia, with astrocytes also serving as a source of MCP-1. Additionally, there have been reports indicating the presence of MCP-1 and CCR2 in neurons. This MCP-1/CCR2 signaling pathway has been implicated in various neuroinflammatory disorders, including Alzheimer's disease, multiple sclerosis, and ischemic brain damage. Beyond its role in the central nervous system, MCP-1 is a versatile chemokine that can be found in various cell types such as endothelial cells, epithelial cells, fibroblasts, monocyte-macrophages, and vascular smooth muscle cells. Its primary function lies in orchestrating inflammation by attracting monocytes and macrophages to sites of inflammation. Furthermore, MCP-1 has the capacity to enhance the expression of proinflammatory cytokines like TNF-α and IL-β, further contributing to the inflammatory response[35,36].

1.3. Role of neuroinflammation in neurological diseases

Degeneration of the CNS is a complex process marked by the chronic and gradual loss of neuronal structure and function. This degeneration leads to various functional and cognitive impairments. Despite ongoing research efforts, the precise causes of neuronal degeneration remain elusive. Notably, the incidence of neurodegeneration tends to rise with age, predominantly affecting individuals in mid-to-late adulthood. This phenomenon is particularly prevalent among older individuals and is associated with various neurodegenerative conditions, including Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and can also be triggered by viral infections[37].

1.3.1. Chronic neuroinflammation: Alzheimer's disease

Alzheimer's disease (AD) is a prevalent neurodegenerative disorder characterized primarily by progressive dementia. This condition results in an inability to form new memories, reflecting dysfunction within the episodic memory system. The neuropathological hallmarks of AD include the formation of tau aggregates seen as intraneuronal neurofibrillary tangles and the presence of extracellular amyloid-beta (Aβ) plaques. In regions of the brain where Aβ deposition and neuronal loss occur, activated microglia are detected, contributing to memory impairment[38]. Neuroinflammation has emerged as a critical determinant of AD prognosis. Increased inflammation and immune responses have been observed in AD patients and animal models. This inflammatory response closely correlates with Aβ burden, particularly in clinical AD stages, as Aβ activates microglia and astrocytes, which then release cytotoxic cytokines, causing neuronal damage. Anti-inflammatory medications have demonstrated efficacy in slowing AD progression, highlighting the potential benefits of controlling inflammation. Furthermore, AD pathology includes the presence of

hyperphosphorylated tau proteins forming neurofibrillary tangles and aggregated Aβ peptides as amyloid β plaques. These pathological features contribute to microtubule destabilization, membrane degeneration, and neuronal injury[39,40]. Neuroinflammation in AD involves proinflammatory cytokines and their receptors, making them promising therapeutic targets. Strategies to address this inflammation include blocking cytokine gene expression, modulating cytokine receptor function, and regulating cells involved in neuroinflammatory responses. Recent studies have shown that neutralizing proinflammatory cytokines like IL-1β or TNF-α/TNF-α receptor through antibodies can reduce tau kinase activity and the accumulation of oligomeric and fibrillary Aβ in murine AD models[41].

1.3.2. Chronic neuroinflammation: Parkinson’s disease

Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder and ranks fourteenth among causes of death. Presently, more than one million Americans are afflicted by PD. The diagnosis of PD hinges on the identification of abnormalities in motor control, such as tremors, bradykinesia, and rigidity, all of which stem from the degeneration of dopaminergic neurons located in the substantia nigra. Research data indicates that susceptible neuronal populations in PD experience mitochondrial dysfunction, oxidative damage, and the accumulation of α-synuclein, a protein prone to self-aggregation. Furthermore, the activation of microglial cells and the production of pro-inflammatory cytokines are associated with neuronal degeneration in PD[42]. Emerging insights from investigations involving both human subjects and animal models suggest that the pathology associated with α-synuclein, and the resulting neuronal dysfunction might initially manifest in peripheral neurons. These include neurons in the enteric nervous system and branches of the vagus nerve responsible for innervating various organs such as the gut and heart[43].

Neuroinflammation is a distinctive hallmark of Parkinson's disease (PD) pathology, yet the precise role of neuroinflammation in either promoting or safeguarding against neurodegeneration remains unclear. Notably, there is a significant upsurge in innate immune components, such as complement and various cytokines (e.g., IL-1, IL-2, IL-6, and TNF), observed in the substantia nigra and cerebrospinal fluid (CSF) of PD patients. Moreover, an elevation of γ/δ + T cells has been reported in both the peripheral blood and CSF of individuals with PD. There also been indications that an autoimmune mechanism, potentially mediated via humoral responses, might be implicated in the pathogenesis of PD. Over the past decade, there has been a growing body of evidence demonstrating the presence of autoantibodies targeting antigens associated with or relevant to the pathogenesis of PD in affected individuals. These antibodies include those directed against melanin, α-synuclein, and GM1 ganglioside[44].

1.3.3. Chronic inflammation: multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disorder affecting the CNS, impacting both white and gray matter. A prominent histopathological feature of MS is the presence of focal demyelinated lesions scattered throughout the white matter. The disease typically follows an episodic course, marked by recurrent exacerbations interspersed with remission periods. During the relapsing-remitting phase, immune-mediated responses are prevalent, featuring widespread microglial activation and significant cellular infiltration into the CNS. Over time, patients often progress to secondary progressive MS, characterized primarily by neuronal and axonal degeneration along with extensive cortical demyelination[45].

MS pathogenesis involves various subtypes of CD4+ T helper lymphocytes, notably Th1 and Th17, as well as cytotoxic CD8+ lymphocytes, all of which contribute to

neuroinflammation. These activated lymphocytes migrate to the brain, recruiting peripheral monocytes/macrophages, ultimately leading to myelin loss and the apoptosis/necrosis of mature oligodendrocytes. The presence of these lymphocytes triggers the activation of resident astrocytes and microglia, resulting in the release of various inflammatory mediators, including cytokines such as chemokines, IL2, IL3, TNFα, IFNγ, and others, in the extracellular compartment, where they exert cytotoxic effects against oligodendrocytes[46]. Additionally, hippocampal pathology in MS, including extensive demyelination, neuronal loss, atrophy, and microglia activation, has been substantiated through postmortem and imaging studies. MRI investigations have further revealed associations between hippocampal volume loss, altered morphology, and the presence of depressive symptoms in individuals with MS[47].

1.4. Microglia and its function

Microglia are the brain's resident macrophages, and they assume a pivotal role in an organism's defense mechanisms as well as tissue repair processes. They are central players in brain inflammation and are closely associated with inflammatory neurodegenerative diseases. The initial sign of neuroinflammation typically manifests as the activation of microglia. Microglia become activated when confronted with various triggers, including pathogens, tissue damage, excessive stimulation, neurotoxins, infections, or injuries. In response, they may even target healthy neurons, either by physically engulfing them through a process called phagocytosis or by releasing factors that induce apoptosis, a form of programmed cell death[12].

1.5. Lipopolysaccharides (LPS)

LPS, the polysaccharide constituent of gram-negative bacteria, binds to Toll-like receptor 4 (TLR4) primarily expressed on microglia within the central nervous system. This interaction initiates an innate immune response, activating TLR4-mediated signaling pathways like nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK), including P38, AKT, ERK, and JNK. Consequently, proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), are released, playing pivotal roles in the neuroinflammatory process[48]. Notably, TLR4 activation in microglia also triggers the production of neurotoxic factors, including prostaglandin E2 (PGE2) and nitric oxide (NO), contributing to extensive neuronal cell death. Additionally, the TLR-4-specific viral inhibitory peptide VIPER shows promise in inhibiting TLR-4-mediated responses induced by LPS. Importantly, the administration of LPS to animals has been associated with cognitive impairment and a spectrum of behavioral changes mirroring symptoms seen in neurodegenerative diseases in humans. This makes LPS administration a valuable approach for studying neuroinflammation-related conditions in mice[49].

1.6. Dextran sodium sulfate (DSS)

DSS is a widely employed chemical agent utilized to induce inflammation within the bowels through its administration in drinking water. This model has found extensive application in various research studies, as DSS is known to provoke damage to the intestinal epithelial barrier[50]. This damage results in heightened intestinal permeability, ultimately leading to the development of DSS-induced IBD. The compromised integrity of the gut barrier can give rise to the translocation of endogenous microbiota[51]. When inflammation originating in the gut extends into the CNS, the resident immune cells, such as astrocytes and microglia, can adopt an inflammatory state. In response, they start to release cytokines and

chemokines. Additionally, immune cells from the bloodstream and various inflammatory factors are drawn to the brain[52]. Several studies have demonstrated that this translocation is associated with cognitive impairment and alterations in serotonin (5-hydroxytryptamine, 5-HT)-producing enterochromaffin cells (EC), as well as inflammation and oxidative stress within the brain[53]. While DSS is commonly employed to investigate IBD, it's important to note that the disruption of the intestinal barrier, which induces endotoxemia, can subsequently lead to neuroinflammation[54].

1.7. Gut-brain axis

Gut microbiota is a major topic of interest in gastrointestinal biology. The gut microbiome undergoes significant changes during ontogeny in both humans and animals. The close proximity of trillions of microorganisms to the mucosa and gut lymphoid tissue underscores the critical role of a balanced microbiota in maintaining mucosal health. Conversely, an imbalanced composition, leading to dysbiosis, can contribute to a range of diseases not limited to the gut mucosa but affecting the entire body. These conditions encompass obesity, colon cancer, autoimmune disorders, allergies, and inflammatory bowel diseases[55].

Microbiota, defined as an ecological community of commensal microorganisms that coexist symbiotically and sometimes pathogenically in the human body, plays an essential role in regulatory functions related to both health and disease. Within the gut microbiota, bacterial strains exhibit remarkable diversity, varying with an individual's age. It was traditionally believed that microbial colonization in the gut primarily pertained to colon-specific activities like carbohydrate fermentation, vitamin synthesis, and xenobiotic metabolism. Moreover, the gut microbiota was recognized as a barrier against invasive

pathogenic bacteria within the gastrointestinal tract. Certain bacterial strains, including *Escherichia, Lactobacillus, Saccharomyces,* and *Bacillus*, possess the ability to synthesize various neurotransmitters such as gamma-aminobutyric acid, 5-hydroxytryptamine, dopamine, butyrate, histamine, and serotonin. These neurotransmitters can significantly influence brain activity, as they can traverse the intestinal mucosal layer and enter the bloodstream. Research has revealed that older individuals with AD tend to have reduced levels of certain bacteria in their microbiota, leading to decreased butyrate levels. This reduction in butyrate could contribute to increased brain inflammation and the progression of cognitive decline[56].

Neuroinflammation-induced imbalances can also disrupt the hypothalamic-pituitary-adrenal (HPA) axis. Certain neurotransmitters, including acetylcholine, dopamine, noradrenaline, and serotonin, regulate peripheral cytokines by affecting cortisol levels, thereby influencing the secretion of corticotropin-releasing hormone (CRH) in the hypothalamus and adrenocorticotropic hormone (ACTH) in the pituitary. Under normal circumstances, peripheral cytokines, which are hydrophilic and possess large molecular weights, cannot readily cross the BBB. However, in pathological states, they can traverse the BBB, contributing to neuroinflammatory processes[57].

1.8. Approaches to anti-inflammatory treatments

Inflammation is a dynamic process in which proinflammatory cytokines like TNF-α, IL-1β, and vascular endothelial growth factor (VEGF) play pivotal roles. Various "biologicals" have been developed to address inflammation, including agents that target specific cytokines or their receptors (anticytokine therapies), hinder lymphocyte migration into tissues, block monocyte-lymphocyte costimulatory molecule interactions, or deplete B lymphocytes.

Anticytokine therapy is now established as part of the treatment for autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis, and more[58]. Additionally, innate immune cells in the brain exhibit heightened responses during aging and in neurodegenerative diseases. Future research should consider systemic comorbidities associated with conditions like AD, systemic inflammation, and aging as important risk factors to comprehend and harness the immunological processes linked to neurodegenerative diseases. Recognizing that immune system modifications contribute to the pathogenesis of chronic neurodegenerative diseases could open new avenues for potential treatments to delay disease onset and progression[59]. Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) represent a significant drug group with valuable applications in treating inflammatory conditions like rheumatoid arthritis and osteoarthritis. This class of drugs has evolved from aspirin to the more recent developments like selective COX-2 inhibitors. While numerous drugs and natural compounds have shown potential in mitigating inflammation, additional research is imperative to firmly establish the preclinical and clinical effectiveness of these substances in combating inflammatory conditions[60]. Ongoing research is dedicated to exploring the therapeutic potential of natural compounds derived from mushrooms and plants, such as psilocybin and eugenol, with the goal of expanding our understanding of their effects on inflammation and potentially leading to the development of effective and promising treatment approaches.

1.9. Eugenol

Eugenol, primarily derived from clove oil and identified as a constituent of *Eugenia caryophyllata* in 1929, became commercially available in the United States in 1940[61]. Eugenol and its related compounds exhibit potent antioxidant properties, offering protection against free radical-mediated diseases like cancer, inflammation, type-2 diabetes mellitus, cardiovascular ailments, neurodegenerative disorders, and periodontal diseases[62].

Antioxidative effects of eugenol can manifest as scavenging free radicals or generating them, depending on their concentration and nature, thus influencing cell viability and anti-inflammatory activity variably. Traditionally employed for treating dental caries and periodontal diseases, eugenol boasts a wide range of beneficial properties, encompassing antibacterial, antifungal, anticancer, anti-inflammatory, and antioxidant activities. Within the central nervous system, eugenol contributes to neuroprotection against delayed neuronal death following ischemic damage and mitigates the toxic effects of amyloid-β peptides in PC-12 cells[63]. In the context of epilepsy, eugenol has demonstrated the potential to reduce seizure severity and neuronal excitability in experimental models. Overall, eugenol exhibits a diverse pharmacological profile, spanning anti-inflammatory, antitumor, antibacterial, antifungal, antipyretic, anesthetic, and analgesic activities[63,64].

1.10. Psylocibin

Research on the anti-inflammatory potential of psychedelics, particularly through the 5-HT2A receptor, is an evolving area of study. While specific investigations on psychedelic compounds are limited, there have been some promising findings. In 2008, a study indicated that psychedelics might have the ability to inhibit TNF-α inflammatory processes by interacting with the 5-HT2A receptor subtype, marking a significant discovery that has inspired further research in this field[65].

In vivo, psilocybin is rapidly converted into psilocin (4-hydroxy-N,N-dimethyltryptamine). The acute pharmacological effects often associated with psilocybin administration are conventionally linked to psilocin's ability to bind to 5-HT2A receptors (5HT2AR). In vitro studies using human receptor-expressing cells have revealed that psilocin exhibits a high affinity for binding to 5HT2AR. Furthermore, it has demonstrated potent anti-

inflammatory effects in animal models of inflammatory disorders. These findings suggest that psilocin, as the active metabolite of psilocybin, may have therapeutic potential not only in the realm of psychedelics but also in addressing inflammatory conditions[65,66].

Additionally, there's a hypothesis suggesting that psychedelics could interrupt ongoing neural processes by resetting resting state functional connectivity in the brain. This interruption of neural processes may contribute to the reduction of neuroinflammation and prevent cascading events from recurring, potentially leading to long-lasting therapeutic effects. Although research in this area is still in its early stages, these findings open up intriguing possibilities for the therapeutic use of psychedelics beyond their traditional applications[67,68]. Research on psilocybin-containing mushrooms has indeed primarily focused on their potential antidepressant effects and their use in treating mental health conditions. However, there is emerging interest in investigating their broader therapeutic potential, including possible anti-inflammatory properties. While the majority of studies are still in their early stages, ongoing research may reveal new insights into the various effects and applications of compounds found in these mushrooms.

1.11. Summary

In summary, inflammation's role in neurodegenerative diseases covers various aspects. It begins with the cellular processes involved in inflammation, particularly the recruitment of immune cells like neutrophils, macrophages, and lymphocytes, along with the vital role played by endothelial cells at the blood-brain barrier. There are several key inflammatory mediators such as IL-1β and TNF-α, which significantly contribute to chronic neurodegenerative conditions like Alzheimer's and Parkinson's diseases.

Moreover, there is intriguing connection between gut microbiota and neuroinflammation, demonstrating how imbalances can lead to a range of diseases, including neurodegenerative disorders. The gut-brain axis is showing the profound influence of microbiota on brain health. Notably, potential anti-inflammatory treatments are encompassing anticytokine therapies and natural compounds like eugenol, known for their antioxidant and anti-inflammatory properties. The emerging field of research on psychedelics such as psilocybin and their potential to mitigate inflammation is introduced, suggesting novel therapeutic avenues.

Based on the literature, we hypothesized that:

1. DSS and LPS will induce inflammation in the brain of animals

2. Psilocybin and eugenol will be able to alleviate this inflammation in a pre- and post-treatment regime.

To test these hypotheses, we decided to use an animal (mice)-based model of induction of inflammation through the exposure of animals to DSS or LPS. We planned to evaluate inflammation by analyzing the mRNA and protein levels of various cytokines. We further planned to test the effect on psilocybin and eugenol alone and in combination on DSS- and LPS-induced inflammation (expression of cytokines) in a pre- and post-treatment mice model.

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Chapter 2 The Effect of Combined Treatment of Psilocybin and Eugenol on Lipopolysaccharide-Induced Brain Inflammation in Mice

2.1. Abstract

Inflammation is an organism's biological defense mechanism. Acute and chronic inflammation of the body triggers the production of pro- and anti-inflammatory pathways that can affect the content of cytokines in the brain and thus cause brain inflammation. Disorders such as depression and posttraumatic stress disorder (PTSD) are often associated with elevated inflammation. Recently, positive promising clinical results of psilocybin for the treatment of depression and PTSD were reported. Thus, we decided to test whether psilocybin alone or in combination with eugenol, an anti-inflammatory and antioxidant agent, would prevent the increase in or decrease the content of cytokines in the brain of C57BL/6J mice injected with lipopolysaccharides (LPS). Two experiments were performed, one with pre-treatment of mice through gavage with psilocybin (0.88 mg/kg), eugenol (17.6 mg/kg), or combinations of psilocybin and eugenol (1:10, 1:20, or 1:50), followed by intraperitoneal injection of LPS, and the second, post-treatment, with initial injection with LPS, followed by treatment with psilocybin, eugenol, or their combination. Brain tissues were collected, and cytokines were analyzed by qRT-PCR, Western blot, and ELISA. Data were analyzed with a one-way ANOVA followed by Tukey's post hoc test or with multiple unpaired Student's t-tests. LPS upregulated mRNA expression of *COX-2*, *TNF-α*, *IL-1β*, and *IL-6*. All pre-treatments decreased the expression of *COX-2* and *TNF-α*, with psilocybin alone and in 1:50 combination, with eugenol being the most effective. In the post-treatment, all combinations of psilocybin and eugenol were effective in reducing inflammation, with the 1:50 ratio displaying the most prominent results in reducing the mRNA content of tested cytokines. Western blot analysis confirmed the effect on COX-2 and IL-1β proteins. Finally, the ELISA

showed that post-treatment with psilocybin + eugenol (1:50) demonstrated the best results, decreasing the expression of multiple markers including IL-6 and IL-8. This demonstrates the anti-inflammatory effects of a combination of psilocybin and eugenol in the brain of animals with systemically induced inflammation.

Keywords: psilocybin; eugenol; LPS; inflammation; brain

2.2. Introduction

Neuroinflammation is an inflammatory response within the brain or spinal cord. Various factors can trigger neuroinflammation, including traumatic brain injury, infections, toxins and toxic metabolites, and immune dysregulation[1]. Multiple pro-inflammatory cytokines, chemokines, secondary messengers (NO and inositol trisphosphate), and reactive oxygen species (ROS) contribute to this inflammatory response. In the central nervous system (CNS), many of these mediators are produced by activated cells, such as microglia and astrocytes, endothelial cells, and immune cells derived from the peripheral nervous system[2,3].

Neuroinflammation protects the brain against insults by removing or inhibiting noxious agents and reversing their effects[4]. Additionally, the inflammatory response promotes tissue repair, supports the blood–brain barrier, and removes cellular debris that would otherwise contribute to neurodegeneration and disease progression. Early inflammation is vital to the healing and regeneration of tissue following some insults and can thereby lead to neurodegeneration[5,6].

There are various mechanisms by which neuroinflammation arises, depending on its underlying cause. During the inflammation that occurs after a concussion, pro-inflammatory cytokines are released, which can worsen the damage already caused by the physical injury and cause DNA fragmentation and cell death. The release of additional cytokines can also

compromise the blood–brain barrier, which reduces its ability to prevent pathogens and other toxins from passing through it.

Both cognitive degeneration and neurodegenerative diseases are associated with inflammation and with aging. In a healthy, aging brain, pro-inflammatory cytokines are chronically increased, and anti-inflammatory cytokines are reduced. Moreover, research has revealed that the aging brains exhibit an increased number of activated microglia, indicating that the immune system is activated. Clearly, there is a link between neuroinflammation and the aging brain. Inflammation is responsible for much of the neurodegeneration associated with Alzheimer's disease. Microglia in AD are associated with amyloid plaque formation[7].

Gut inflammation is associated with the pathogenesis of Parkinson's[8] and is thought to influence the brain, impacting the substantia nigra, thereby disrupting dopamine production and leading to disease progression. Evidence suggests that inflammation plays a significant role in psychiatric illnesses as well. Brain inflammation has been linked to a wide range of diseases, including depression, schizophrenia, PTSD, and mood disorders[9].

In Gram-negative bacteria, the outer membrane contains large polysaccharide and lipid molecules known as lipopolysaccharides (LPS). Upon infection with Gram-negative bacteria, they are a major triggering factor for the inflammatory cascade. When LPS interact with their receptors, several intracellular molecules are activated that alter the expression of various inflammation-related mediators. As a result, neurodegenerative processes are triggered. Through the TLR-4 signaling pathway, LPS cause neuroinflammation, which results in cognitive impairment. This makes LPS valuable tools for the study of neuroinflammation in neurodegenerative diseases[10,11].

Eugenol, an aromatic compound commonly used as a topical pain reliever during dental procedures, is found in plants such as cloves, bay leaves, and allspice. Pharmacological

studies have demonstrated that eugenol is an effective free radical scavenger with anticonvulsant, bactericidal, antifungal, analgesic, antiseptic, hepatoprotective, and antioxidant properties. The mechanism of eugenol's anti-inflammatory action involves the inhibition of tumor necrosis factor α (TNF-α) and reduced production of nitrous oxide radicals[12–14].

In 2017, Said and Rabo examined the protective effects of eugenol supplementation against aluminum (Al)-induced neural damage in rats. Co-administration of Al and eugenol restored brain-derived neurotrophic factor (BDNF) and 5-HT (serotonin) levels and enhanced total antioxidant status (TAS) in the brain. Eugenol co-administration also decreased upregulated TNF-α expression[15]. Another study, performed by Parween et al. showed that eugenol significantly improved healthy ageing and slowed neurodegeneration in a CL4176 worm (Caenorhabditis elegans) Alzheimer's model by enhancing oxidative stress resistance and slowing paralysis[16]. A different group of scientists Taheri et al. studied the effects of eugenol on an Alzheimer's model in rats. Rats treated with 0.01 mg/kg eugenol showed improved memory and had a significant decrease in amyloid plaques[17].

Akbar et al. examined the effects of eugenol in healthy mice and found it improved hippocampal dendritic complexity and memory performance, increased neurogenesis, and decreased the number of apoptotic cells in the dentate gyrus and cornu ammonis 1 basal regions[18]. As reported by Revi and Rengan, eugenol had the ability to polarize microglia from a pro-inflammatory to an anti-inflammatory state[19]. Due to its antioxidant, anti-apoptotic, and neurotrophic properties, eugenol has strong potential to be a neuroprotective agent.

Psilocybin-containing mushrooms have been used for their healing properties throughout history. Most of the research on psilocybin has focused on its antidepressant properties[20,21]. Psilocybin's success in treating various mental health disorders, such as

anxiety, depression, and obsessive–compulsive disorder, has changed how it is perceived in the medical community.

Psilocybin is a 5-HT2A (serotonin) receptor agonist. Despite serotonin's pro-inflammatory effects on 5-HT2A receptors, psilocybin has been shown to exert strong anti-inflammatory effects in animal models of inflammatory disorders[22,23]. Additionally, psychedelics have been shown in cell and animal models to inhibit inflammation induced by TNF-α.

There is great potential for psychedelics, psilocybin especially, to be used for treating neuroinflammatory disease. For example, all known genetic and environmental risk factors for AD are associated with inflammation, suggesting that reducing inflammation could be a target for disease prevention[24].

The administration of 5-HT1A and 5-HT2A receptor agonists to rats with streptozotocin-induced AD demonstrated significant neuroprotective effects in hippocampal neurons through anti-apoptotic and anti-inflammatory pathways[25]. In particular, activation of 5-HT2A receptors in rodent neurons increases Sirtuin 1 expression, which protects against reactive oxygen species as changes to SIRT1 expression and activity have been linked to inflammatory diseases[26,27]. Psilocybin and other psychedelics have been shown to stimulate neurogenesis, induce neuroplastic changes, and reduce neuroinflammation[28].

To determine whether psilocin has advantages for neural tissue homeostasis and promotes anti-inflammatory and regenerative effects, Kozkowska and colleagues examined its effects on activated microglia in a mouse model. Post-psilocin, pro-inflammatory proteins (TLR4, p65, and CD80) were downregulated, and TREM2, which is linked to neuroprotection and proper microglial phagocytosis, was upregulated. In addition, psilocin inhibited the phagocytosis of healthy neurons by microglia and caused a reduction in

microglial pro-inflammatory responses[29]. It can be concluded that psilocybin is an effective therapeutic molecule for treating multiple neural conditions characterized by inflammatory pathogenic processes.

The potential for psychedelic compounds to influence and enhance functional neuronal connectivity, stimulate neurogenesis, restore brain plasticity, reduce inflammation, and enhance cognition provides a new therapeutic target and compelling argument for further investigation of the potential for psychedelics as a disease-modifying compound in conditions where currently none exists.

Psilocybin has the capacity to function as an antioxidant and may therefore be effective at reducing inflammation-induced oxidative stress. Despite its anti-inflammatory properties, psilocybin has not yet been extensively examined in terms of its effects on brain inflammation. In this study, we aimed to investigate whether the application of eugenol and psilocybin, both separately and together, would have anti-inflammatory features in murine brains. We tested the effects of eugenol and psilocybin on LPS-induced brain inflammation in mice.

2.3. Results

2.3.1. Induction of Inflammation with LPS

We injected mice (i.p.) with 0.83 mg/kg LPS to induce inflammation in the brain[30]. This dose was previously shown to be effective in stimulating the expression of pro-inflammatory cytokines in the periphery within 1–2 h of injection and in the brain within 3–4 h; the effect in the brain lasted for at least 24 h. We collected brains from animals at 4, 24, and 48 h post-injection to analyze cytokine content.

2.3.1.1. Upregulation of Cytokines as Shown by qRT-PCR

mRNA was isolated from brain tissue and analyzed via RT-qPCR. The expression of pro-inflammatory cytokines and enzymes was quantified. All four cytokines were upregulated at 4h ($p < 0.001$). *TNF-α* and *IL-1β* were upregulated at all 3 time points ($p < 0.001$, **Figure 2.1.**B,C, respectively), while *COX-2* was downregulated after 48 h ($p < 0.01$, **Figure 2.1.**A).

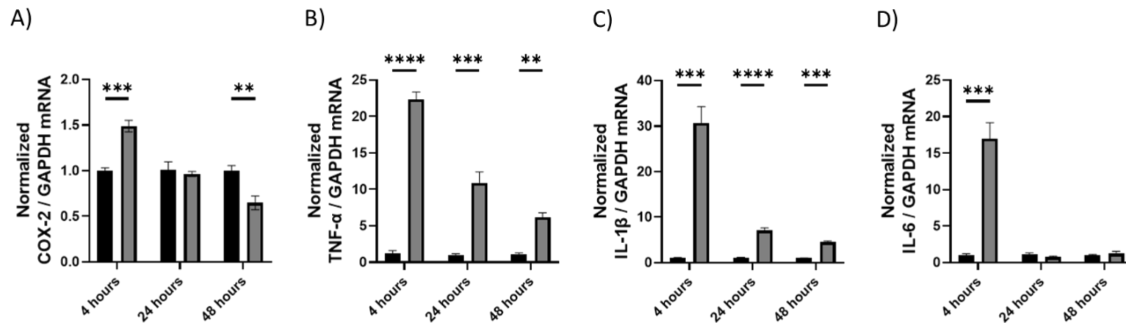

Figure 2.1. The effect of LPS on mRNA expression in brain tissue at 4, 24, and 48 h. Changes in mRNA expression as measured by RT-qPCR for: (**A**) *COX-2*, (**B**) *TNF-α*, (**C**) *IL-1β*, (**D**) *IL-6*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. Data were analyzed with (**A**–**D**): Multiple unpaired Student's t-tests were performed with a false discovery rate correction (Q = 5%, n = 3–6). Significance (p) is indicated within the figures using the following scale: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Bars represent mean ± SEM.

2.3.1.2. Changes in Cytokines Revealed by Western Blot

Protein was isolated from brains collected from mice exposed to LPS, and the expression of COX-2 and IL-1β proteins was measured. No statistically significant changes were observed (**Figure 2.2.**).

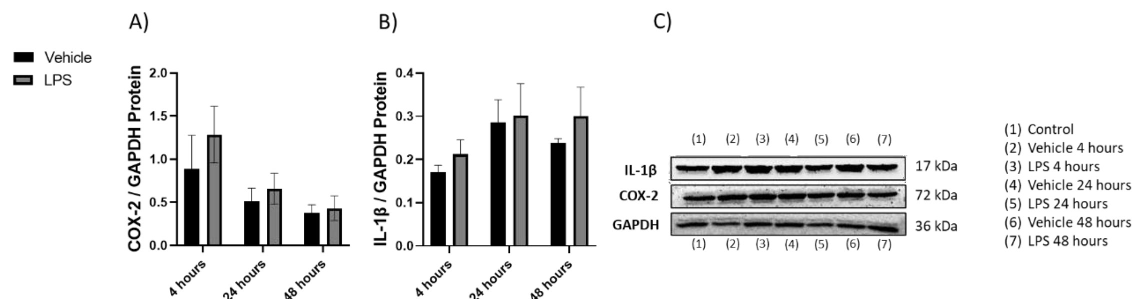

Figure 2.2. The effect of LPS and vehicle treatments on expression of COX-2 and IL-1β in 4,

24, and 48 h time points. Figures represent changed protein expression for selected genes measured by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative densitometry is presented as a ratio to GAPDH. (**A**) COX-2 expression, (**B**) IL-1β expression, (**C**) representative images blots with each protein detected. Original membranes can be seen in **Figures S2.3–S2.5**. Multiple unpaired Student's *t*-tests were performed with a false discovery rate correction (Q = 5%, $n = 3$). The samples for each protein were run on the same gel. No image enhancements were applied.

2.3.2. Pre-Treatment and Post-Treatment with Psilocybin and Eugenol

Based on the data from **Figure 2.1.**, we decided to harvest tissues 24 h after LPS treatment; at that time point, the inflammation induced at 4 h persisted within the brain. We performed pre- and post-treatment experiments. In the pre-treatment experiment, we gave animals psilocybin, eugenol, or a combination at 48 and 24 h prior to injecting them with the LPS.

2.3.2.1. Body Weight Measurements

Ther statistically significant differences were noted in the change in body weight (**Figure 2.3.**, **Table S2.3**). In the psilocybin + LPS pre-treatment group, mice gained up to 0.8 and lost up to 5.9 g of body weight during the treatment period. Notably, mice pre-treated with eugenol + LPS and mice pre-treated with psilocybin + eugenol (1:10) + LPS showed a similar decrease in weight between 2.5 to 4 g. LPS + eugenol showed interesting results for the post-treatment compared to the pre-treatment condition. Not only did this result in less severe weight loss, which was between 1.5 to 2.4 g, but some of the mice in the group gained between 0.8 and 1.7 g of weight.

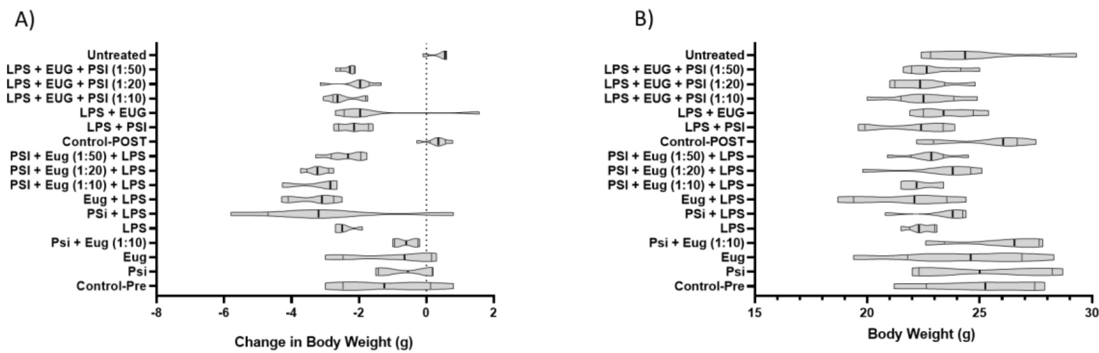

Figure 2.3. The effect of treatments on body weight (**A**) and body weight change (**B**) in the mouse model. Truncated violin plots. ANOVA and Tukey (n = 4–6). Significance of differences for (**A**) can be seen in **Table S2.3**.

2.3.2.2. RT-qPCR Analysis of Cytokines in Pre- and Post-Treatment Animals

To understand the effects of psilocybin and eugenol as well as their combinations on brain inflammation, we analyzed mRNA expression by RT-qPCR. mRNA levels of *IL-1β* trended higher than the control (p = N.S.), while only the psilocybin pre-treatment (Psi + LPS) significantly reduced *IL-1β* compared to the LPS group ($p < 0.01$, **Figure 2.4.**A). In contrast, *IL-6* mRNA levels were upregulated compared to the control ($p < 0.0001$) and downregulated by Psi + LPS ($p < 0.0001$), Psi + Eug (1:10) + LPS ($p < 0.0001$), Psi + Eug (1:20) + LPS ($p < 0.0001$), and Psi + Eug (1:50) + LPS ($p < 0.0001$) compared to the LPS group (**Figure 2.4.**B). *COX-2* mRNA levels were significantly increased compared to the control ($p < 0.01$), while all pre-treatment groups significantly reduced relative *COX-2* levels ($p < 0.01$, **Figure 2.4.**C). Interestingly, dual psilocybin and eugenol treatment resulted in similar relative *COX-2* levels to the LPS group (**Figure 2.4.**C). Similar to *COX-2*, LPS significantly increased *TNF-α* mRNA levels compared to the control ($p < 0.0001$), while all pre-treatment groups significantly reduced *TNF-α* mRNA levels compared to the LPS group ($p < 0.001$, **Figure 2.4.**D).

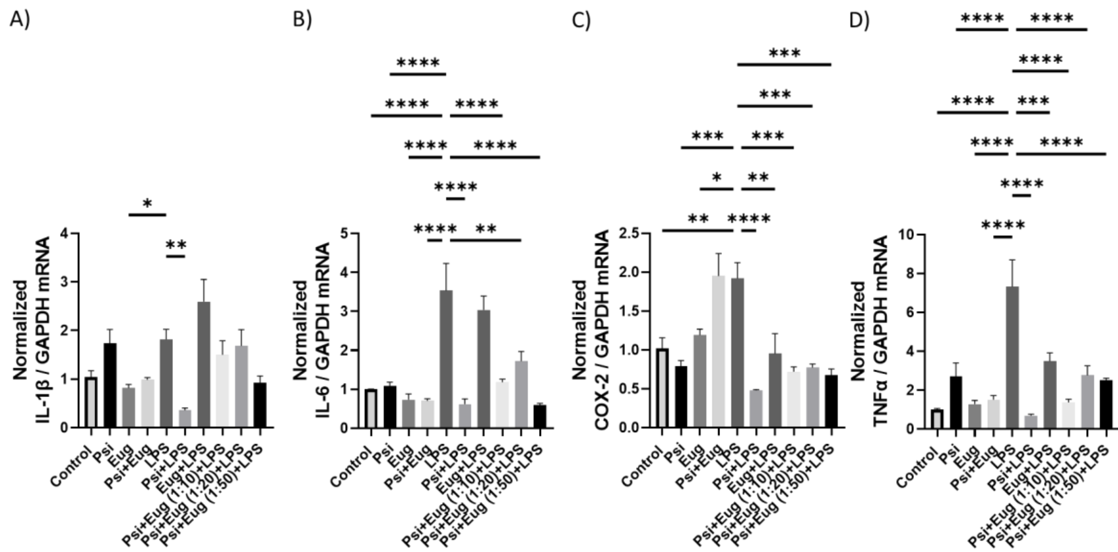

Figure 2.4. The effect of pre-treatment on mRNA expression in brain tissue. Changes in mRNA expression as measured by RT-qPCR for: (**A**) *IL-1β*, (**B**) *IL-6*, (**C**) *COX-2*, (**D**) *TNF-α*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. Data were analyzed with ANOVA and Dunnett's post hoc test compared to LPS (n = 3–6). Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Bars represent mean ± SEM.

While LPS treatment resulted in *IL-1β* levels that trended higher than the control (p = N.S.), no differences were seen in any post-treatment compared to the LPS group (p = N.S., **Figure 2.5.**A). In contrast, relative *IL-6* mRNA levels were significantly upregulated in the LPS group compared to the control ($p < 0.0001$), and all post-treatment groups significantly downregulated *IL-6* mRNA levels compared to LPS group ($p < 0.0001$, **Figure 2.5.**B). Surprisingly, LPS did not upregulate *COX-2* mRNA levels (p = N.S.), while LPS + Psi upregulated *COX-2* levels compared to the LPS group ($p < 0.05$, **Figure 2.5.**C). Similarly, *TNF-α* was significantly upregulated in the LPS group compared to the control ($p < 0.001$), while all post-treatments significantly downregulated *TNF-α* levels compared to the LPS group ($p < 0.001$, **Figure 2.5.**D).

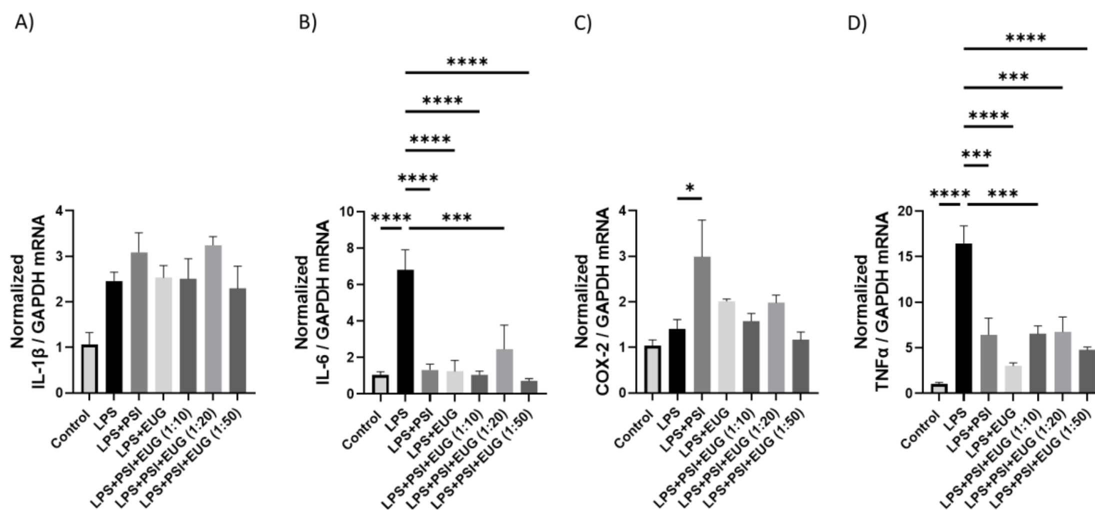

Figure 2.5. The effect of post-treatment on mRNA expression in brain tissue. Changes in mRNA expression as measured by RT-qPCR for: (**A**) *IL-1β*, (**B**) *IL-6*, (**C**) *COX2*, (**D**) *TNF-α*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. Data were analyzed with ANOVA and Dunnett's post hoc test compared to LPS (n = 3–6). Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$. Bars represent mean ± SEM.

2.3.2.3. Protein Analysis of Pre- and Post-Treatment Groups by Western Blot

Relative protein levels of COX-2 in the LPS group were shown to be upregulated compared to the control ($p < 0.05$, **Figure 2.6.**A). While pre-treatment may lower COX-2 protein abundance, no significant changes were seen compared to the LPS group (p = N.S., **Figure 2.6.**A). In contrast, IL-1β protein levels were not significantly altered across any of the pre-treatment groups; however, LPS appear to upregulate IL-1β levels (p = N.S.), while eugenol + LPS and combined treatment groups appear to decrease IL-1β levels compared to the LPS group (p = N.S., **Figure 2.6.**B).

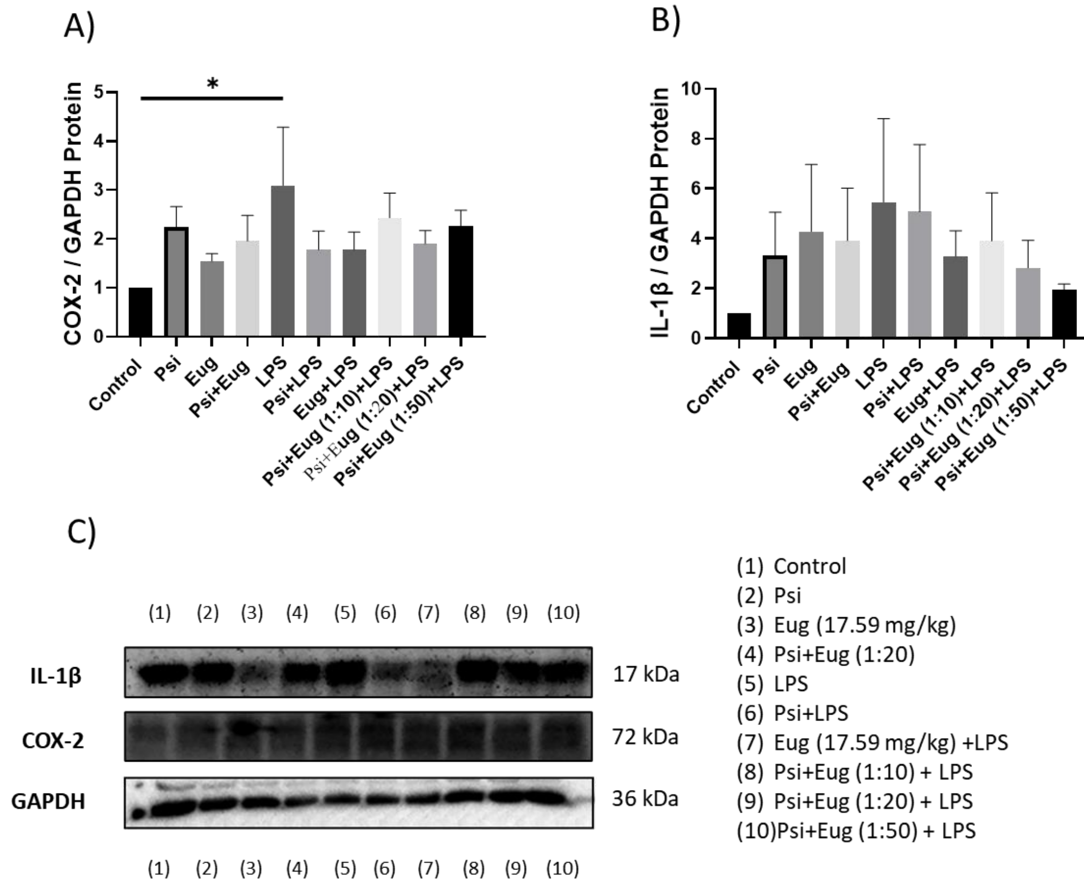

Figure 2.6. The effect of psilocybin and eugenol on the expression of (**A**) COX-2 and (**B**) IL-1β. (**C**) Representative images blots with each protein detected. Original membranes can be seen in **Figures S2.6–S2.8**. Figures represent changed protein expression for selected genes measured by Western blot in the pre-treatment group. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative densitometry is presented as a ratio of the target protein to GAPDH. Data were analyzed with ANOVA and Dunnett's post hoc test compared to LPS (*n* = 5–6). Significance (*p*) is indicated within the figures using the following scale: *, $p < 0.05$. Bars represent mean ± SEM.

For post-treatment, both COX-2 and IL-1β levels appeared to be higher in the LPS groups compared to the controls (*p* = N.S., **Figure 2.7.**A,B, respectively). While all treatments demonstrated a small trend to lower COX-2 protein levels, no significant differences were observed (*p* = N.S., **Figure 2.7.**A). In contrast, the post-treatment group Psi + Eug (1:20) + LPS significantly lowered IL-1β levels compared to the LPS group ($p < 0.05$),

while other psilocybin and eugenol post-treatment combination (1:10 and 1:50) groups showed a trend to lower IL-1β levels, but this was not significant (p = N.S., **Figure 2.7.**B).

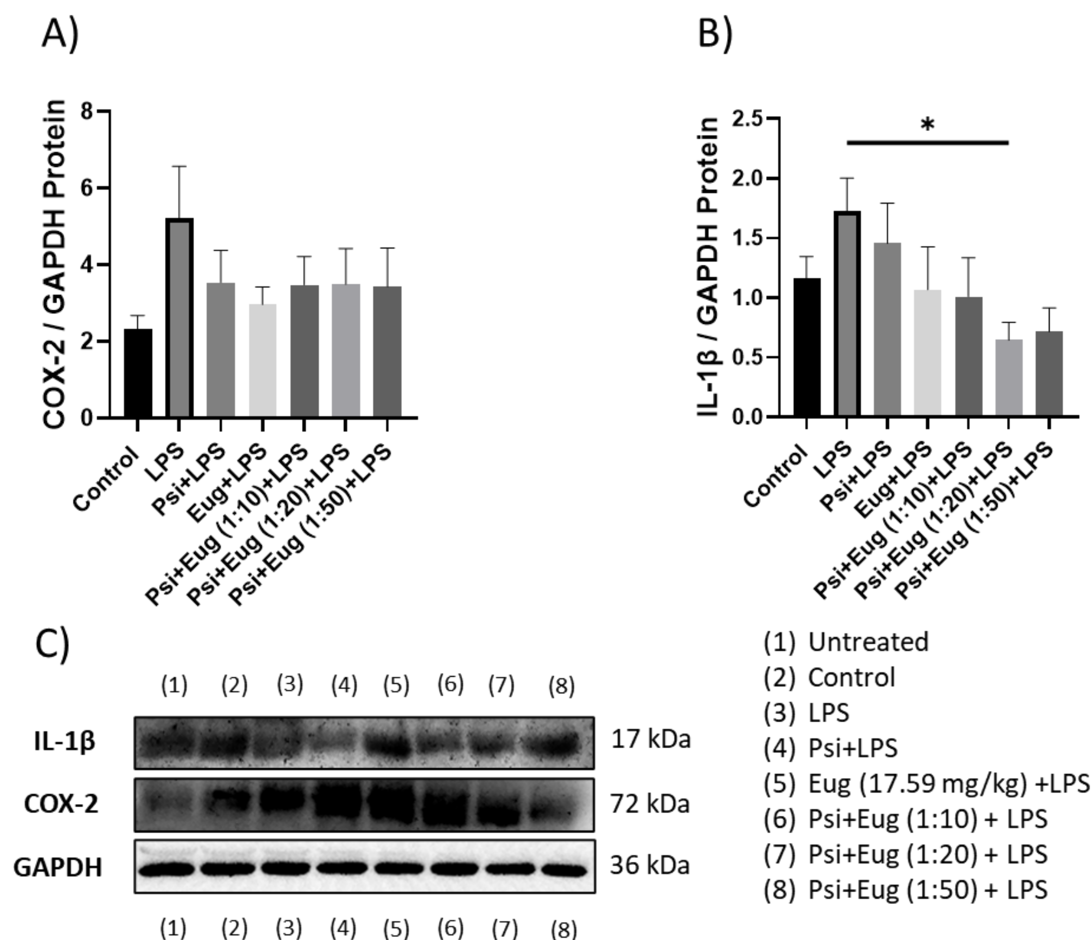

Figure 2.7. The effect of post-treatments on the expression of (**A**) COX-2 and (**B**) IL-1β. (**C**) Representative images blots with each protein detected. Original membranes can be seen in **Figures S2.9–S2.11**. Figures represent changed protein expression for selected genes measured by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative densitometry is presented as a ratio of target protein to GAPDH. Data were analyzed with ANOVA and Dunnett's post hoc test compared to LPS (n = 5–6). Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$. Bars represent mean ± SEM.

2.3.2.4. Cytokines in Pre- and Post-Treatment Groups Measured by ELISA

Among all treatment groups, LPS + psilocybin + eugenol (1:50) demonstrated a significant decrease in expression of IL-6 compared to the LPS + psilocybin + eugenol (1:20)

(**Figure 2.8.**E, $p < 0.01$) and LPS + eugenol (**Figure 2.8.**E, $p < 0.05$) groups. On the other hand, LPS + psilocybin + eugenol (1:50) demonstrated a significant increase in expression of TNF-α compared to the control (**Figure 2.8.**L, $p < 0.05$). The same results were shown for the combined treatment group (1:50) compared to the LPS group (**Figure 2.8.**L, $p < 0.01$). No other significant differences were observed (**Figure 2.8.**).

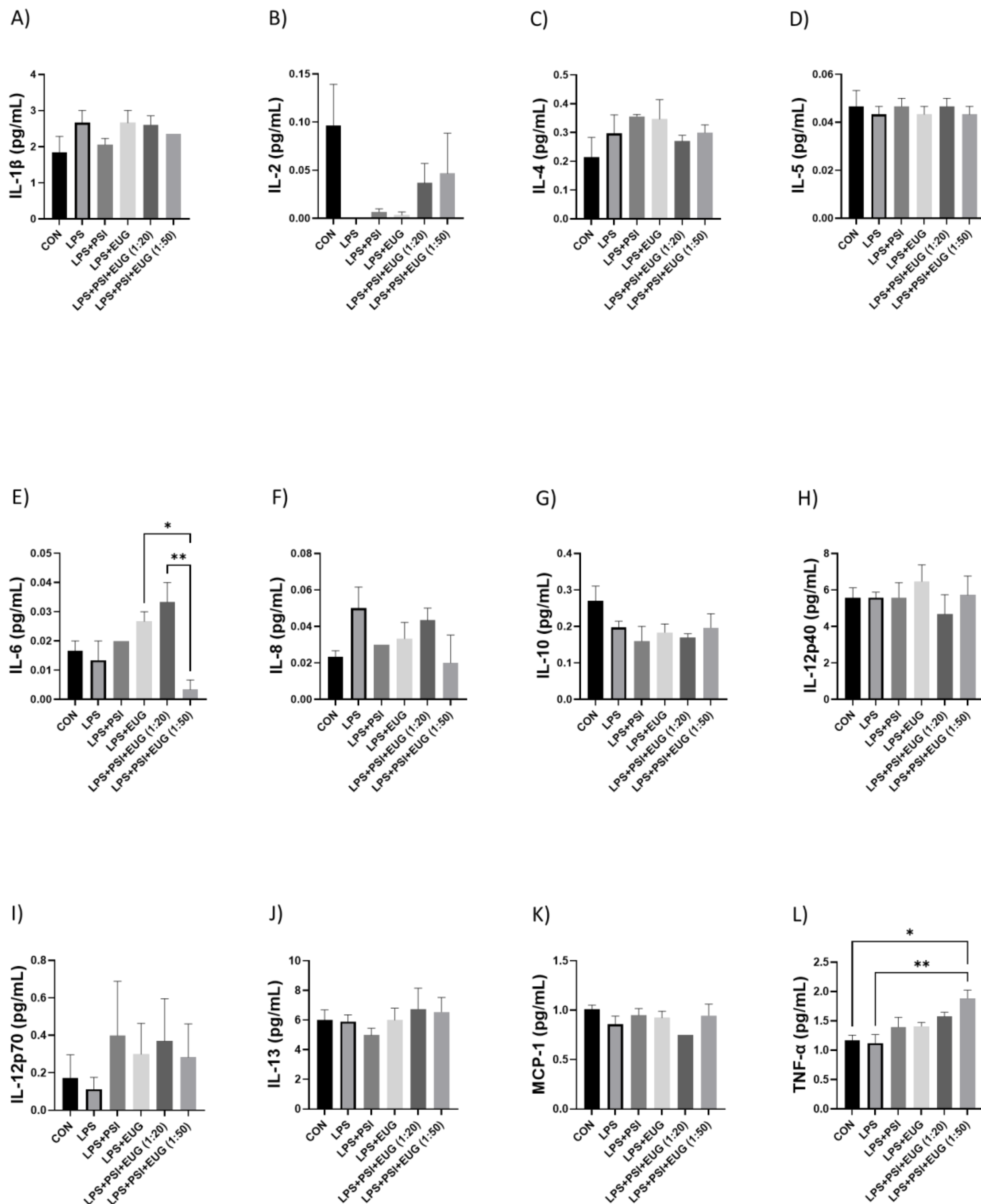

Figure 2.8. Pro-inflammatory cytokine levels in the post-treatment LPS-induced brain inflammation. The levels of (**A**) IL-1β, (**B**) IL-2, (**C**) IL-4, (**D**) IL-5, (**E**) IL-6, (**F**) IL-8, (**G**)

IL-10, (**H**) IL-12p40, (**I**) IL-12p70, (**J**) IL-13, (**K**) MCP-1, and (**L**) TNF-α were measured by

an ELISA. Data were analyzed with ANOVA and Tukey's post hoc test ($n = 3$). Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$; **, $p < 0.01$. Bars represent mean ± SEM.

Next, we measured pro-inflammatory cytokines collected in blood using the ELISA. As shown in **Figure 2.9.**A, LPS + eugenol has a significantly higher expression of IL-13 compared to the control and LPS + Eug + Psi (1:20 and 1:50) groups ($p < 0.05$). On the other hand, the control group has significantly higher expression of IL-12p70 ($p < 0.0001$) and monocyte chemotactic protein-1 (MCP-1, $p < 0.05$) compared to all other groups (**Figure 2.9.**B,C, respectively). Additional ELISA data can be found in the **Supplementary Materials (Figures S2.12 and S2.13)**.

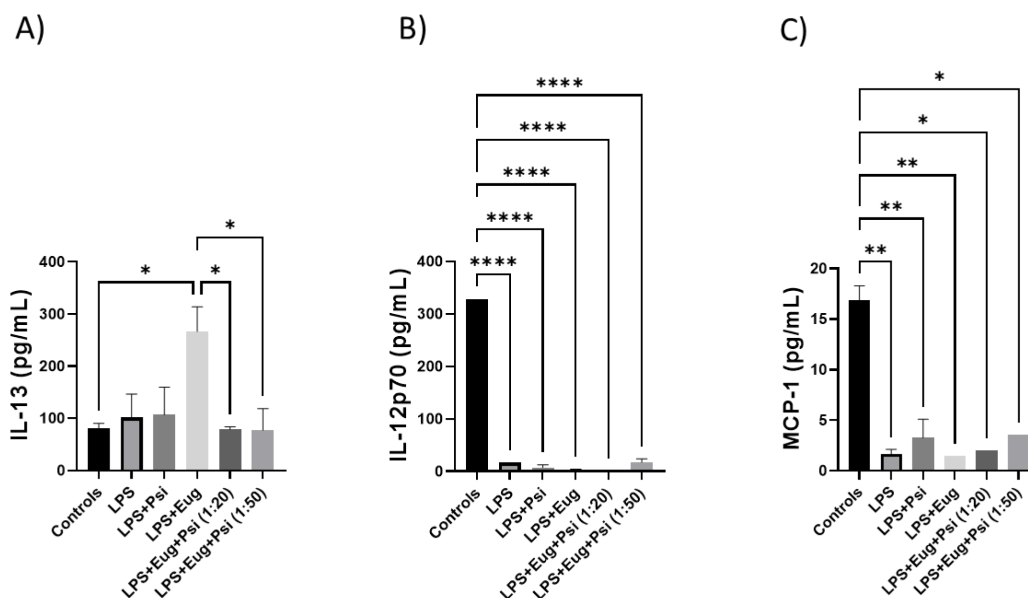

Figure 2.9. The content of pro-inflammatory cytokines in post-treatment LPS-induced inflammation in blood. The amounts of (**A**) IL-13, (**B**) IL-12p70, (**C**) MCP-1 were determined by ELISA. Data were analyzed with ANOVA and Tukey ($n = 1–3$). Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. Bars represent mean ± SEM.

2.4. Discussion

The prevention and treatment of inflammation have been the main focus of many researchers. During multiple studies across several decades, we have accumulated knowledge

about the role of inflammation in the development of chronic pathologies such as neurodegeneration, Alzheimer's disease, Huntington's disease, and Parkinson's disease, as well as their underlying signaling pathways. In addition, the anti-inflammatory properties of natural compounds of mushrooms and plants have been the subject of interest in recent studies. In this study, we focused on psilocybin and eugenol and their effects on inflammation in the brain.

Neuroinflammatory responses have profound implications for immunity, physiological function, biochemistry, and psychological well-being. Moreover, the degree of neuroinflammation depends on the context, duration, and progression of the primary stimulus or insult. An inflammatory reaction may result in the recruitment of immune cells, edema, tissue damage, and the potential for cell death. There is, however, no universal agreement on what constitutes neuroinflammation.

In our research, we used LPS to induce inflammation in mice. LPS are among the major components of the outer membrane of Gram-negative bacteria and are widely used as inflammation-inducing agents. LPS are commonly used to model sepsis and inflammation in mammals due to their potent pro-inflammatory properties and well-studied TLR4-dependent mechanisms of activation[31,32]. One of the studies reports that administration of LPS contributes to increased neuroinflammation along with damage to the blood–brain barrier (BBB)[11]. To support that point, another study states that the level of cytokines in the central nervous system and peripheral system can be elevated by the combination of LPS and toll-like receptor 4 (TLR4), which simultaneously activates the hypothalamus–pituitary–adrenal axis. Subsequently, the release of inflammatory factors, such as IL-1β, IL-6, and TNF-α, is induced by the activation of the NF-κB signaling pathway[33].

There are many applications of the LPS model, including the study of acute lung injury, endometriosis, and acute renal injury. The use of the LPS model in neural research is also widespread[34]. We established LPS-induced inflammation models in mice, which are supported by the enhanced expression of pro-inflammatory cytokines in the brain. Afterwards, we demonstrated the effect of administrating different treatments of eugenol and psilocybin, as well as their combinations, prior to and after inflammation induction.

The anti-inflammatory properties of psilocybin and psychedelic mushrooms have been the object of recent studies. Evidence suggests that psychedelics could be useful for treating the behavioral and psychological symptoms of dementia. By upregulating neurotrophic factors, they enhance neuronal survival, promote neuronal growth, and have a profound effect on immune function. The use of psychedelics may be able to modify the progression of neurodegenerative diseases[35]. In 2020, Nkadimeng et al. investigated the antioxidant and anti-inflammatory properties of *Psilocybe natalensis* on LPS-stimulated RAW 264.7 macrophages[21]. The mushroom extracts decreased LPS-induced TNF-α and inhibited pro-inflammatory cytokines IL-1β and IL-10. The reduction in these pro-inflammatory cytokines is associated with improved health outcomes in chronic inflammation[36]. Additionally, the extracts decreased LPS-induced TNF-α and inhibited pro-inflammatory cytokines IL-1β and IL-10. In chronic inflammation, reducing these pro-inflammatory cytokines is associated with improved health outcomes[21]. They also showed that mushroom extracts contained components, such as n-hexadecanoic acid, 4h-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 3-octanone, and dibutyl phthalate, can induce natural anti-inflammatory and antioxidant effects. Additionally, LPS-induced nitric oxide and prostaglandin E2 production, which plays a role in inflammatory diseases, were inhibited by extracts of *Psilocybe natalensis*. Psychedelics were shown to be potent 5-HT2A receptor antagonists and to have anti-inflammatory effects via inhibition of *TNF-α* induced inflammation[22] and several

downstream markers, such as *IL-6*, *IL-5*, *IL-1β*, *IL-13*, *GM-CSF*, and *MCP-1*[37]. IL-5 is a homodimeric cytokine, and its most important role lies in the differentiation, growth, activation, and survival of eosinophils as well as their recruitment to airways[38]. IL-4 and IL-13 are members of the Th2-type cytokines and play a critical role in the type II inflammatory response triggered by allergy or parasite infection. They stimulate B cell proliferation and activation of eosinophils, basophils, and mast cells. Additionally, IL-4 and IL-13 participate in fibrosis of skin and internal organs. These cytokines can switch immunoglobulin (Ig) class of IgE and IgG4[39–41].

Nkadimeng et al. conducted further research in 2021 on the anti-inflammatory effects of four psilocybin-containing mushrooms on LPS-induced inflammation in human macrophage cells in vitro. *Cyclooxygenase-2* (*COX-2*) is an inducible early response gene, activated in response to various stimuli, such as LPS, IL-1, and TNF-α. The COX-2 enzyme is able to synthesize pro-inflammatory mediators, prostaglandins, which have been reported to function as immuno-suppressors. Upregulation of COX-2 has been shown to be associated with inflammation[42]. LPS stimulation significantly increased the content of COX-2, TNF-α, IL-1β, and IL-6. Treatment with the extracts reversed the LPS-induced increase in COX-2, TNF-α, IL-1β, and IL-6 in a dose-dependent manner. Researchers found that the extracts contained anti-inflammatory properties similar to those of quercetin, a well-known antioxidant flavanol found in a variety of fruits and plants[36]. Psilocybin's role in inflammation has yet to be studied in full, and more research is needed to elucidate its therapeutic potential.

The effects of eugenol on inflammation have been studied for longer compared to psilocybin. As such, it is known that eugenol has an anti-inflammatory effect on acute lung injury induced by LPS. Eugenol pre-treatment downregulates the expression of pro-inflammatory cytokines *IL-6* and *TNF-α* and the signaling of inflammatory enzyme markers such as *COX-2* and *NF-κB*, which lead to inflammatory response inhibition[43]. Moreover,

eugenol can be used as a damage-preventing agent from oxidative stress[44]. Another study reports that eugenol's anti-apoptotic and anti-inflammatory effects can mediate the side effects of gemcitabine by increasing the activity of caspase-3 and reducing *COX-2* and *IL-1β* gene expression[45]. That study was conducted with HeLa cells, a human cervical cancer line. However, there are no studies describing the effect of combined treatment involving both eugenol and psilocybin.

After establishing the LPS-induced inflammation model in male mice, we administered psilocybin and eugenol as a pre-treatment or as a post-treatment. IL-1β is a pro-inflammatory cytokine that is involved in the regulation of pain, inflammation, homeostasis, and autoimmune conditions. It promotes the differentiation of monocytes into conventional dendritic cells[46,47]. TNF-α is a multifunctional cytokine that participates in the regulation of immune–inflammatory reactions involved in host defense against infectious, autoimmune, and endocrine diseases and cancer, and its actions help determine the survival or death of various cells[48]. Similar to IL-10, it has a pleiotropic effect on inflammation and immune response. Due to the trans-signaling mechanism of IL-6, it has a wide range of target cells, making it a key cytokine in inflammation[49]. The effect of psilocybin alone in the pre-treatment group showed higher expression of *TNF-α* and *IL-1β*, but not *IL-6* and *COX-2*, relative to the control group (**Figure 2.4.**). The results of RT-qPCR (**Figure 2.4.**) associate with the results of Western blotting, which demonstrated elevated content of IL-1β and COX-2 compared to the control group (**Figure 2.6.**). On the other hand, RT-qPCR results demonstrated that administrating psilocybin post-treatment resulted in a notable reduction in expression of *IL-6*, and *TNF-α*, shown in **Figure 2.5**.

The main function of MCP-1 is to recruit monocytes and macrophages to sites of inflammation. It has also been found that MCP-1 can increase the expression of both TNF-α and IL-β[50]. IL-8 plays an important role in inflammation and wound healing and can recruit T

cells and nonspecific inflammatory cells into sites of inflammation by activating neutrophils. IL-8 is mainly secreted from leukocytes and endothelial cells under special conditions such as exposure to IL-1 or TNF-α. IL-8 is chemotactic for fibroblasts; it accelerates their migration, and stimulates the deposition of tenascin, fibronectin, and collagen I during wound healing *in vivo*[38,39]. ELISA results, compared to the control group, demonstrated an increase in the amount of IL-6, IL-12p70, and TNF-α (**Figure 2.8.**E,I,L, respectively), and a decrease in IL-2 and IL-10, (**Figure 2.8.**B,G, respectively). Apart from that, post-treatment did not appear to affect the expression of IL-1β, IL-4, IL-5, IL-8, IL-10, IL-12p40, IL-13, and MCP-1 (**Figure 2.8.**A, C,D,F,G,H,J,K, respectively). IL-2 cytokine is involved in the activation and regulation of the immune response. It is produced by T cells, specifically CD4+ helper cells. It takes part in effector T-cell differentiation and provides T cells with a long-lasting competitive advantage, resulting in the optimal survival and function of memory cells. Not only can IL-2 induce the proliferation of T cells and T-helper 1 and Th2 effector, but it is also crucial in the development of T memory cells[51,52]. IL-10, a cytokine, can affect the activity of multiple cell types. It inhibits the production of pro-inflammatory cytokines by inhibiting T-helper 1 (Th1) lymphocytes and stimulating B lymphocytes and Th2 lymphocytes. This leads to the downregulation of the inflammatory response[53]. IL-12p40 is a subunit of IL-12 and acts as a chemoattractant for macrophages; it also promotes the migration of bacterially stimulated dendritic cells. IL-12p40 is associated with several pathogenic inflammatory responses such as silicosis, graft rejection, and asthma. However, it is protective in a mycobacterial model[54]. Another subunit of IL-2 is IL-12p70, a pro-inflammatory cytokine composed of p35 and p40. It enhances Th1, cytotoxic CD8+ T, and NK cell responses by increasing IFN-γ production. IL-12p70 also promotes the proliferation of IL-2-dependent T cells and enhances the expression of CD25 on CD4+ Th1 cells[55]. However, despite psilocybin

showing better results in post-treatment groups, psilocybin alone may not be enough to be used as an anti-inflammatory agent.

We observed changes in inflammatory markers in pre- and post-treatment eugenol groups. After using eugenol as a pre-treatment, RT-qPCR showed no significant difference between LPS and either eugenol or psilocybin pre-treatment groups, except for lower expression of *COX-2* and *TNF-α* (**Figure 2.4.**C,D). The expression of COX-2 (**Figure 2.6.**A) in Western blot supports results for the RT-qPCR in the pre-treatment. On the other hand, the amount of IL-1β was similar for both psilocybin and eugenol pre-treatment groups (**Figure 2.6.**B). However, these results do not align with previously described studies of eugenol's anti-inflammatory effects in the pre-treatment group. Interestingly, the post-treatment results of RT-qPCR for eugenol showed significant downregulation of *IL-6* and *TNF-α*, while no significant differences in *COX-2* or *IL-1β* expression compared to the LPS group (**Figure 2.5.**A–D). The ELISA results also showed no difference between psilocybin and eugenol groups, except for slightly lower content of *IL-12p70* (**Figure 2.8.**I). The cause of these results needs further investigation.

The effect of combined treatment of eugenol + psilocybin varied not only in pre- and post-treatment but depended on different ratios of these two drugs. Pre-treated mice with psilocybin + eugenol in a ratio of 1:50 demonstrated the best results compared to all other groups with no significant decrease of *IL-1β* (**Figure 2.4.**A). The Western blots, however, showed a slight decrease in COX-2 and IL-1β (p = N.S., **Figure 2.6.**A, B). Besides that, LPS + psilocybin + eugenol (1:50) demonstrated a significant decrease in IL-6 expression (**Figure 2.8.**E) but not IL-1*β*. This may be due to eugenol's influence, as similar results were described for eugenol in one of the studies[56].

2.4.1. Summary

The research involves administering psilocybin and eugenol as pre-treatment and post-treatment in a mouse model of LPS-induced inflammation. Results show varying effects on the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, as well as other inflammatory markers. Results showed that psilocybin alone, especially in post-treatment, reduced the expression of inflammatory markers like IL-6 and TNF-α. Eugenol demonstrated varying effects, with notable downregulation of IL-6 and TNF-α in post-treatment. The combined treatment of psilocybin and eugenol at a specific ratio (1:50) showed promising results in reducing IL-1β and IL-6 expression. The study suggests that both psilocybin and eugenol have anti-inflammatory potential, with the combination showing varying effects depending on the drug ratio. Further research is needed to fully understand the therapeutic potential of these compounds in treating neuroinflammatory conditions.

2.5. Materials and Methods

2.5.1. Animals

For this study, we used C57BL/6J mice (Charles River Laboratories, Laval, QC, Canada) in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care, which was approved by the Animal Care Committee at the University of Lethbridge, AB, Canada.

Our study was conducted in 2 parts: part 1, investigating the effect of the i.p. injection of lipopolysaccharides (LPS) on inflammation, and part 2, investigating how treatment with psilocybin, eugenol, and their combination affects inflammation.

2.5.2. Animal Handling

For the 1st part, 8–10-week-old mice were assigned into 4 groups: 0, 4, 24, and 48 h. Mice received either an i.p. LPS injection at a dose of 0.83 mg/kg or a saline solution as a vehicle. The number of hours refers to the time between injection and tissue harvesting for each group. Mice were weighed daily. After each time point, mice were anesthetized with isoflurane. Each mouse was decapitated using the mouse guillotine. The brains were extracted, cut, washed in 1× PBS, and placed in 1.5 mL microtubes. All tissues after collection were frozen using liquid nitrogen or dry ice and stored at −80 degrees Celsius until utilized for molecular analysis.

For part 2, 8–10-week-old mice were assigned to 2 major groups: the 1st group received a pre-treatment and then LPS, while the 2nd group received LPS and then a post-treatment. Pre- and post-treatments were distributed to mice via gavage. Group 1 mice received treatments at 2 time points, 48 and 24 h before LPS injection, and tissues were harvested 24 h post-LPS treatment. Group 2 received treatments 20 h after LPS injection, and tissues were harvested 4 h later.

2.5.3. Chemicals and Apparatus

The dose of psilocybin (CAS No. 520-52-50, Applied Pharmaceutical Innovation, Edmonton, AB, Canada) used was calculated from the common dose used in humans (5 mg, based on the average weight of 70 kg) and prorated to mice using a factor of 12.3[57]. For the combination with eugenol (CAS No. 97-53-0, Sigma-Aldrich, Saint Louis, MI, USA), three different ratios were used: 1:10, 1:20, and 1:50. LPS–L-4391-1MG, serotype 0111:B4 (Lot No. 059M4173V, SIGMA Life Science, Rehovot, Israel) was administered via intraperitoneal injection at a concentration of 0.83 mg/kg.

Table 2.1. The pre- and post-treatment groups for the LPS model

Pre-treatments included:	Post-treatments included:
1. Control—vehicle;	1. Control—vehicle;
2. Psilocybin (0.88 mg/kg);	2. LPS (0.83 mg/kg);
3. Eugenol (17.6 mg/kg);	3. LPS + psilocybin (0.88 mg/kg);
4. Psilocybin + eugenol (1:20; 0.88 mg/kg psilocybin and 17.59 mg/kg eugenol);	4. LPS + eugenol (17.6 mg/kg);
5. LPS (0.83 mg/kg);	5. LPS + psilocybin + eugenol (1:10);
6. Psilocybin (0.88 mg/kg) + LPS;	6. LPS + psilocybin + eugenol (1:20);
7. Eugenol (17.6 mg/kg) + LPS;	7. LPS + psilocybin + eugenol (1:50).
8. Psilocybin + eugenol (1:10; 0.88 mg/kg psilocybin and 8.8 mg/kg eugenol) + LPS;	
9. Psilocybin + eugenol (1:20; 0.88 mg/kg psilocybin and 17.6 mg/kg eugenol) + LPS;	
10. Psilocybin + eugenol (1:50; 0.88 mg/kg psilocybin and 44.0 mg/kg eugenol) + LPS.	

The remaining 4 mice did not receive any treatment or gavage and acted as a control without receiving the vehicle.

Additional equipment and supplies used:

- NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific,

Wilmington, DE, USA).

- ECL Prime Western Blotting System (Cat No. GERPN2232, GE Healthcare, Chicago, IL, USA).
- TRIzol® Reagent (Cat No. 15596018, Invitrogen, Carlsbad, CA, USA).
- FluorChem HD2 Imaging System (Cell Biosciences, Santa Clara, CA, USA).
- iScriptTM Select cDNA synthesis kit (Cat No. 1708897, BioRad, Hercules, CA, USA).
- SsoFastTM EvaGreen® Supermix (Cat No. 1725202, BioRad, Hercules, CA, USA).
- C1000TM Thermal Cycler equipped with a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA).

2.5.4. Protein Extraction and Quantification

The brain tissue was crushed by pestle with 400 µL of RIPA lysis buffer in 1.5 mL microtubes. The mixture was homogenized on the shaker with Zirconium beads (Cat No. D1032-15, Cole-Parmer, QC, Canada) 3 times for 3 min with 2 min on ice in between sets. Next, 200 µL of RIPA lysis buffer was added to each microtube and put on the shaker at 4 °C for 2 h. The microtubes were then centrifuged for 10 min at 13,500 rpm. The supernatant was collected. Using the Bradford protein assay with bovine serum albumin as the standard, protein concentrations were determined via a NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.5.5. Western Immunoblotting

Western immunoblotting was conducted with 150 µg/µL of protein. Samples were prepared with 4× loading buffer (0.0625 M Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-mercaptoethanol) and RIPA lysis buffer and heated at 95 °C for 5 min. The protein sample and PageRuler Plus Prestained Protein Ladder (Cat No. 26620, Thermo

Scientific, MA, USA) were loaded and electrophoretically separated by SDS-PAGE into gels with a combination of 8% (top) and 12% (bottom) polyacrylamide at 60 V for 30 min and then switched to 75 V for 1 h. Polyvinylidene difluoride membranes (Amersham Biosciences, Baie d'Urfé, QC, Canada) were used to transfer resolved proteins. Then, membranes were incubated for 1 h in a blocking solution (5% skim milk in PBS, 0.5% Tween 20) at room temperature and incubated with primary antibodies COX2, IL-1β and GAPDH at 4 °C overnight.

After incubation, the membranes were washed three times with 0.1% Tween-20 in PBS (PBS-T). The membranes were incubated with 1:5000 dilution of either bovine anti-mouse secondary antibodies or donkey anti-rabbit secondary antibodies for two hours at room temperature.

Next, membranes were washed 3 times with PBS-T and then exposed to the ECL Prime Western Blotting System (Cat No. GERPN2232, GE Healthcare, Chicago, IL, USA). Chemiluminescence was detected using the FluorChem HD2 Imaging System (Cell Biosciences, Santa Clara, CA, USA). Unaltered PVDF membranes were stained with Coomassie blue (BioRad, Hercules, CA, USA) to confirm equal protein loading. Signals were quantified using the NIH Image J64 software and normalized relative to GAPDH or Coomassie staining as indicated.

2.4.2. RNA Isolation

RNA was isolated from brain tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), purified using an RNAesy kit (Qiagen), according to the manufacturer's instructions, and quantified using a NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4.3. Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed on brain tissue from all experimental groups. According to the manufacturer's instructions, cDNA was generated with 500 ng RNA using the iScriptTM Select cDNA synthesis kit (Cat No. 1708897, BioRad, Hercules, CA, USA). PCR reactions were based on the SsoFastTM EvaGreen® Supermix (Cat No. 1725202, BioRad, Hercules, CA, USA) and 500 nM of forward and reverse primers specific for target sequences of interest. Primers were designed using the **https://www.idtdna.com/Primerquest** platform, accessed on 2 June 2022. Primers were checked before on dilution series of normal brain tissue cDNA. The reference gene (*GAPDH*) was analyzed with the GeNorm method. The reactions were analyzed on a C1000TM Thermal Cycler equipped with a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The PCR programs were run according to the SsoFastTM guidelines with annealing temperatures as specified for the specific primer pairs. Expression analysis was performed with the BioRad Software (CFX Manager) and was based on the ΔΔCt method with the reference genes that were stably expressed in the GeNorm analysis. Each experiment included three biological replicates for each group and two technical replicates per sample. The genes used for qRT-PCR were *IL-1β*, *TNF-α*, *IL6*, *COX2*, and *Amylase A* (**Figure S2.1**).

2.5.6. Enzyme-Linked Immunoassay (ELISA)

Samples for the ELISA were prepared using extracted proteins. Three samples from each post-treatment group, except for the psilocybin + eugenol group, were selected randomly, for a total of 18 samples. After thawing, protein samples were centrifuged for 10 min at 5000 rpm. Supernatant was aliquoted into 0.2 µL labeled microtubes, put in a box, and sent to Eve Technologies (Calgary, AB, Canada) for the enzyme-linked immunoassay. All procedures were carried out on ice.

2.5.7. Statistical Analysis

Data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) and are presented as means with standard error of the mean (SEM) error bars. Multiple unpaired Student's *t*-tests with a false discovery rate correction (Q = 5%) were used for comparisons between two groups. A one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's post hoc test were used for the analysis of three and more groups. p values < 0.05 were considered statistically significant.

2.6. Conclusions

This study established a model of LPS-induced inflammation in the brains of male mice and demonstrated the effects of treatment with eugenol, psilocybin, and their combination on the expression of pro-inflammatory cytokines. In addition, our study demonstrated the anti-inflammatory effects of combined treatment with psilocybin and eugenol in brain tissue, which have not previously been described. With growing interest in psilocybin applications for medical purposes, this study provides useful insights into its effect on inflammation, which will help guide future research in this area.

2.7. Supplementary Materials

The following supporting information can be downloaded at: **https://www.mdpi.com/article/10.3390/molecules28062624/s1**, or seen in the Appendices section; Figure S2.1: The effect of LPS i.p. injections on mRNA expression in brain tissue in **Figure 2.1**; Figure S2.2: The effect of vehicle treatments on expression of COX2, IL-1β, GAPDH in 4, 24, and 48 h time points in **Figure 2.2**; Figure S2.3: Original Western blots of brain tissue proteins showing COX-2 (molecular weight is 72 kDa) in **Figure 2.2**; Figure S2.4: Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa) in **Figure 2.2**; Figure S2.5: Original Western blots of brain tissue proteins showing GAPDH (molecular weight is 36 kDa) in **Figure 2.2**; Figure S2.6: Original Western blots of brain tissue proteins showing COX-2 (molecular weight is 72 kDa) in **Figure 2.6**; Figure S2.7: Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa) in **Figure 2.6**; Figure S2.8: Original Western blots of brain tissue proteins showing GAPDH (molecular weight is 36 kDa) in **Figure 2.6**; Figure S2.9: Original Western blots of brain tissue proteins showing COX-2 (molecular weight is 72 kDa)

in **Figure 2.7**; Figure S2.10: Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa) in **Figure 2.7**; Figure S2.11: Original Western blots of brain tissue proteins showing GAPDH (molecular weight is 36 kDa) in **Figure 2.7**; Figure S2.12: The content of inflammatory cytokines in post-treatment LPS-induced inflammation in brain tissue in **Figure 2.8**; Figure S2.13: The content of pro-inflammatory cytokines in post-treatment LPS-induced inflammation in blood in **Figure 2.9**; Table S2.1: Antibodies used for Western blots; Table S2.2: Primer sequences for qPCR analysis; Table S2.3: Significance Matrix for Body Weight Change in **Figure 2.3**.

2.8. Institutional Review Board Statement

This study used C57/BL/6J mice (Charles River Laboratories, Laval, QC, Canada) in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care. This study was approved by the Animal Care Services at the University of Lethbridge (Protocol No. 2113, approved 25 April 2022).

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Chapter 3 Psilocybin and Eugenol Prevent Dextran-Induced Neuroinflammation in Mice

3.1. Abstract

Neuroinflammation has emerged as a central pathology common to several acute and chronic brain diseases. Recent studies have displayed the anti-inflammatory properties of naturally occurring compounds derived from mushrooms and plants could potentially reduce neuroinflammation and disease progression. In this study, we aimed to investigate the impact of psilocybin and eugenol, as well as their combinations, on neuroinflammation. To induce inflammation through the gut-brain axis, we employed a colitis mouse model via oral feeding of dextran sulfate sodium (DSS). By administering various concentrations and combinations of treatments, both before and after inducing inflammation, we sought to assess the combined anti-inflammatory effects of psilocybin and eugenol. Our findings revealed oral psilocybin and eugenol post-treatment significantly reduced the expression of pro-inflammatory cytokines and inflammatory mediators in the brain, including IL-1β, IL-6, and COX-2. Notably, combined treatment of psilocybin and eugenol exhibited the strongest reduction in IL-6 levels when compared to the DSS group. While both psilocybin and eugenol possess anti-inflammatory effects, the combined treatment demonstrated additive reduction in neuroinflammation. This study adds to the growing body of evidence supporting the therapeutic potential of psilocybin and eugenol in psychiatric and neurodegenerative inflammatory disorders. Further research is necessary to elucidate the underlying mechanisms of their anti-inflammatory effects and to evaluate their efficacy in clinical settings.

Keywords: *Psilocybin, Eugenol, Dextran, Inflammation, Brain*

3.2. Introduction

Neuroinflammation is an inflammatory response within the brain or spinal cord involving the activation of neuroimmune cells into pro-inflammatory states, accompanied by the release of multiple pro-inflammatory cytokines, chemokines, secondary messengers (Ca2+, inositol trisphosphate, and NO), and reactive oxygen species (ROS) [1–3]. Although the primary function of neuroinflammation is to inhibit the harmful effects of brain insults and is neuroprotective [4] by repairing tissue, supporting the blood-brain barrier, and facilitating the removal of cellular debris [5], excessive or prolonged inflammation can induce and exacerbate neurogenic disease progression, and even cause nerve damage, DNA fragmentation, and cell death [6]. While neuroinflammation can be induced via brain or spine injuries, other factors including toxins, infections, and immune system dysregulation [7] can lead to cognitive degeneration and neurodegenerative diseases, like Alzheimer's disease (AD). In addition, there is evidence indicating that inflammation has a notable impact on psychiatric disorders and is associated with depression, schizophrenia, post-traumatic stress disorder (PTSD), and mood disorders [6,8].

Intestinal inflammation can impact brain function and contribute to neuroinflammatory processes through the gut-brain axis. Dextran sodium sulfate (DSS) is the most widely used chemical to induce inflammation of the bowels [9] causing damage to the intestinal epithelium barrier function and DSS-induced inflammatory bowel disease (IBD) [10]. In addition, DSS causes a disruption in the integrity of the gut barrier resulting in endotoxemia. Multiple studies demonstrate IBD and endotoxemia can induce cognitive impairment, alterations in serotonin (5-hydroxytryptamine, 5-HT)-producing enterochromaffin cells (EC), inflammation and oxidative stress within the brain [11,12]. While DSS is commonly used to study IBD, DSS can be used to study neuroinflammation due to the disruption of the intestinal barrier causing endotoxemia, neuroinflammation and

inflammatory-related neurodegenerative diseases [13]. Ongoing research is focused on investigating the therapeutic potential of natural compounds derived from mushrooms [14] and plants [15] as possible treatment options due to their anti-inflammatory and antioxidant potential.

Eugenol (4-allyl-2-methoxyphenol) (Eug) is an aromatic compound, commonly found in essential oils of plants such as cloves, bay leaves, and allspice. Some studies demonstrated that eugenol is usually used as a treatment for dental caries and periodontal disease [16]. Moreover, pharmacological studies reported that eugenol also includes anticonvulsant, bactericidal, antifungal, analgesic, antiseptic, hepatoprotective, and antioxidant properties. Eugenol inhibits tumor necrosis factor α (TNF-α) and reduces production of nitrous oxide radicals [17,18]. Eugenol, in addition to its effects on the CNS, has been shown to act as a neuroprotector, mitigating delayed neuronal death caused by ischemic damage in the CNS. [19]. Additionally, rats treated with 0.01 mg/kg eugenol showed improved memory and had a significant decrease in amyloid plaques, suggesting an effect on AD [20]. In the experimental epilepsy models, eugenol is capable of reducing severity of seizures and neuronal excitability [21]. Eugenol has been shown to effectively modulate microglia, inducing a shift from a pro-inflammatory to an anti-inflammatory state, thereby demonstrating its effects on microglial polarization [22].

Psychedelic mushrooms have been used for centuries due to their purported healing properties, however, has not been thoroughly studied for their anti-inflammatory potential. Psychedelic mushroom extracts have demonstrated the ability to alter the inflammatory profile of macrophages [23], suggesting psilocybin (Psi), the principal bioactive compound found in psychedelic mushrooms, may have anti-inflammatory potential. Despite this finding, little research has been performed on if psilocybin can prevent neuroinflammation.

In vivo, psilocybin undergoes rapid dephosphorylation to form psilocin (4-hydroxy-N,N-dimethyltryptamine). The acute pharmacological effects commonly associated with the administration of psilocybin/psilocin have traditionally been attributed to psilocin's binding properties to 5-HT2A receptors (5HT2AR). *In vitro* studies conducted on human receptor-expressing cells have demonstrated that psilocin exhibits a strong affinity for binding to 5HT2AR. Additionally, activation of 5HT2AR has shown robust anti-inflammatory effects in animal models [24,25]. Moreover, psychedelics have been shown in human macrophages to inhibit inflammation induced by TNF-α *in vitro* [23].

Due to the lack of research on the effects of psychedelic mushrooms and their components on neuroinflammation, this study was performed to elucidate if psilocybin and eugenol can potentially be utilized as novel anti-inflammatory therapeutics for brain diseases. In our previous study we studied the effects of eugenol and psilocybin treatments at relevant doses on LPS-induced inflammation in mice brains [26], however, this study examines the effects on the growing gut-brain etiology of neuroinflammatory diseases [27]. We hypothesized that psilocybin and eugenol can have neuro-protective effects in DSS-induced neuroinflammation in mice.

3.3. Results

3.3.1. RT-qPCR Analysis of Cytokines in Pre- and Post-Treatment Animals

mRNA was isolated from brain tissue and analyzed via RT-qPCR. The expression of pro-inflammatory cytokines and enzymes was quantified. mRNA levels of *IL-1β* were significantly higher in the DSS group than the control ($p < 0.05$, Figure 3.1.A). Similar to IL-1β, expression of Cyclooxygenase-2 (COX-2) was significantly higher for the DSS group compared to the control ($p < 0.01$, Figure 3.1.C). Changes in expression of the other two cytokines were insignificant, although TNF-α trended lower for all treatments and IL-6

trended higher for all treatments compared to the DSS, except DSS + psilocybin (p = N.S., Figure 3.1.D and 3.1.B respectively).

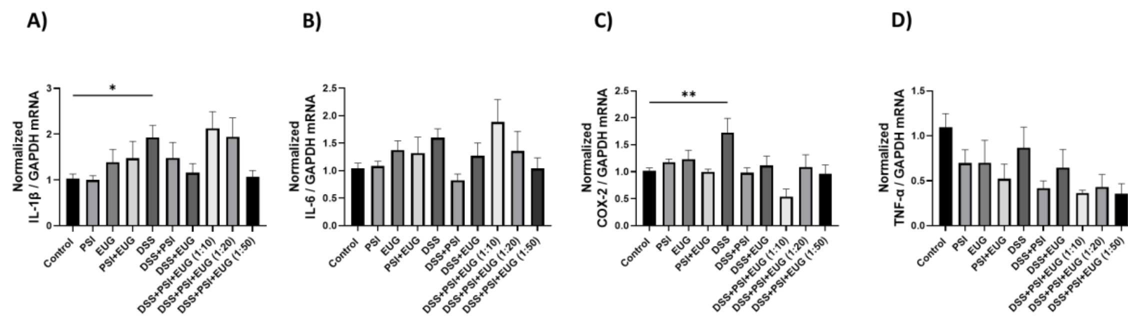

Figure 3.1. The effect of pre-treatment on mRNA expression in brain tissue. Changes in mRNA expression as measured by RT-qPCR for: (**A**) *IL-1β*, (**B**) *IL-6*, (**C**) *COX-2*, (**D**) *TNF-α*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. Data were analyzed with ANOVA and Dunnett's post hoc test. Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$, **, $p < 0.01$. Bars represent mean ± SEM. PSI, psilocybin; EUG, eugenol; DSS, Dextran Sodium Sulfate.

All post-treatment groups significantly downregulated *IL-1β* expression including DSS + Psi ($p < 0.001$), DSS + Eug ($p < 0.0001$), DSS + Psi + Eug (1:10) ($p < 0.01$), DSS + Psi + Eug (1:20) ($p < 0.001$), DSS + Psi + Eug (1:50) ($p < 0.01$) compared to the DSS group (Figure 3.2.A). Similar results shown for the *IL-6* and *COX-2* levels of in the DSS group compared to the control ($p < 0.01$) and all post-treatment groups compared to the DSS group ($p < 0.01$, Figure 3.2.B and 3.2.C). DSS did not upregulate *TNF-α* and there was no significant difference in expression of *TNF-α* in any of the treatment groups (Figure 3.2.D).

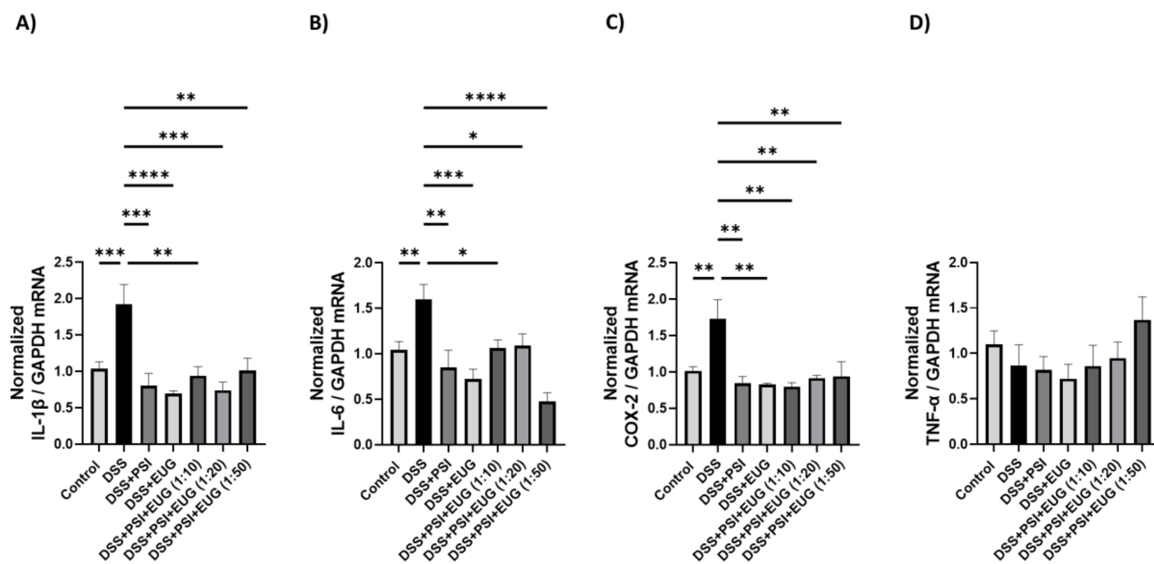

Figure 3.2. The effect of post-treatment on mRNA expression in brain tissue. Changes in mRNA expression as measured by RT-qPCR for: (**A**) *IL-1β*, (**B**) *IL-6*, (**C**) *COX2*, (**D**) *TNF-α*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. Data were analyzed with ANOVA and Dunnett's post hoc test. Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Bars represent mean ± SEM. PSI, psilocybin; EUG, eugenol; DSS, Dextran Sodium Sulfate.

3.3.2. RT-qPCR Analysis of MCP-1 and Amylase A in Pre- and Post-Treatment Animals

We also measured levels of *MCP-1* and *Amylase A* in the pre-treatment and the post-treatment groups. Psi + Eug + DSS (1:10) group showed the most notable change in levels of *MCP-1* expression in the pre-treatment compared to the DSS group ($p < 0.0001$, Figure 3.3.A). On the other hand, Psi + Eug + DSS (1:50) demonstrated significantly lower levels of *MCP-1* compared to the DSS group ($p < 0.01$, Figure 3.3.A). Interestingly, expression of *MCP-1* was significantly lower in the control, psilocybin, and eugenol groups, compared to the DSS group ($p < 0.0001$, Figure 3.3.A).

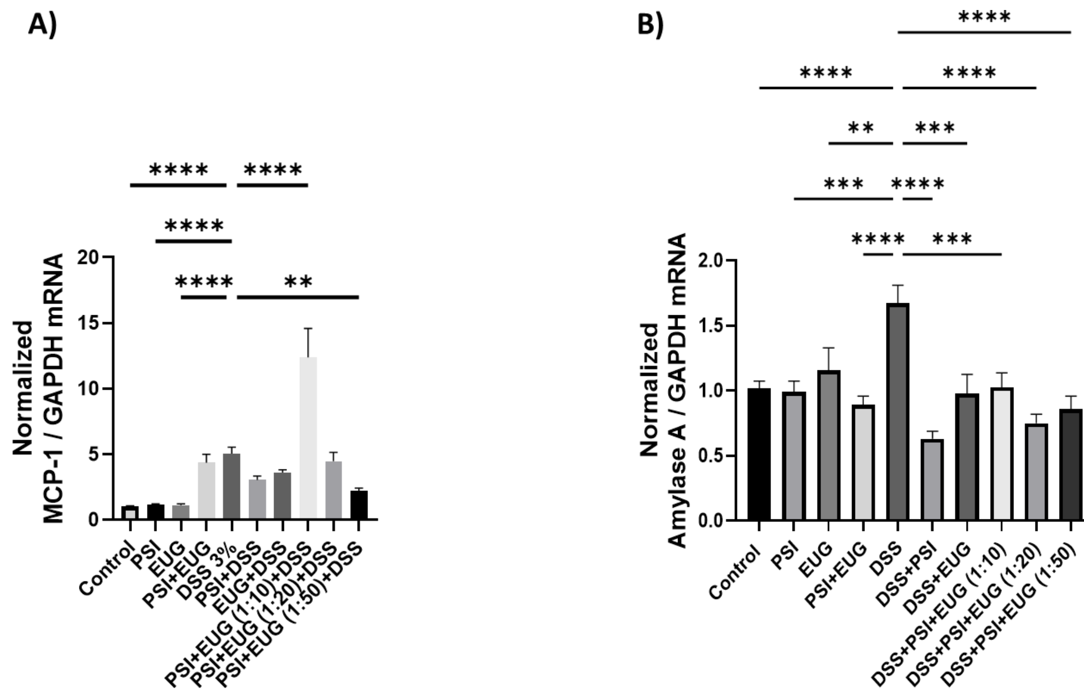

Figure 3.3. The effect of pre-treatment on mRNA expression in brain tissue. Changes in mRNA expression as measured by RT-qPCR for: (A) *MCP-1*, (B) *Amylase A*. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as a housekeeping gene. Data were analyzed with ANOVA and Dunnett's post hoc test. Significance (p) is indicated within the figures using the following scale: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Bars represent mean ± SEM. PSI, psilocybin; EUG, eugenol; DSS, Dextran Sodium Sulfate.

The highest levels of *Amylase A* were demonstrated in the DSS group compared to the control ($p < 0.0001$, Figure 3.3.B). All other treatment groups showed significantly lower expression of *Amylase A* compared to the DSS group ($p < 0.001$, Figure 3.3.B).

DSS demonstrated the highest levels of *MCP-1* compared to the control ($p < 0.01$, Figure 3.4.A). Post-treatment groups demonstrated significantly lower levels of *MCP-1*, with DSS + Eug and Psi + Eug + DSS (1:10) being the most significant ($p < 0.0001$, Figure 3.4.A). Similar results were shown in the *Amylase A* expression levels ($p < 0.001$, Figure 3.4.B).

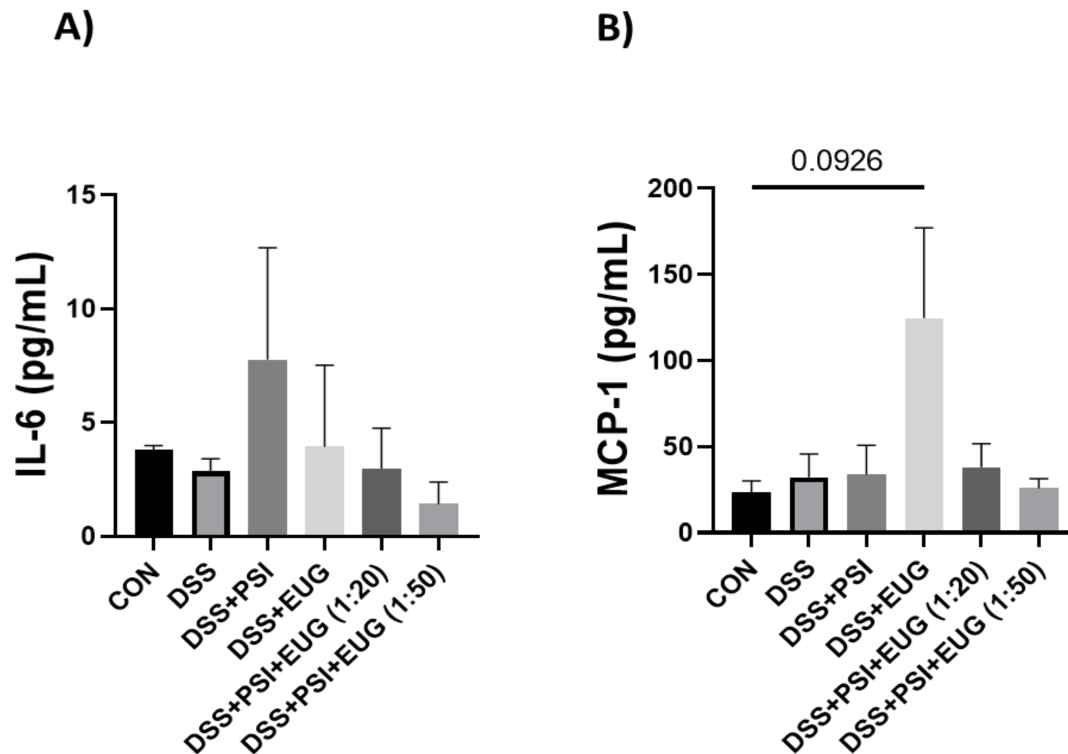

Figure 3.4. The effect of post-treatment on mRNA expression in brain tissue. Changes in mRNA expression as measured by RT-qPCR for: (A) *MCP-1*, (B) *Amylase A*. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as a housekeeping gene. Data were analyzed with ANOVA and Dunnett's post hoc test. Significance (p) is indicated within the figures using the following scale: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Bars represent mean ± SEM. PSI, psilocybin; EUG, eugenol; DSS, Dextran Sodium Sulfate.

3.3.3. Cytokines in Pre- and Post-Treatment Groups Measured by ELISA

Relative levels of Interleukin 2 (IL-2) in the DSS + Eug group were shown to be downregulated compared to the DSS group ($p < 0.05$, Figure 3.5.A). No significant changes were shown for expression of Interleukin 4 (IL-4) or Interleukin 10 (IL-10), although IL-10 post-treatment groups tended to be lower than the DSS group (p = N.S., Figure 3.5.B and 3.5.C).

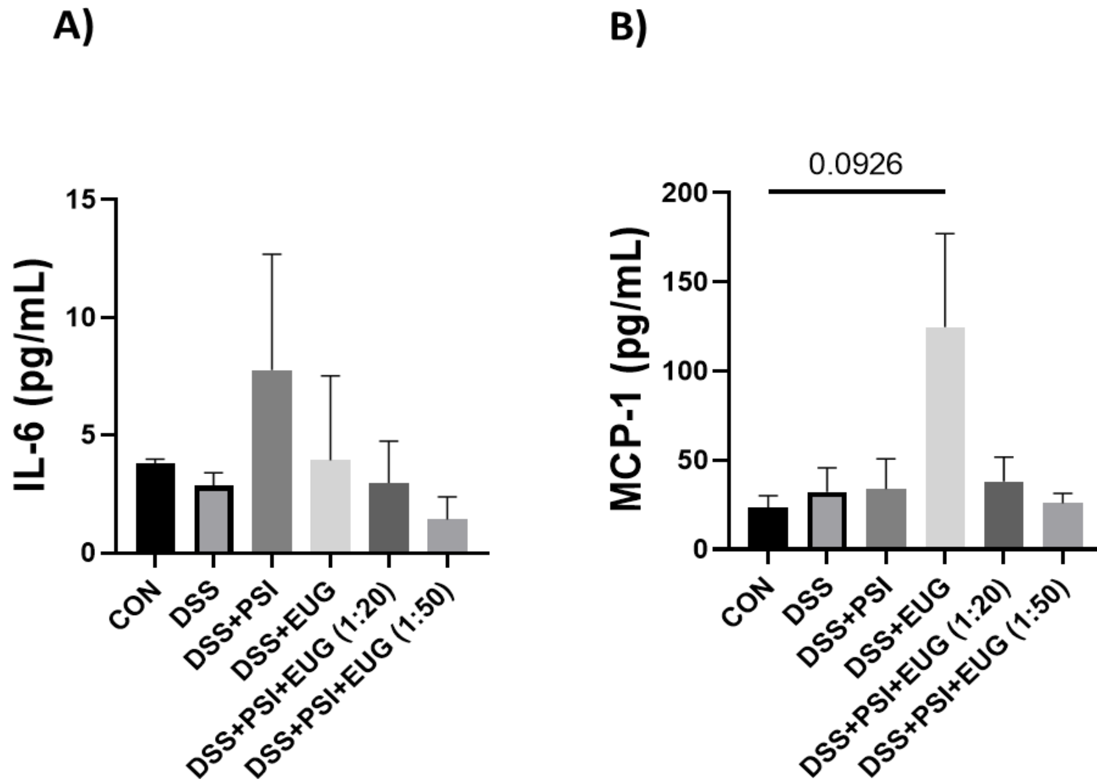

Figure 3.5. Pro-inflammatory cytokine levels in the post-treatment DSS-induced brain inflammation. The levels of (**A**) IL-2, (**B**) IL-4, (**C**) IL-10 were measured by an ELISA. Data were analyzed with ANOVA and Tukey's post hoc test (n = 3). Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$. Bars represent mean ± SEM. PSI, psilocybin; EUG, eugenol; DSS, Dextran Sodium Sulfate.

Next, we measured levels of IL-6 and MCP-1 (Figure 3.6.A and 3.6.B respectively) in blood using ELISA. Although no significant differences were shown, MCP-1 levels in DSS + Eug group appears to be higher compared to the control group (p = 0.0926, Figure 3.6.B).

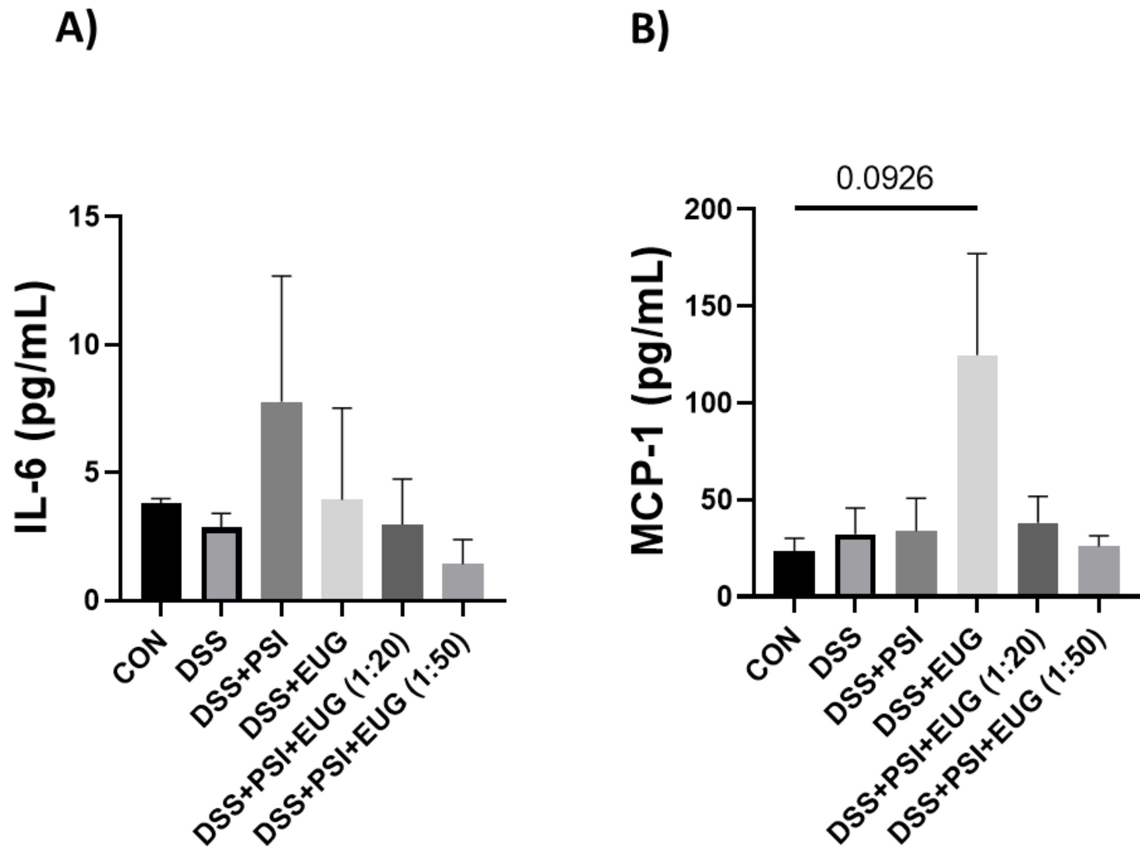

Figure 3.6. The content of pro-inflammatory cytokines in post-treatment DSS-induced inflammation in blood. The amounts of (**A**) IL-6, (**B**) MCP-1 were determined by ELISA. Data were analyzed with ANOVA and Tukey (n = 3). Bars represent mean ± SEM. PSI, psilocybin; EUG, eugenol; DSS, Dextran Sodium Sulfate.

3.4. Discussion

The inflammatory response in the brain and spinal cord has been a central concern of many researchers. Aspects of neuroinflammation, such as context, course, duration, and progression of primary cause, affect degree of inflammatory response. That response may result in recruitment of immune response, tissue damage or potential cell death. In addition, properties of natural compounds of mushrooms and plants and their effect on inflammation have been subject of interest in recent studies. In our study, we used psilocybin and eugenol, as well as their combination and their impact on neuroinflammation.

In this research, our first step was to induce inflammation in mice's intestines, using DSS as an induction agent. DSS is a chemical that is widely used to induce colitis in animal models. Inflammatory bowel disease can lead to the neuroinflammation through the gut-brain axis [28]. One study shows that DSS treatment elevates expression of IL-6, and TNF-α in hippocampus [29]. It was also reported that inflammation in the bowel is associated with alterations in the central nervous system by the activation of TNF-α signaling and microglia in the brain[30]. We established DSS-induced inflammation models in mice, which are supported by the enhanced expression of pro-inflammatory cytokines in the brain. Afterwards, we demonstrated the effect of administrating different treatments of eugenol and psilocybin, as well as their combinations, prior to and after inflammation induction.

Evidence suggests that psychedelics can stimulate neurogenesis and gliogenesis, reduce inflammation, and ameliorate oxidative stress, which could be used for therapeutics in psychiatric, neurodegenerative, and movement disorders [31]. One study demonstrated anti-inflammatory and antidepressant effects of psilocybin in the human U937 macrophage cell line, by decreasing levels of TNF–α and IL–1β [23]. IL-1β is a pro-inflammatory cytokine with multiple roles. It is involved in the regulation of pain, inflammation, homeostasis and differentiation of monocytes into conventional dendritic cells [32,33]. TNF-α is a multifunctional cytokine, it has a pleiotropic effect on inflammation and immune response. TNF-α is involved in the regulation of immune–inflammatory reactions of host defense against infectious, autoimmune, and endocrine diseases and cancer, and its actions help determine the survival or death of various cells [34]. The reduction of such pro-inflammatory cytokines is associated with improved health outcomes in chronic inflammation [35]. Authors of this study also demonstrated that mushroom extracts contained components, such as n-hexadecanoic acid, 4h-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 3-octanone, and dibutyl phthalate, can induce natural anti-inflammatory and antioxidant effects. Psilocybin's role in

inflammation is a new topic and has yet to be fully studied, therefore more research is needed to explore its therapeutic potential.

Compared to psilocybin, eugenol's effects on inflammation have been more thoroughly studied. For instance, eugenol can alleviate DSS-induced colonic inflammation by downregulating pro-inflammatory cytokine expression, such as TNF-α, recover intestinal barrier and beneficially affect gut microbiota [36]. Eugenol shows antidepressant effects by promoting metallothionein-III production in the hippocampus [37]. Eugenol can also be used as a damage-preventing agent in oxidative stress [38]. Since eugenol ameliorates DSS-induce colitis, there is a possibility that the observed reduction in brain inflammation is not a direct result of addressing brain inflammation. Instead, it may be due to alleviating colitis, which would prevent endotoxemia resulting in the failure to induce brain inflammation. Therefore, this study does not provide explanation of the precise mechanism of action of psilocybin and eugenol in relation to the location of anti-inflammatory action. These effects could be mediated at either the gut or the brain. That being said, our previous research in an LPS model of brain inflammation demonstrated similar results, suggesting psilocybin and eugenol do indeed act on the brain directly. Further research should study the contributions of anti-inflammatory effects on each the gut and the brain.

It is worth noting that there are neuropsychiatric and neurological diseases, in which gut-brain axis plays important role, such as depression, schizophrenia, Parkinson's disease, epilepsy and migraine [39]. These diseases, whereby inflammation plays a large role in the pathogenesis of the disease, would likely benefit from psilocybin and eugenol co-treatment. While other neurological and gut diseases may be of benefit as well, this study in particular indicates that psilocybin and eugenol can beneficially influence the gut-brain axis.

Some studies report eugenol's ability to inhibit apoptosis and secretion of pro-inflammatory cytokines [40] and can reduce *IL-1β* and *COX-2* gene expression [41]. COX-2 is

activated in response to various factors, such as TNF-α. COX-2 can synthesize pro-inflammatory mediators and prostaglandins, which can function as immune-suppressors. It was also shown that upregulation of COX-2-associated with inflammation [42]. In our previous study we described first effects of combined treatment involving both eugenol and psilocybin on LPS-induced brain inflammation in mice model [26].

After establishing the DSS-induced inflammation model in male mice, we administered psilocybin and eugenol as a pre-treatment or as a post-treatment. Apart from upregulation of *IL-1β* and *COX-2* in the DSS, relative to the control, RT-qPCR results for the pre-treatment did not show any significant changes (Figure 3.1.A and C, respectively). Although not significant, *TNF-α* trends to be downregulated for all groups compared to the control (Figure 3.1.D). On the other hand, post-treatment results of RT-qPCR demonstrated notable downregulation of all treatment groups, relative to the DSS, for *IL-1β*, *IL-6* and *COX-2* (Figure 3.2.A, B and C, respectively). The most significant change was shown for downregulation of pro-inflammatory cytokine *IL-6* in the DSS + psilocybin + eugenol (1:50), relative to the DSS groups (Figure 3.2.B). Interestingly, all treatment groups showed similar downregulation of *COX-2* compared to the DSS (Figure 3.2.C).

We also measured the effect of treatments for markers *MCP-1* and *Amylase A*. MCP-1 is a chemokine and its main role in inflammation is to attract monocytes, memory T cells and dendric cells to the inflammatory sites. MCP-1 can also increase expression of cytokines TNF-α and IL-β[43]. Surprisingly, DSS + psilocybin + eugenol (1:10) showed the most notable upregulation of *MCP-1* in the pre-treatment relative to the DSS group (Figure 3.3.A). Apart from that, all other groups showed a decrease in expression of MCP-1 and most noticeable it was demonstrated for psilocybin, eugenol and DSS + psilocybin + eugenol (1:50) groups compared to the DSS (Figure 3.3.A). On the other hand, the DSS + psilocybin + eugenol (1:10) and DSS + eugenol groups demonstrated lowest levels of *MCP-1* in the post-treatment

compared to the DSS (Figure 3.4.A). All other groups also demonstrated a decrease in amounts of *MCP-1* compared to the DSS (Figure 3.4.A). Amylase A is an enzyme that participates in the degradation of polysaccharides. It is used for the prognosis and treatment of inflammatory diseases, actinomycetes infection and against some bacteria as the ones that cause caries [44]. Pre-treatment group of psilocybin + DSS demonstrated the most significant decrease of *Amylase A* compared to the DSS group, while all other groups also showed relative downregulation of this marker (Figure 3.3.B). On the other hand, DSS + psilocybin + eugenol (1:50) group in the post-treatment demonstrated similar upregulation of *Amylase A* to the DSS group (Figure 3.4.B).

ELISA results demonstrated a decrease in levels of IL-2 compared to the DSS group (Figure 3.5.A). Apart from that, post-treatment did not show any effect on levels of IL-4 and IL-10 (Figure 3.5.B and C, respectively). IL-2 is a cytokine that is involved in the activation and regulation of the immune response [45]. It is a key growth and survival factor of T-cells, it promotes differentiation of the memory T cell phenotype [46]. It is produced by T cells, specifically CD4+ helper cells and IL-2 can induce the proliferation of T cells and T-helper 1 (Th1) and Th2 effector[47]. IL-4 is a signature type II inflammatory response cytokine. It belongs to the Th2-type cytokines and is a key player in the inflammatory response triggered by allergy or parasite infection. It stimulates B cell proliferation and activation of eosinophils, basophils, and mast cells. Moreover, it also can immunoglobulin (Ig) class of IgE and IgG4, and it also plays a role in the fibrosis of internal organs and skin [48]. IL-10 is a cytokine that can affect multiple cell types. Its anti-inflammatory role is to suppress monocytes and macrophage ability, as well as production of pro-inflammatory cytokines, such as IL-6, by inhibiting Th1 and stimulating B lymphocytes and Th2 lymphocytes. Thus, leading to the suppression of inflammatory response [49]. We also found a significant increase of MCP-1 amounts in blood for the DSS + Eug group compared to the control (Figure 3.6.B).

3.5. Materials and Methods

3.5.1. Animals

For this study, we used C57BL/6J mice (Charles River Laboratories, Laval, Canada) following the Guide to Care and Use of Animals of the Canadian Council of Animal Care, which was approved by the Animal Care Committee at the University of Lethbridge, Canada.

3.5.2. Animal Handling

8–10-week-old mice were assigned into groups that received doses of DSS or vehicle for 5, 6 and 7 days. Mice received either vehicle, 2.5% and 3% DSS dissolved in drinking water and were weighed daily. The number of days refers to the time between the starting day of treatment and tissue harvesting for each group. After each time point, mice were anesthetized with Isoflurane and decapitated using the mouse guillotine. The brains were extracted, cut, washed in 1x PBS and placed in 1.5 ml microtubes, frozen using liquid nitrogen or dry ice and stored at -80 degrees C.

In addition, 8–10-week-old mice were assigned to 2 groups and received either treatment prior to or after receiving DSS. Gavage was the chosen method of treatment distribution. Pretreated mice received treatments 24 hours and 1 hour before providing DSS in drinking water and tissues were harvested 6 days post DSS treatment. Post-treated mice received the treatment once after providing DSS for 6 days and tissues were harvested 4 hours later. The dose of psilocybin to be used was calculated from the common dose used in humans – 5 mg, based on the average weight of 70 kg, and prorated it to mice using a factor of 12.3. We collected brains from animals at 5, 6, and 7 days after providing the solution to analyze cytokine content.

3.5.3. Chemicals and treatment groups

The dose of psilocybin (CAS No. 520-52-50, Applied Pharmaceutical Innovation, Edmonton, AB, Canada) used was calculated from the common dose used in humans (5 mg,

based on the average weight of 70 kg) and prorated to mice using a factor of 12.3. For the combination with eugenol (CAS No. 97-53-0, Sigma-Aldrich, Saint Louis, MI, USA), three different ratios were used: 1:10, 1:20, and 1:50.

Table 3.1. The pre- and post-treatment groups for the DSS model

Pre-treatments included:	**Post-treatments included:**
Control—vehicle;	Control—vehicle;
Psilocybin (0.88 mg/kg);	3% DSS (0.83 mg/kg);
Eugenol (17.6 mg/kg);	3% DSS + psilocybin (0.88 mg/kg);
Psilocybin + eugenol (1:20; 0.88 mg/kg psilocybin and 17.59 mg/kg eugenol);	3% DSS + eugenol (17.6 mg/kg);
DSS (0.83 mg/kg);	3% DSS + psilocybin + eugenol (1:10);
Psilocybin (0.88 mg/kg) + 3% DSS;	3% DSS + psilocybin + eugenol (1:20);
Eugenol (17.6 mg/kg) + 3% DSS;	3% DSS + psilocybin + eugenol (1:50)
Psilocybin + eugenol (1:10; 0.88 mg/kg psilocybin and 8.8 mg/kg eugenol) + 3% DSS;	
Psilocybin + eugenol (1:20; 0.88 mg/kg psilocybin and 17.6 mg/kg eugenol) + 3% DSS;	
Psilocybin + eugenol (1:50; 0.88 mg/kg psilocybin and 44.0 mg/kg eugenol) + 3% DSS.	

3.5.4. Protein Extraction

Protein extraction was performed with 400 µL of RIPA lysis buffer in 1,5 ml microtubes. The shaker with Zirconium beads (Cat# D1032-15, Cole-Parmer, QC, Canada) was used to homogenize mixtures 3 times for 3 minutes with 2 minutes on ice in between sets. Next, 200 µL of RIPA lysis buffer was added to each microtube and put on the shaker at 4°C for 2 hours, then centrifuged and supernatant was collected. NanoDrop 2000/2000c

Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used for protein quantification, using the Bradford protein assay with bovine serum albumin as the standard.

3.5.5. RNA Isolation

RNA was isolated from brain tissue using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA); purified using an RNAesy kit (Qiagen), according to the manufacturer's instructions, and quantified using NanoDrop 2000c (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was converted to cDNA using iScriptTM Select cDNA synthesis kit (Cat# 1708897, BioRad, Hercules, CA, USA).

3.5.6. Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was performed on brain tissue from all experimental groups with SsoFastTM EvaGreen® Supermix (Cat# 1725202, BioRad, Hercules, CA, USA) in a C1000TM Thermo Cycler equipped with a CFX96 Touch™ Real-Time PCR Detection System (BioRad). Primers were designed using the https://www.idtdna.com/Primerquest platform. Reference gene (*GAPDH*) were analyzed with the GeNorm method. The comparative CT method (ΔΔCt method) was used to calculate relative fold expression levels using the BioRad Software (CFX Manager). Each experiment included three biological replicates for each group and two technical replicates per sample. The genes used for qRT-PCr were *IL-1β*, *TNFα*, *IL6*, *COX2*, *Amylase A*, *TRP1, TRPM8, TRPV1, HTR2A, HTR2B, MCP1, GAPDH.*

3.5.7. Enzyme-linked immunoassay (ELISA)

Three samples from each group were selected randomly for ELISA and were prepared using extracted protein from brain tissue. Samples were centrifuged for 10 minutes at 5000 rpm, supernatant was aliquoted into 0.2 µL labeled microtubes, put in a box and sent to Eve Technologies (Calgary, AB, Canada) for enzyme-linked immunoassay. All procedures were done on ice.

3.5.8. Statistical Analysis

GraphPad Prism 9 (GraphPad Software, San Diego, CA) was used for statistical analysis and is presented as means with standard error of the mean (SEM) error bars. A one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used for the analysis of three and more groups. Multiple unpaired Student's t-tests with a false discovery rate correction (Q=5%) were used for comparisons between two groups. P-Values < 0.05 were considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$.

3.6. Conclusions

This study established DSS-induced inflammation in the brains of male mice model. It demonstrated the effects of eugenol, psilocybin, and their combinational treatment on the expression of pro-inflammatory cytokines and some other inflammatory markers. In addition, our study demonstrated the anti-inflammatory effects of combined treatment with psilocybin and eugenol in brain tissue. With growing interest in psilocybin applications for medical purposes, this study provides useful insights into its effect on inflammation, which will help guide future research in this area.

3.7. Institutional Review Board Statement

This study used C57/BL/6J mice (Charles River Laboratories, Laval, QC, Canada) in accordance with the Guide to Care and Use of Animals of the Canadian Council of Animal Care. This study was approved by the Animal Care Services at the University of Lethbridge (Protocol No. 2113, approved 25 April 2022).

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Chapter 4 General Discussion and Future Directions

4.1.1. Model of LPS

LPS model is one of the most crucial and widely used in both *in vitro* and *in vivo* animal models for studying neuroinflammation and neurodegeneration. Furthermore, LPS-induced model of neuroinflammation has been demonstrated to facilitate amyloid deposition *in vivo*[50]. Numerous studies in animals have extensively reported that the administration of LPS results in a range of central effects, partly mediated by pro-inflammatory cytokines that primarily released from microglia[51,52]. While it is widely acknowledged that cytokines released in the periphery typically do not pass through the blood-brain barrier (BBB), they can still transmit signals to the brain. Studies have consistently shown that LPS can induce the synthesis of pro-inflammatory cytokines within the brain[51]. Certain research findings have linked AD neuropathology to the presence of LPS within the brain[1]. One of the applications of the LPS-induced neuroinflammation model is in investigating the role of neuronal mitochondria in the modulation of neuroinflammation[53].

In our study, we established LPS-induced model of neuroinflammation in male C57BL/6J mice and used it to analyze cytokine content after 4-, 24- and 48-hours post-injection of LPS. Using this model, we demonstrated the effects of eugenol and psilocybin on neuroinflammation before and after administration of the LPS. The novel finding of our research was introduction of combined treatment of psilocybin and eugenol in 3 different ratios of (1:10), (1:20) and (1:50), with psilocybin + eugenol (1:50) demonstrating the most significant downregulation of pro-inflammatory cytokines, such as COX-2, IL-6 and IL-1β induced by LPS. Similar results were reported in the study performed by[54], where they used IPEC-J2 cell model to study the effect of eugenol on LPS-induced inflammation in porcine intestinal epithelial cells. They demonstrated the increase of pro-inflammatory biomarkers,

such as IL-8 and TNF-α in groups treated with LPS and decrease in LPS + eugenol treatment groups[54].

In our research, we also observed the impact of LPS and various treatments on alterations in body weight. Notably, the pre-treatment group receiving LPS + psilocybin + eugenol (in a ratio of 1:10) exhibited a reduced weight loss, amounting to up to 4 grams ($p < 0.001$), in contrast to the LPS + psilocybin group, which experienced weight loss of up to 5.9 grams ($p < 0.05$). The LPS group alone demonstrated consistent weight loss between 2 and 3 grams ($p < 0.01$). From here we can suggest psilocybin further contributes to the weight loss and eugenol has ameliorating effect on the wight loss, demonstrated in the combined treatment with psilocybin. Interestingly, one of the reports states that even when using a low dose of LPS, weight loss in treated subjects remains unavoidable[55]. As a result, the clinical application of LPS-induced blood-brain barrier (BBB) opening, especially for AD patients, would necessitate strategies to mitigate the weight loss and elevated mortality observed in the preclinical study. This could potentially be achieved by exploring alternative pro-inflammatory compounds, adjusting the dosing regimen, or combining LPS with an anti-inflammatory agent that can mitigate unintended effects without inhibiting BBB opening[55]. Another group of researchers, that have been studying LPS-induced experimental acute lung injury, discovered that LPS-instilled animals experienced a notable weight loss of approximately 12% from their initial weight, the administered treatments did not appear to mitigate this effect[56].

4.1.2. Model of DSS

The model of DSS-induced inflammation is considered an ideal model to study IBD, including ulcerative colitis (UC) disease and Crohn's disease. Although DSS is frequently

used for the study of IBD, it's crucial to recognize that the disruption of the intestinal barrier, resulting in endotoxemia, can trigger subsequent neuroinflammation[13]. In terms of mechanisms, it has been reported that proinflammatory cytokines associated with IBD can disrupt the integrity of the BBB and increase its permeability. Consequently, cytokines and proinflammatory genes have the potential to enter the brain, where they can locally influence the behavior of microglia. When microglia are activated, they produce and release a wide range of neurotoxic pro-inflammatory mediators, including cytokines, which can have a detrimental impact on hippocampal neurogenesis[57–59]. Numerous studies conducted in animal models of colitis have detected inflammatory markers, such as TNF-α, IL-1β, TLR-4 and IL-6, within both the hippocampal region and various areas of the brain[57,58,60].

In our research, after establishing DSS-induced neuroinflammation model in male C57BL/6J mice, we used this model to demonstrate the effect of psilocybin and eugenol on the expression of pro-inflammatory markers in the brain tissues. We used RT-qPCR to measure levels of pro-inflammatory markers in the brain tissues prior and after the administration of DSS into the drinking water for all of the treatment groups. As expected, we noticed the increase of expression of *IL-1β* and *COX-2* in the DSS group, relative to the control. The downregulation of *IL-1β*, *IL-6* and *COX-2* was demonstrated for all of the post-treatment groups. Like with the LPS model, the interesting result was demonstrated by DSS + psilocybin + eugenol (1:50) group with the most significant decrease of pro-inflammatory cytokine *IL-6*, compared to the DSS group ($p < 0.0001$). One of the studies also showcased the anti-inflammatory properties of the plant-based compound - sophorae decoction, which is a Chinese medicinal formula comprised of six Chinese herbs. It effectively ameliorated the pathological damage and symptoms observed in mice with DSS-induced colitis, impacting immune function by modulating the balance between T-helper (Th17) and regulatory T (Treg) cells. The treatment resulted in increased body weight for the mice and minimized the

Disease Activity Index (DAI), with fewer and less severe ulcers compared to the DSS group. Additionally, it reduced the levels of inflammatory factors such as IL-1β, TNF-α, and phosphorylated NF-κB p65. Furthermore, it decreased the proportion of Th17 cells in the spleen and mesenteric lymph nodes, along with the expression of ROR-γt, IL-17A, STAT3, and IL-6 in colon tissues[61].

The exact mechanisms through which colonic inflammation can initiate neuroinflammation remain unclear. Colonic inflammation is believed to induce neuroinflammation through either a specific communication pathway between the gut and the brain, potentially involving the gut microbiota or afferent neurons, or by inciting inflammation in the colon, which may lead to systemic inflammation[28–30,62]. This systemic inflammation can then activate inflammatory processes within the CNS. Importantly, these two pathways may work in tandem rather than being mutually exclusive. The findings from one of the studies reveal a significant rise in the inflammatory chemokine KC, along with inflammatory biomarkers S100A8 and S100A9, observed in the colons, serum, and brains of mice afflicted with colitis. It's well-established that colitis can lead to inflammation beyond the intestinal tract in both mice and humans[63]. When considering the elevation of inflammatory markers in both the bloodstream and the brain, it supports a hypothesis where colitis triggers a widespread inflammatory response that eventually leads to inflammation in the CNS. Gaining a more comprehensive understanding of the mechanisms by which gut-driven inflammation contributes to neuroinflammation is essential.

There is contradictory data regarding the ability of LPS to penetrate the BBB. Some of the studies suggest that LPS can cross BBB[64,65]. For instance, one study revealed a predominant presence of LPS in astrocytes situated near the meninges and meningeal cells within the medulla oblongata in rat brains. Additionally, it was detected in tanycyte-like cells lining the ventricular system, the choroid plexus, endothelial cells, and circumventricular

organs (CVOs). Importantly, LPS consistently co-localized with CD14, TLR-4, and NFκB in these areas. Furthermore, this particular study confirmed the existence of baseline levels of LPS in the bloodstream using the LAL assay[64]. In another study, it is reported that LPS has the capacity to directly impair the integrity of the BBB by causing structural disruptions. This detrimental effect on the BBB is attributed to several mechanisms, including interference with the paracellular pathways regulated by tight junctions (TJs) and adherens junctions (AJs), disturbance of the transcellular pathway, influence on P-glycoprotein (P-gp), and alterations in vesicle trafficking. These fundamental mechanisms collectively contribute to the increased permeability of the BBB and the disruption of its protective barrier function[65].

On the other hand, some studies suggest that LPS has minimal penetration across murine BBB. For example, the research conducted by Banks *et al.* revealed that, in contrast to another well-known effect of LPS, the weight loss, the BBB exhibits a relatively higher resistance to LPS-induced disruption. Furthermore, this study demonstrated that different regions of the BBB have varying sensitivities to LPS-induced disruption. Importantly, the involvement of non-brain endothelial cells (BECs) is not essential for LPS to induce disruption, and this disruption occurs through both paracellular and transcytotic mechanisms, with the transcytotic disruption being dependent on prostaglandin signaling[66]. Therefore, considering the existing evidence, it remains uncertain whether LPS has the ability to breach the BBB and further research is needed in this area. For example, LPS can be tagged with a radioactive isotope, a practice employed in a different study[67]. In that study, LPS was labeled with radioactive iodine (I-LPS) and co-administered alongside radioactively labeled albumin (I-Alb), which served as a vascular space marker.

There are several studies, which demonstrate the effect of eugenol on BBB. For example, in of the studies it was demonstrated that weight-drop-induced traumatic brain injury (TBI) led to brain edema and BBB disruption. Pretreatment with a 100 mg/kg dose of

eugenol mitigated these effects, effectively restoring BBB integrity and reducing brain edema caused by the trauma[68]. A computational analysis of eugenol's inhibitory activity revealed its potential to cross the BBB, likely contributing to its analgesic effects, along with enhanced intestinal absorption and permeability in Caco-2 and MDCK cells. In silico analyses revealed eugenol's enhanced binding to plasma proteins, suggesting a potential transport mechanism in plasma and superior ability to traverse the BBB when compared to diclofenac and aspirin. These combined chemical properties enable eugenol, similar to COX-2-specific nonsteroidal anti-inflammatory drugs (NSAIDs) and conventional NSAIDs, to permeate lipid membranes, including the BBB, and accumulate in acidic tissues such as the stomach, renal medulla, and inflammatory sites[69]. However, the exact mechanism by which eugenol cross BBB is yet to be elucidated.

In this research, we also measured levels of *MCP-1.* Surprisingly, the pre-treatment group receiving DSS + psilocybin + eugenol (1:10) exhibited the most significant increase in *MCP-1* levels when compared to the DSS group ($p < 0.0001$). Conversely, both the DSS + psilocybin + eugenol (1:10) and DSS + eugenol groups displayed the lowest *MCP-1* levels in the post-treatment phase compared to the DSS group ($p < 0.0001$). Interestingly, a group of researches strongly advocated that the idea of peanut shell extract (PSE) possesses anti-inflammatory properties when administrated to mice with DSS-induced colitis. The heightened secretion of pro-inflammatory cytokines such as TNF-α, MCP-1, IL-1β, and IL-6, observed in mice treated with DSS, was significantly diminished in mice that received PSE. Furthermore, the secretion of these cytokines in macrophages, which are recognized as the primary cells responsible for releasing pro-inflammatory cytokines during intestinal inflammatory responses, indicated that PSE was as effective as luteolin in reducing their secretion[70].

4.2. Future Directions and Drawbacks

In this study, we used ELISA to assess the levels of various anti-inflammatory and pro-inflammatory markers within the brain. However, it's important to note that only a limited subset of markers has been examined in the DSS model. As a result, it would be beneficial to expand the analysis using ELISA to include wider specter of the inflammatory markers previously described in this and similar research for future studies.

mRNA sequencing could have also been used to test broader number of pathways targeted by inflammation and responsive to psilocybin and eugenol. This method is also instrumental in drug development, as it allows for the assessment of drug efficacy by identifying changes in the activity or the up- or down-regulation of disease markers within the target tissues[71]. Recent studies in mammals have indicated that microRNAs (miRNAs) play a role in neurodegenerative diseases (NDs) and neuroprotection; impairing miRNAs can lead to neurodegeneration[72]. So, the analysis of miRNAs and other non-coding RNAs could have been useful for our studies.

We are suggesting using *in vitro* BBB models for investigating drug interactions with the BBB within a controlled laboratory setting[73], or *in vivo* pharmacokinetic studies[74] to help understand mechanisms of BBB penetration.

One other subject that would be interesting to study is serotonin receptors and Transient receptor potential (TRP) channels. Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter produced mainly by peripheral activated intestinal pigment cells, mast cells and platelets. Seven serotonin receptors (5-HTR1-7) have been identified thus far, including 5-HT receptor 2A (5-HTR2A) and 5-HT receptor 2B (5-HTR2B). 5-HT involved in the regulation of blood vessels and bronchi activity via activation 5-HTR2A/2B[75,76]. Serotonin has a role in immune response. It stimulates lymphocytes and monocytes, which lead to the

secretion of cytokines. Like this, vascular smooth muscle cells will synthesize IL-6 as a response to serotonin[77]. On the other hand, specific activation of 5-HTR2A in primary aortic smooth muscle cells leads to inhibition of TNF-α-induced inflammation[24]. TRP channels are involved in the systematic response to inflammation. TRP channels are voltage-gated cation channels such as TRPV1, which is involved in the thermoregulation and pain regulation; activation of TRPA1 and TRPM8, induces cold/pain defense[78]. TRPV1 plays an important role in the development of signs and symptoms of chronic inflammatory diseases such as arthritis, DSS-induced colitis, chronic airway inflammation, chronic pancreatitis, autoimmune diabetes, or in fibrotic scarring reactions[79–81].

In vivo, psilocybin rapidly undergoes dephosphorylation to convert into psilocin. Furthermore, *in vitro* research conducted on human receptor-expressing cells has shown that psilocin possesses a high affinity for binding to 5-HT2AR[24,25]. Remarkably, when 5-HT1A and 5-HT2A receptor agonists were administered to rats afflicted with streptozotocin-induced Alzheimer's disease (AD), substantial neuroprotective effects were observed in hippocampal neurons. These effects were attributed to the activation of anti-apoptotic and anti-inflammatory pathways[82].

To enhance comprehension of psilocybin's receptor-mediated mechanisms, we are suggesting to conduct focused neuroimaging and behavioral investigations to explore the effects of both 5HT2A agonists and antagonists. Additionally, in the current field of pharmaceutical research on psilocybin, there's a notable absence of studies that simultaneously incorporate biomarkers for genotypes and expression levels of 5-HT2A receptors. Such comprehensive studies have the potential to provide insights into the therapeutic role of 5-HT2A receptors in psilocybin's applications, potentially paving the way for the development of personalized medicines for various conditions like anxiety and stress-related disorders.

Finally, we are suggesting conducting immunohistochemistry (IHC) of brain tissue. IHC can be used for the assessment of predictive and prognostic biomarkers in various malignancies, including those affecting the breast, gastrointestinal tract, lung, hematolymphoid system, and central nervous system[83]. Additionally, IHC is a valuable tool for quantifying neuroinflammation by detecting markers of activated microglia and astrocytes, such as ionized calcium-binding adapter molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP), respectively. Alterations in the expression or morphology of these markers can signify the anti-inflammatory effects of natural compounds[84]. In one study IHC was used to investigate the presence of fungal and bacterial infections in brain tissue obtained from both AD patients and control subjects. That study offered a comprehensive insight into the mycobiome within the CNS of AD patients. Moreover, the researchers presented the initial evidence showcasing the presence of fungal DNA from multiple species in CNS samples obtained from seemingly healthy control subjects[85].

Interestingly, another study used IHC to explore the activity of microglia in different brain regions, both in the context of healthy aging and AD-related pathology. The investigation of that group of researchers encompassed both human and mouse samples. Their study reveals elevated microglia-induced neuroinflammation in the white matter of individuals with early-onset Alzheimer's disease (EOAD), in comparison to a group of young controls. However, it's essential to acknowledge that their present investigation, which relied on IHC for post-mortem sample analysis, has limitations in determining whether white matter neuroinflammation indeed initiates cognitive decline in EOAD. Nevertheless, the early detection of neuroinflammation could potentially serve as a valuable predictor for the onset of dementia and neurodegeneration in EOAD. The use of IHC staining in their study provided a high level of confidence in distinguishing the presence or absence of lymphocytes and enabled precise measurements of inflammatory cells[86].

In the future, as further research unfolds, we may acquire deeper insights into the potential of psychedelics and natural compounds, as well as their combined effects. This knowledge could pave the way for the integration of these compounds into various medical fields, potentially offering valuable contributions to finding the treatment of neurodegenerative diseases such as AD, PD and MS.

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Appendices

Appendix 1: Supplementary materials for Chapter 2

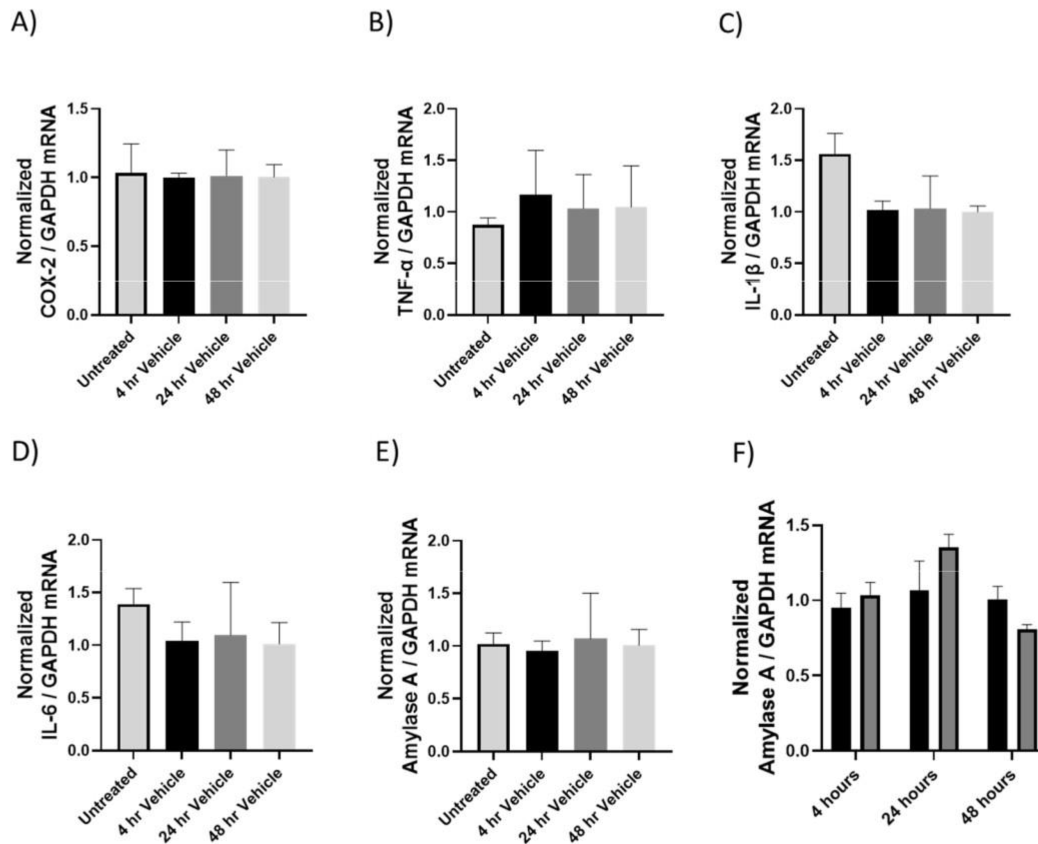
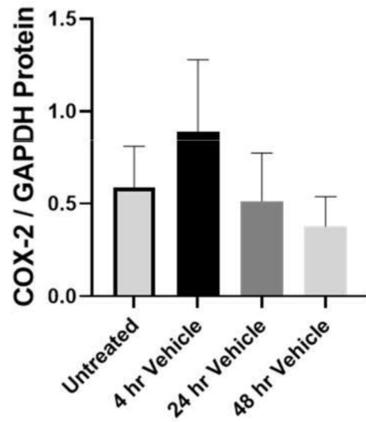


Figure S2.1. The effect of LPS i.p. injections on mRNA expression in brain tissue in Figure 1. Changes of mRNA expression as measured by RT-qPCR for: (**A**) COX-2 (**B**) TNFα (C) *IL-1β* (**D**) *IL-6* (**E, F**) *Amylase A*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. Data were analyzed with (**A-E**): Multiple unpaired Student's t-tests (FDR: Q=5%, n=5-6) and (**F**): t-test (n=3-6). Bars represents Mean ± SEM

A)



B)

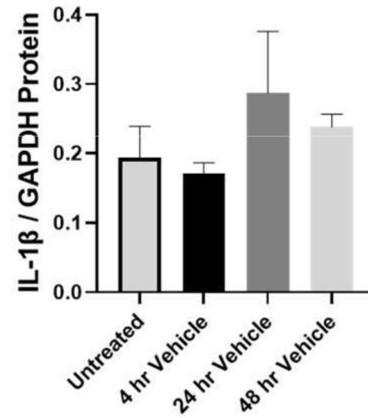


Figure S2.2. The effect of vehicle treatments on expression of COX2, IL-1β, GAPDH in 4h, 24h and 48h time points in Figure 2. Figures represents changed protein expression for selected genes measured by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Relative densitometry was presented as a ratio of target protein to GAPDH. (A) COX 2 expression (B) IL-1β expression. (A-B): ANOVA / Tukey (n=3). Bars represent Mean ± SEM.

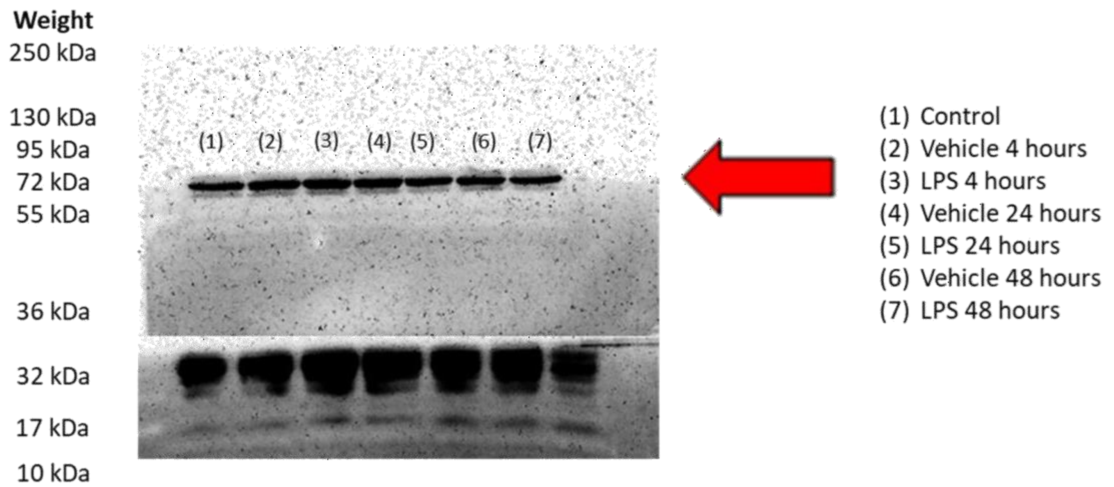


Figure S2.3. Original Western blots of brain tissue proteins showing COX-2 (molecular weight is 72 kDa). Red arrow indicates bands shown in Figure 2. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

IL-1β 17 kDa in Figure 2

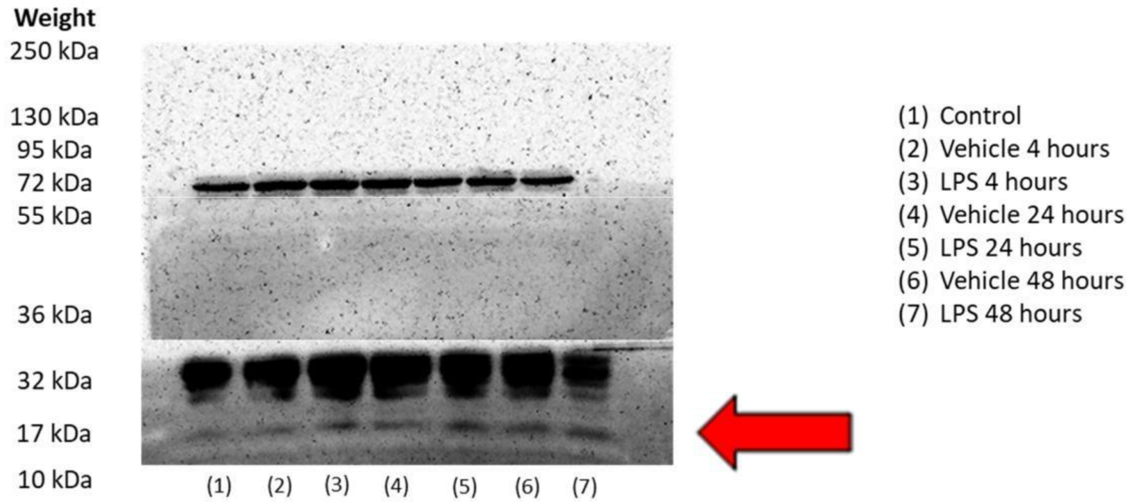


Figure S2.4. Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa). Red arrow indicates bands shown in Figure 2. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

GAPDH 36 kDa in Figure 2

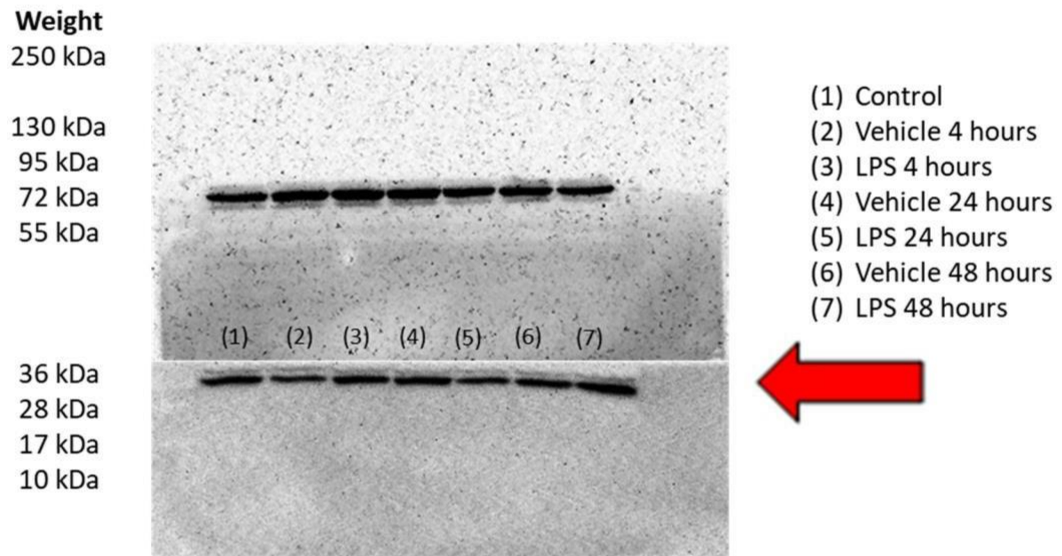


Figure S2.5. Original Western blots of brain tissue proteins showing GAPDH (molecular weight is 36 kDa). Red arrow indicates bands shown in Figure 2. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately. Bottom piece has been re-blotted and re-incubated with GAPDH.

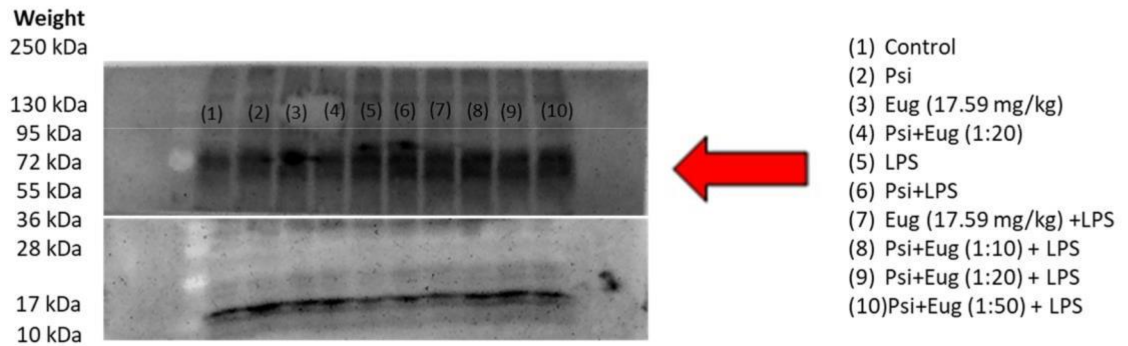


Figure S2.6. Original Western blots of brain tissue proteins showing COX-2 (molecular weight is 72 kDa). Red arrow indicates bands shown in Figure 6. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

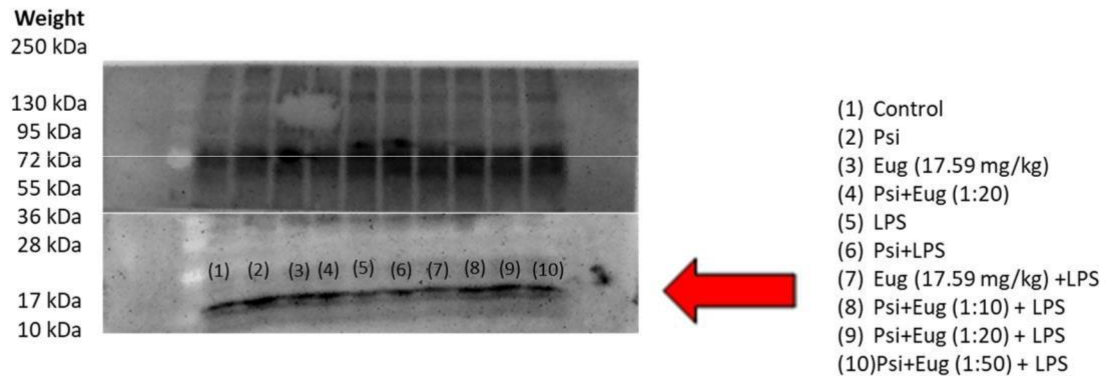


Figure S2.7. Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa). Red arrow indicates bands shown in Figure 6. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

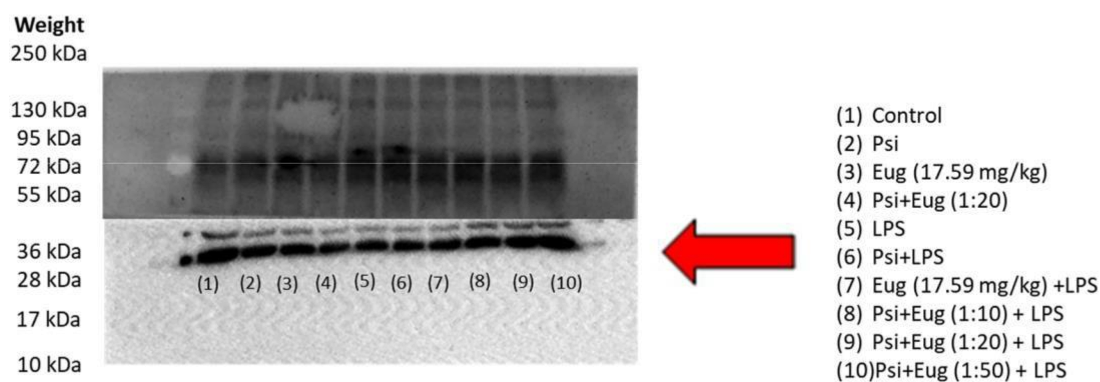


Figure S2.8. Original Western blots of brain tissue proteins showing GAPDH (molecular weight is 36 kDa). Red arrow indicates bands shown in Figure 6. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately. Bottom piece has been re-blotted and re-incubated with GAPDH.

COX-2 72 kDa in Figure 7

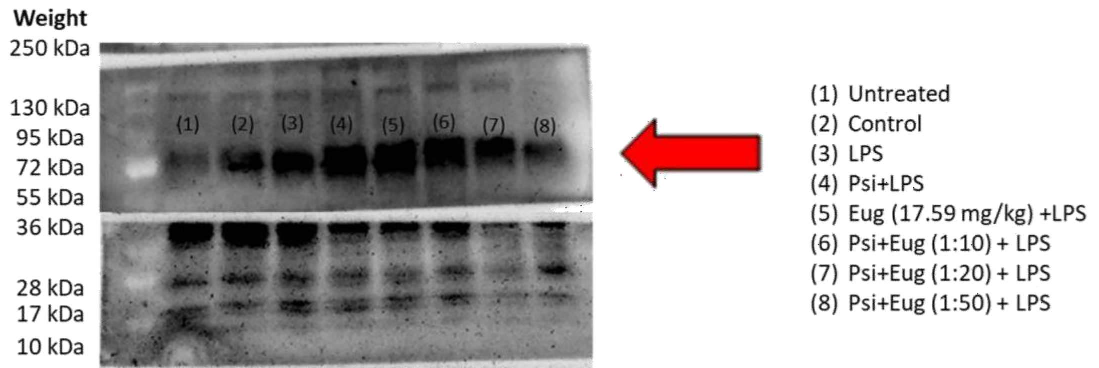


Figure S2.9. Original Western blots of brain tissue proteins showing COX-2 (molecular weight is 72 kDa). Red arrow indicates bands shown in Figure 7. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

IL-1β 17 kDa in Figure 7

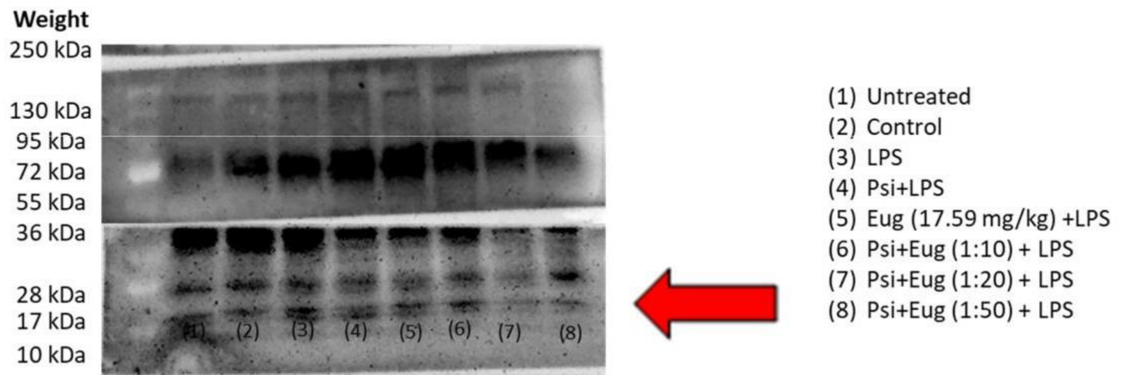


Figure S2.10. Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa). Red arrow indicates bands shown in Figure 7. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

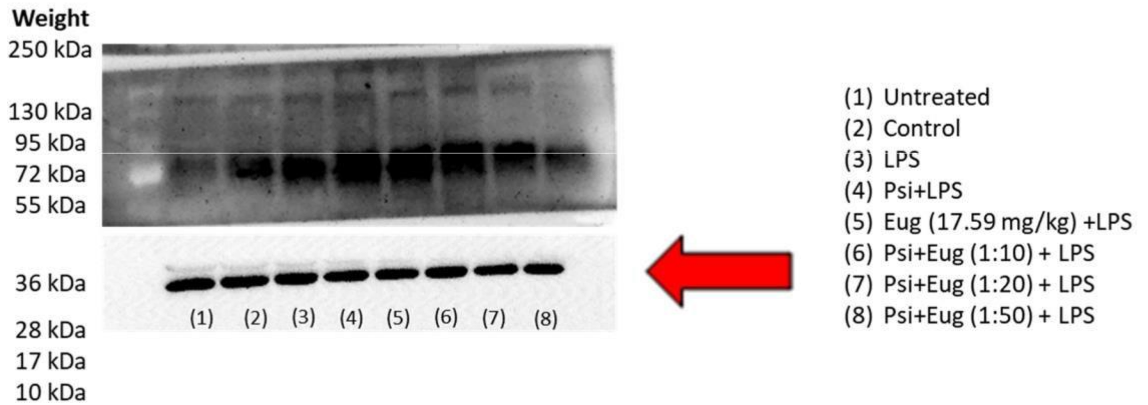


Figure S2.11. Original Western blots of brain tissue proteins showing GAPDH (molecular weight is 36 kDa). Red arrow indicates bands shown in Figure 7. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately. Bottom piece has been re-blotted and re-incubated with GAPDH.

Table S2.1. Antibodies used for Western blots

Antibody	**Supplier, Cat No**	**Dilution**
Mouse anti-GAPDH (0411)	Santa Cruz, sc-47724	1:5000 in 5% milk (PBST)
Bovine anti-Mouse	Santa Cruz, sc-2371	1:5000 in 5% milk (PBST)
Donkey anti-Rabbit	Santa Cruz, sc-2313	1:5000 in 5% milk (PBST)
COX-2 (29)	Santa Cruz, sc-19999	1:5000 in 5% milk (PBST)
IL-1β (B122)	Santa Cruz, sc-12742	1:5000 in 5% milk (PBST)

Abcam, Abcam Inc, Cambridge, United Kingdom; **BSA**, Bovine Serum Albumin; **PBST**, 1x Phosphate-Buffered Saline, 0.1 % Tween® 20; **Santa Cruz**, Santa Cruz Biotechnology, Inc., Texas, United States; **Cell Signaling**, Cell Signaling Technologies, Massachusetts, United States

Table S2.2. Primer sequences for qPCR analysis

Target Gene	Sequence Forward (5' → 3')	Sequence Reverse (5' → 3')
IL1B **ACC# NM_008361.4**	CAGGCAGGCAGTATCACTCATT	AAGAAGGTGCTCATGTCCTCATC
TNFA **ACC# NM_001278601.1**	GCCTCTTCTCATTCCTGCTTGT	TGGGAACTTCTCATCCCTTTGG
IL6 **ACC# NM_001314054.1**	GACTTCCATCCAGTTGCCTTCT	TATCCTCTGTGAAGTCTCCTCTCC
COX2 **ACC# NM_011198.4**	CCTTCTCCAACCTCTCCTACTACA	AGCTCCTTATTTCCCTTCACACC
Amylase a **ACC# nm-011198.4**	GGACTTTCCTGGAGTTCCCTATTC	CCTGAGCAGCATCTTGGTAGTT
GAPDH **ACC# XM_036165840.1**	CATCACTGCCACCCAGAAGA	AGTGGATGCAGGGATGATGTT

Table S2.3. Significance Matrix for Body Weight Change in Figure 3. ANOVA & Tukey (*n*=4-6) Significance (p) is indicated within the figures using the following scale: *, $p < 0.05$; **, p<0.01; ***, p<0.001;****, p<0.0001.

	Untreated	LPS + Psi + Eug (1:50)	LPS + Psi + Eug (1:20)	LPS + Psi + Eug (1:10)	LPS + EUG	LPS + Psi	Control - Post	PSI + Eug (1:50) + LPS	PSI + Eug (1:20) + LPS	PSI + Eug (1:10) + LPS	Eug + LPS	Psi + LPS	LPS	Psi + Eug (1:10)	Eug	Psi	Control - Pre
Untreated		**	*	**	**	*	ns	**	****	***	****	***	**	ns	ns	ns	ns
LPS + Psi + Eug (1:50)	**		ns	ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
LPS + Psi + Eug (1:20)	*	ns		ns	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
LPS + Psi + Eug (1:10)	**	ns	ns		ns	ns	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
LPS + EUG	**	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
LPS + Psi	*	ns	ns	ns	ns		**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Control - Post	ns	**	**	***	ns	**		***	****	***	****	****	**	ns	ns	ns	ns
PSI + Eug (1:50) + LPS	**	ns	ns	ns	ns	ns	***		ns	ns	ns	ns	ns	ns	ns	ns	ns
PSI + Eug (1:20) + LPS	****	ns	ns	ns	ns	ns	****	ns		ns	ns	ns	ns	*	*	*	ns
PSI + Eug (1:10) + LPS	***	ns	ns	ns	ns	ns	***	ns	ns		ns	ns	ns	ns	ns	ns	ns
Eug + LPS	****	ns	ns	ns	ns	ns	****	ns	ns	ns		ns	ns	**	*	**	*
Psi + LPS	***	ns	ns	ns	ns	ns	****	ns	ns	ns	ns		ns	ns	ns	ns	ns
LPS	**	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns		ns	ns	ns	ns
Psi + Eug (1:10)	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	**	ns	ns		ns	ns	ns
Eug	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns		ns	ns
Psi	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	**	ns	ns	ns	ns		ns
Control - Pre	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	

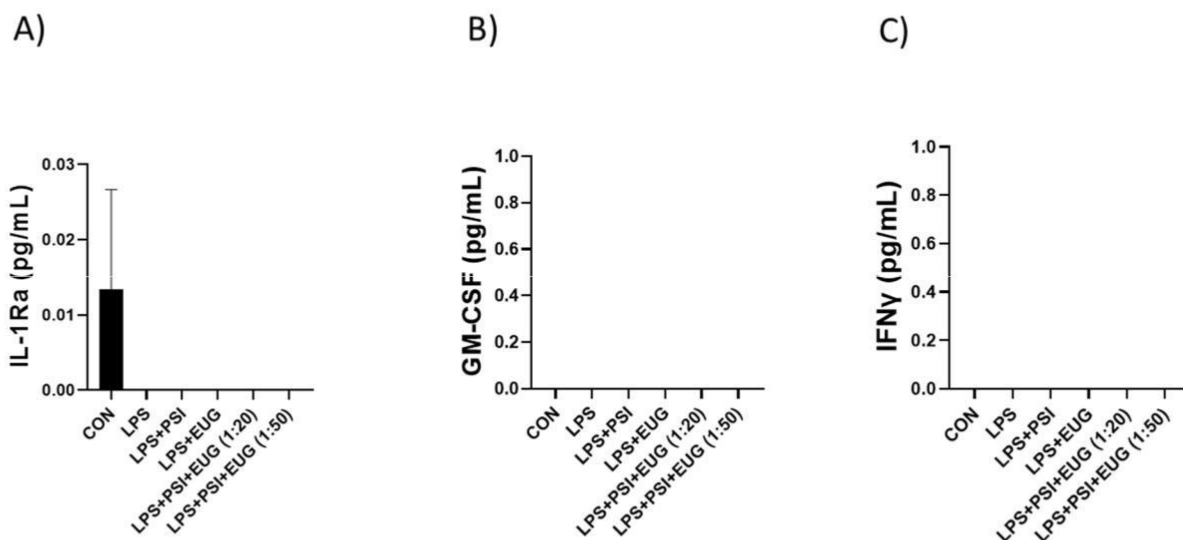


Figure S2.12. The content of inflammatory cytokines in post-treatment LPS-induced inflammation in brain tissue in Figure 8. The levels of (A) IL-1Ra, (B) GM-CSF, (C) IFNy. Data were analyzed with ANOVA & Tukey (*n*=3). Bars represent Mean ± SEM.

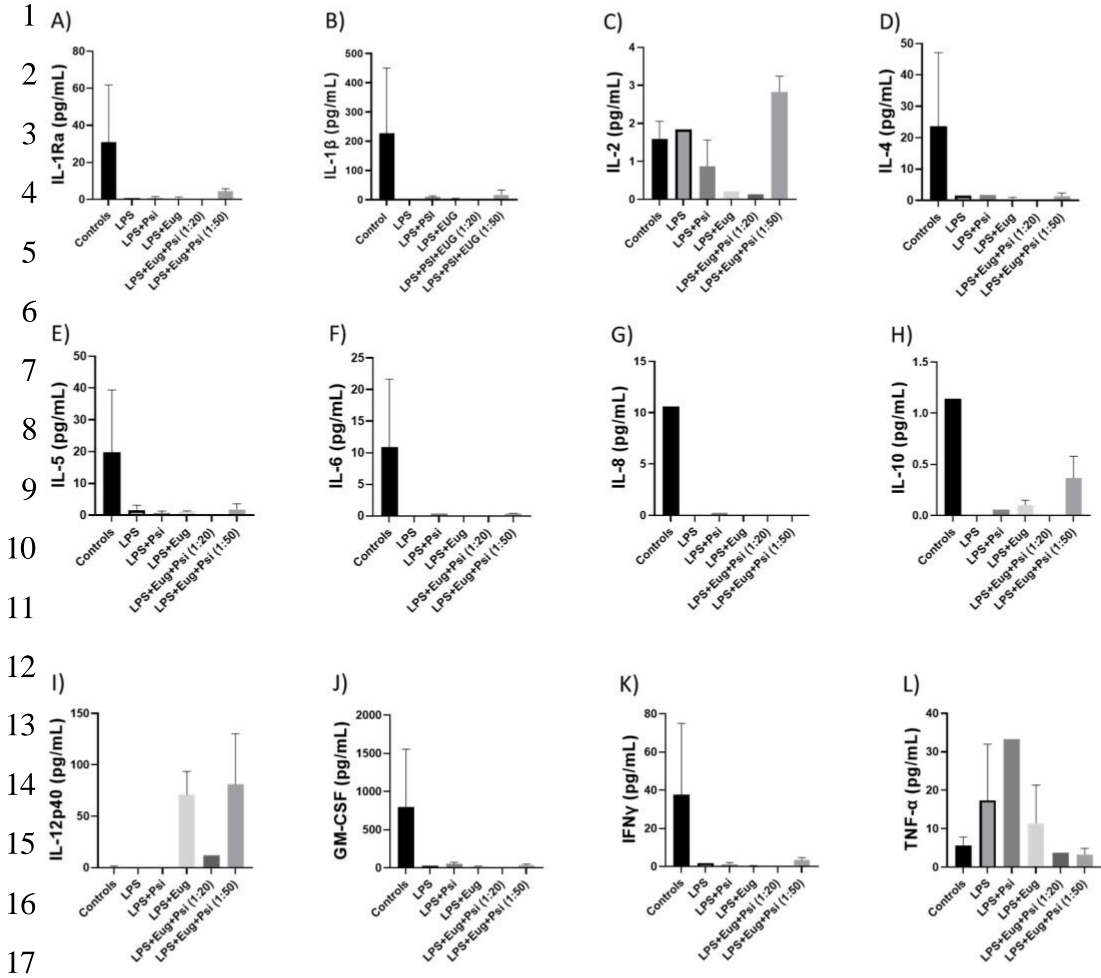


Figure S2.13. The content of proinflammatory cytokines in post-treatment LPS-induced inflammation in blood in Figure 9. The levels of (A) IL-1Ra, (B) IL-1β, (C) IL-2, (D) IL-4, (E) IL-5, (F) IL-6, (G) IL-8, (H) IL-10, (I) IL-12p40, (J) GM-CSF, (K) IFNy, (L) TNFα. Data were analyzed with ANOVA & Tukey (*n*=1-3). Bars represent Mean ± SEM.

Appendix 2: Supplementary materials for Chapter 3

24 **Table S3.1.** Antibodies used for Western blots.

Antibody	Supplier, Cat No	Dilution
Mouse anti-GAPDH (0411)	Santa Cruz, sc-47724	1:5000 in 5% milk (PBST)
Bovine anti-Mouse	Santa Cruz, sc-2371	1:5000 in 5% milk (PBST)
Donkey anti-Rabbit	Santa Cruz, sc-2313	1:5000 in 5% milk (PBST)
COX-2 (29)	Santa Cruz, sc-19999	1:5000 in 5% milk (PBST)
IL-1β (B122)	Santa Cruz, sc-12742	1:5000 in 5% milk (PBST)

25 **Abcam**, Abcam Inc, Cambridge, United Kingdom; **BSA**, Bovine Serum Albumin; **PBST**,
26 1x

27 Phosphate-Buffered Saline, 0.1 % Tween® 20; **Santa Cruz**, Santa Cruz Biotechnology,
28 Inc., Texas, United

29 States; **Cell Signaling**, Cell Signaling Technologies, Massachusetts, United States

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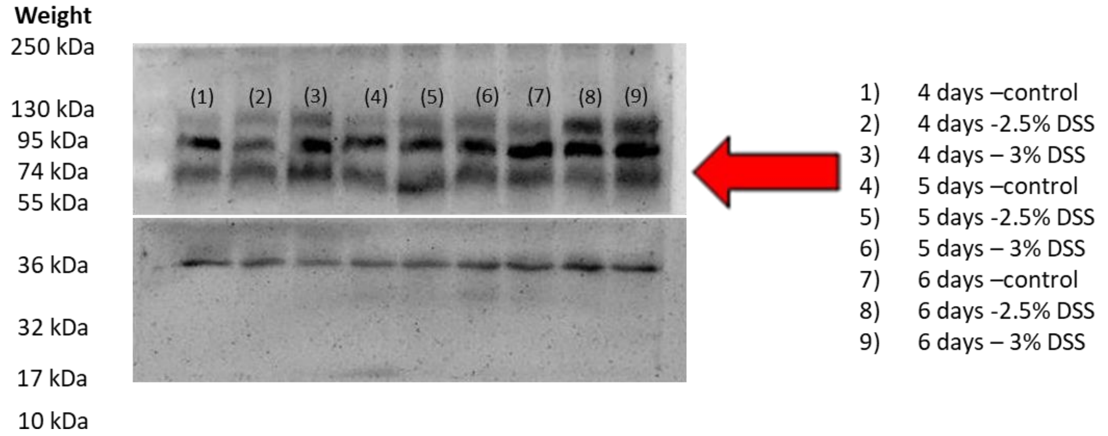
32

33 **Table S3.2.** Primer sequences for qPCR analysis

Target Gene	Sequence Forward (5' → 3')	Sequence Reverse (5' → 3')
IL1B ACC# NM_008361.4	CAGGCAGGCAGTATCACTCATT	AAGAAGGTGCTCATGTCCTCATC
TNFA ACC# NM_001278601.1	GCCTCTTCTCATTCCTGCTTGT	TGGGAACTTCTCATCCCTTTGG
IL6 ACC# NM_001314054.1	GACTTCCATCCAGTTGCCTTCT	TATCCTCTGTGAAGTCTCCTCTCC
COX2 ACC# NM_011198.4	CCTTCTCCAACCTCTCCTACTACA	AGCTCCTTATTTCCCTTCACACC
Amylase a ACC# nm-011198.4	GGACTTTCCTGGAGTTCCCTATTC	CCTGAGCAGCATCTTGGTAGTT
GAPDH ACC# XM_036165840.1	CATCACTGCCACCCAGAAGA	AGTGGATGCAGGGATGATGTT
MCP-1 *ACC#* NM_011333(1)	CTCGGACTGTGATGCCTTAAT	TGGATCCACACCTTGCATTTA
HTR2A *ACC#* NM_008311(1)	GACTCGAGGCTCTACCCTAAT	CAGGAGAGGTTGGTTCTGTTT
HTR2B *ACC#* NM_134252(1)	AGGCGAATGGCTTCATCTT	CAGAACGGTTAGTCAGGATCAG
TRPM8 *ACC#* NM_011643(1)	CTCCTGCTGTTTGCCTATGT	CATCACAGAAGAGGACGAAGAC
TRPV1 *ACC#* NM_001001445(1)	GGCTGTCTTCATCATCCTGTTA	GTTCTTGCTCTCTTGTGCAATC
TRPA1 *ACC#* NM_177781(1)	GAAGGACGCTCTCCACTTATTT	TGTCTACTTTGGCACCTTTACA

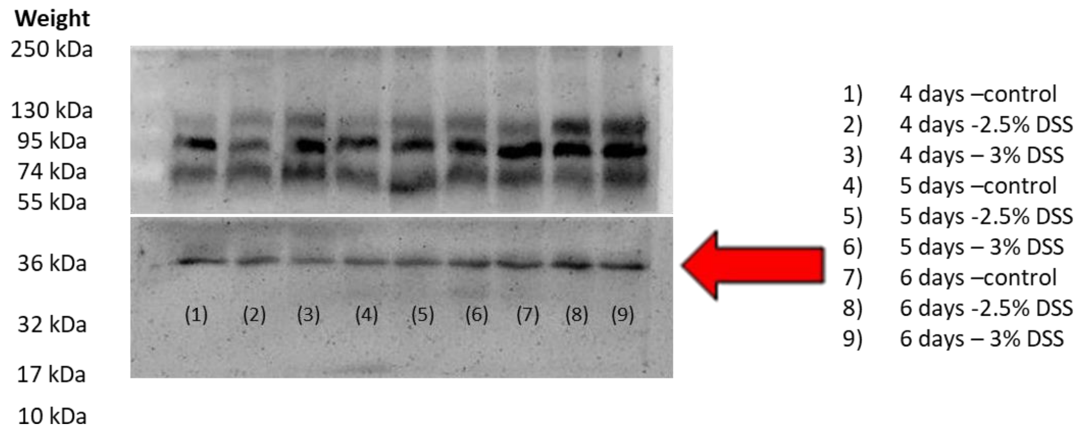
34



35

36 **Figure S3.1**. Original Western blots of brain tissue proteins showing COX-2 (molecular
37 weight is 74 kDa). Red arrow indicates bands for the part 1 of the study. This blot
38 membrane has been cut in 2 pieces. Each piece received different exposure time and
39 images has been taken separately.

40



41

42 **Figure S3.2**. Original Western blots of brain tissue proteins showing GAPDH (molecular
43 weight is 36 kDa). Red arrow indicates bands for the part 1 of the study. This blot
44 membrane has been cut in 2 pieces. Each piece received different exposure time and
45 images has been taken separately.

46

IL-1β 17 kDa for the part 1 of the study

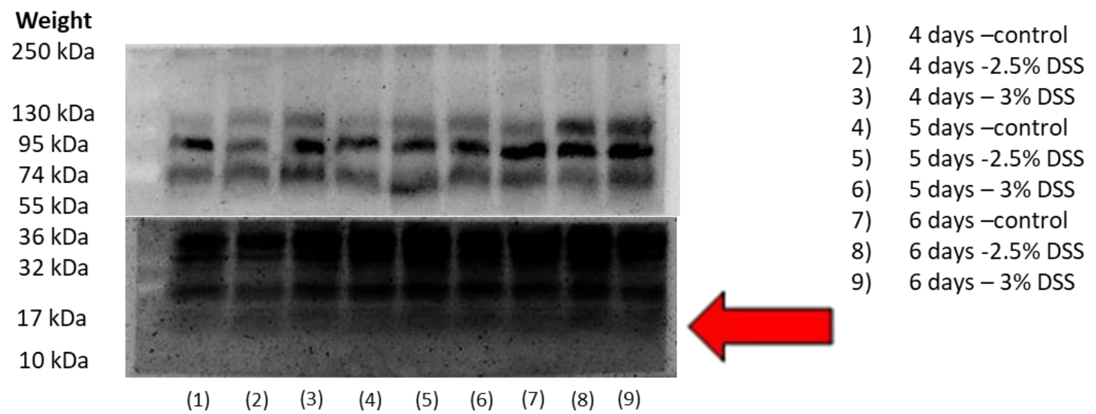
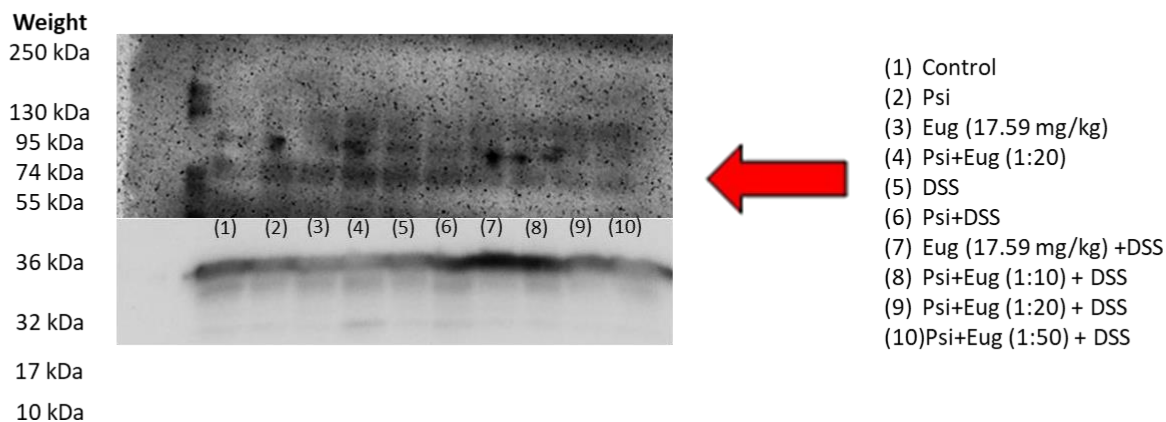


Figure S3.3. Original Western blots of brain tissue proteins showing IL-1β (molecular weight is 17 kDa). Red arrow indicates bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece received different exposure time and images has been taken separately.

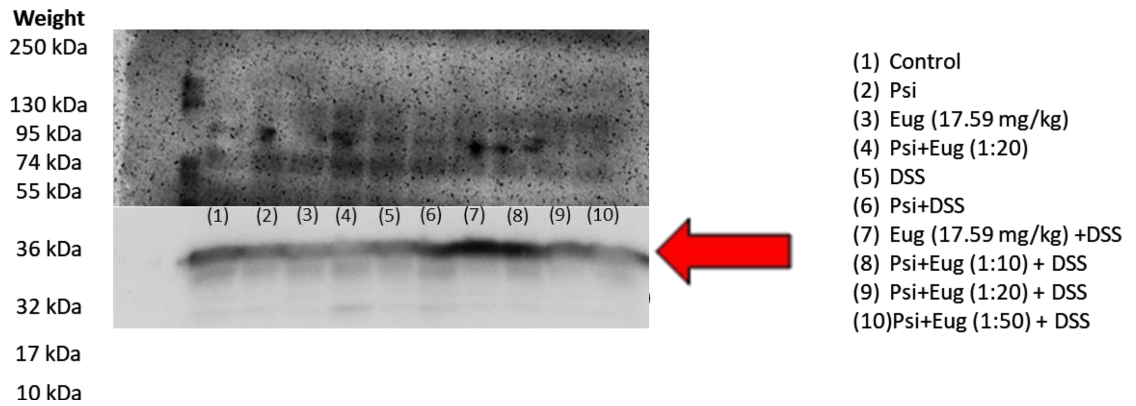


54

55 **Figure S3.4**. Original Western blots of brain tissue proteins showing COX-2 (molecular
56 weight is 74 kDa) in the pre-treatment of the 2nd part of the study. Red arrow indicates
57 bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece
58 received different exposure time and images has been taken separately.

59

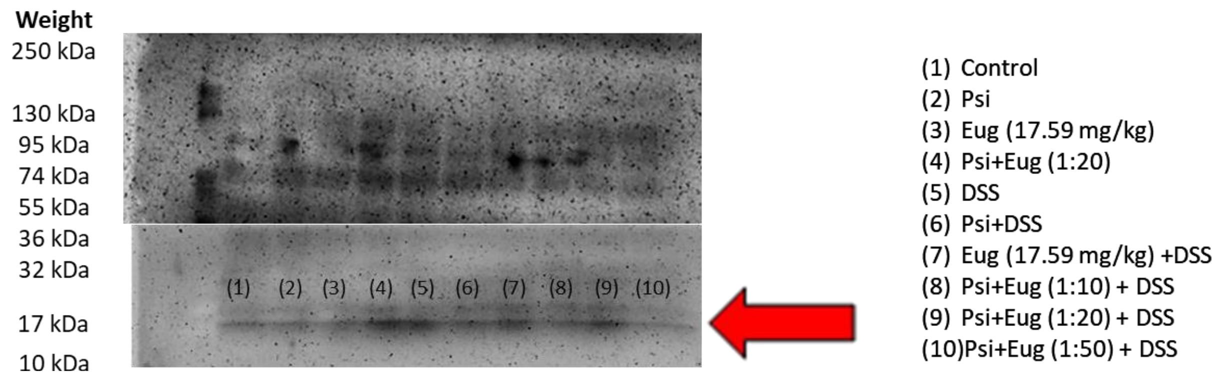
GAPDH 36 kDa, Pre-treatment (part 2 of the study)



60

61 **Figure S3.5**. Original Western blots of brain tissue proteins showing GAPDH (molecular
62 weight is 36 kDa) in the pre-treatment of the 2nd part of the study. Red arrow indicates
63 bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece
64 received different exposure time and images has been taken separately.

65



66

67 **Figure S3.6**. Original Western blots of brain tissue proteins showing IL-1β (molecular
68 weight is 17 kDa) in the pre-treatment of the 2nd part of the study. Red arrow indicates
69 bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece
70 received different exposure time and images has been taken separately.

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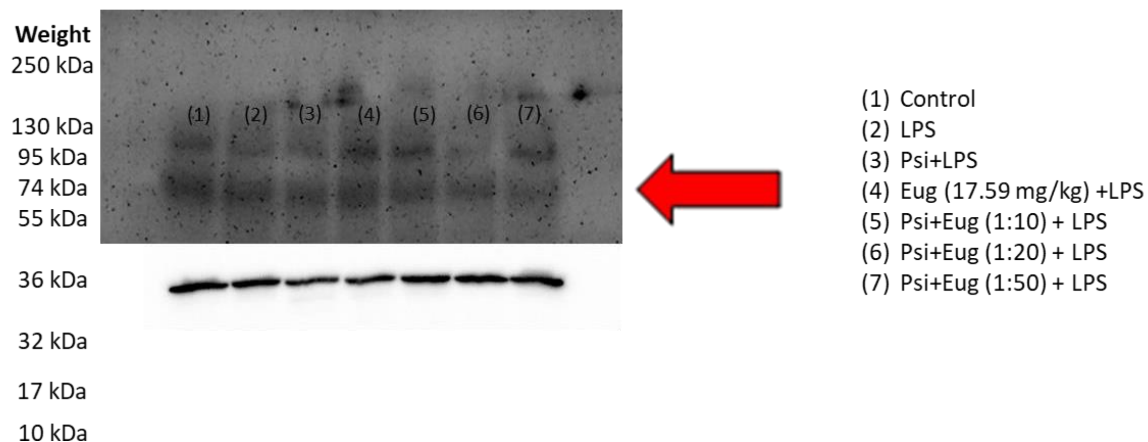
72



73 **Figure S3.7**. Original Western blots of brain tissue proteins showing COX-2 (molecular
74 weight is 74 kDa) in the post-treatment of the 2nd part of the study. Red arrow indicates
75 bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece
76 received different exposure time and images has been taken separately.

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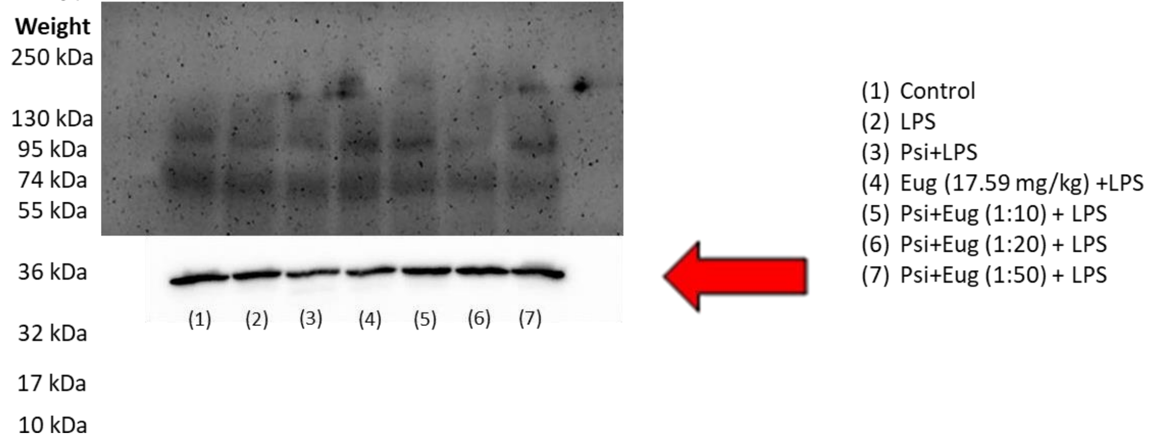
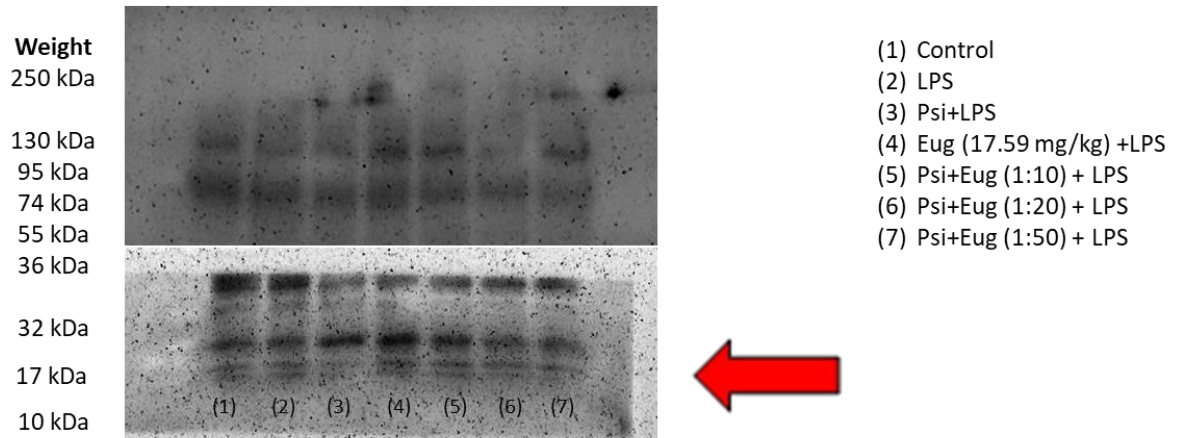


79 **Figure S3.8**. Original Western blots of brain tissue proteins showing GAPDH (molecular
80 weight is 36 kDa) in the post-treatment of the 2nd part of the study. Red arrow indicates
81 bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece
82 received different exposure time and images has been taken separately.

83



84

85 **Figure S3.9**. Original Western blots of brain tissue proteins showing IL-1β (molecular
86 weight is 17 kDa) in the post-treatment of the 2nd part of the study. Red arrow indicates
87 bands for the part 1 of the study. This blot membrane has been cut in 2 pieces. Each piece
88 received different exposure time and images has been taken separately.

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