Charles University

Faculty of Science

Akash Shivling Mali, MSc.

Exploring the role of opioid signaling in modulation of microglial function

Zkoumání úlohy opioidní signalizace v modulaci funkce mikroglií

Doctoral thesis

Supervisor: doc. RNDr. Jiří Novotný, DSc.

Prague, 2023

DECLARATION

I hereby declare that this PhD dissertation entitled "Exploring the role of opioid signaling in modulation of microglial function" submitted to the Department of Physiology, Faculty of Science, Charles University in partial fulfilment of the requirements for degree of PhD (Animal Physiology), is the result of my own work and has not formed previously the basis for the award of any degree, diploma, associateship or fellowship. All publications and other information sources used in this thesis have been duly referenced.

Prague / /2023 ……

Akash S. Mali

Dedicated to my mother

List of publications related to this thesis

Statement about the extent of participation

1. Mali AS, Novotny J. Opioid receptor activation suppresses the neuroinflammatory response by promoting microglial M2 polarization. Mol Cell Neurosci. 2022;121:103744. doi:10.1016/j.mcn.2022.103744 (IF 4.626)

Contribution- A.S.M. carried out viability and cytotoxicity assays, and determined NO and growth of microglial neurites. A.S.M. also performed phagocytosis and migration assays and used Western blotting techniques. In addition, A.S.M. prepared the draft of the manuscript.

2. Mali AS, Honc O, Hejnova L, Novotny J. Opioids alleviate oxidative stress via Nrf2/Ho-1 pathway in LPS-stimulated microglia. Int J Mol Sci. 2023;24(13):11089. http://doi.org/10.3090/ijms231311089 (IF 5.6)

Contribution- A.S.M. carried out viability and cytotoxicity assays. A.S.M. evaluated intracellular and mitochondrial ROS and also studied mitochondrial respiration. Moreover, A.S.M. measured ATP levels and GSH content, performed glucose uptake and NADPH assays, and performed Western blot analyzes.

I confirm, on behalf of all co-authors, that the above stated information about contribution of Akash S Mali to all articles is correct.

Doc. RNDr. Jiří Novotný, DSc

Abstract

Microglial activation is the most important component of neuroinflammation. It appears that opioids may affect microglial M1/M2 polarization in different ways depending on the type of receptor employed. In addition to opioid receptors, Toll-like receptor 4 (TLR4) of the innate immune system can also be activated by some opioid ligands and thus elicit specific cellular responses. Although opioid receptors (ORs) are known to regulate neurotransmission in various peptidergic neurons, their potential role in modulation of microglial function remains largely unknown. In this study, we investigated the effects of OR agonists, namely DAMGO, DADLE, and U-50488, on polarization and metabolic modulation of C8-B4 microglial cells. Our findings have revealed that opioids effectively suppress lipopolysaccharide (LPS)-triggered M1 polarization and promote the M2 polarization state. This was evidenced by decreased phagocytic activity, decreased production of nitric oxide (NO), diminished expression of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-86, and IL-12 beta p40, along with an increased migration rate and elevated expression of anti-inflammatory markers such as IL-4, IL-10, IL-13 arginase 1, and CD206 in microglia compared to cells influenced by LPS. Furthermore, we have demonstrated that opioids exert their influence on microglial polarization via the TLR4/TREM2/NF-κB signaling pathway. There is increasing evidence for a role of metabolic reprogramming in regulating microglial behavior. Nevertheless, the potential role of opioids in modulating mitochondrial function and energy metabolism in microglia has not been explored. Our findings that opioid ligands exert cytoprotective effects via the mechanism affecting LPS-induced ROS production, NADPH synthesis, and glucose uptake contribute to a better understanding of the link between the modulatory effects of opioids, metabolic states, and inflammatory responses in microglia. Interestingly, opioids elevated the level of reduced glutathione, increased ATP content, and enhanced mitochondrial respiration in microglial cells exposed to LPS. These beneficial effects were associated with the upregulation of the Nrf2/HO-1 pathway. These results indicate that activation of opioid signaling can regulate anti-inflammatory and antioxidant effects by preserving mitochondrial function while eliminating ROS in microglia. Inhibition of inflammatory stimuli may therefore be part of the future development of therapeutic approaches to promote proper microglial function, which is crucial for the prevention and treatment of neurodegenerative diseases.

Abstract (Czech)

Mikrogliální aktivace je hlavní složkou neurozánětu. Zdá se, že opioidy mohou ovlivňovat mikrogliální polarizaci M1/M2 různým způsobem v závislosti na použitém typu receptoru. Kromě opioidních receptorů může být některými opioidními ligandy aktivován vrozený imunitní toll-like receptor 4 (TLR4), a tak vyvolat specifické buněčné reakce. Zatímco je známo, že opioidní receptory (OR) regulují neurotransmisi v různých peptidergních neuronech, jejich potenciální role v řízení mikrogliální funkce zůstává do značné míry neznámá. V této studii jsme se rozhodli prozkoumat vliv agonistů OR, jmenovitě DAMGO, DADLE a U-50488, na polarizaci a metabolickou modulaci mikrogliálních buněk C8-B4. Naše zjištění ukázala, že opioidy účinně potlačují polarizaci M1 spouštěnou lipopolysacharidem (LPS) a podporují stav polarizace M2. Důkazem toho byla snížená fagocytární aktivita, snížená produkce oxidu dusnatého (NO), snížená exprese prozánětlivých cytokinů, jako je TNF-α, IL-1β, IL-6, IL-86 a IL-12 beta p40, spolu se zvýšenou rychlostí migrace a zvýšenou expresí protizánětlivých markerů IL-4, IL-10, arginása 1 a CD206 v mikrogliích ve srovnání s buňkami ovlivněnými LPS. Dále jsme prokázali, že opioidy uplatňují svůj vliv na na polarizaci microglií prostřednictvím signální dráhy TLR4/TREM2/NF-κB. Přibývají důkazy naznačují roli metabolického přeprogramování v regulaci chování mikroglií. Potenciální role opioidů v modulaci mitochondriální funkce a energetickho metabolism však dosud nebyla zkoumána. Naše nálezy týkající se schopnosti opioidních ligandů vykazovat cytoprotektivní účinky prostřednictvím mechanismu ovlivňujícího produkci ROS indukovaných LPS, syntézu NADPH a vychytávání glukózy přispívají k lepšímu porozumění spojitosti mezi modulačními účinky opioidů, metabolickými stavy a zánětlivými reakcemi v mikrogliích, Je zajímavé, že opioidy zvýšily hladinu sníženého glutathionu, zvýšily obsah ATP a zvýšily mitochondriální dýchání v mikrogliích vystavených LPS. Tyto příznivé účinky byly spojeny s upregulací dráhy Nrf2/HO-1. Tyto výsledky naznačují, že aktivace opioidní signalizace může regulovat protizánětlivé a antioxidační účinky zachováním mitochondriální funkce se současnou eliminací ROS v mikrogliích. Inhibice prozánětlivých regulátorů se může stát součástí budoucího vývojr terapeutických přístupů k podpoře správného fungování mikroglií, které je klíčové v prevenci a léčbě neurodegenerativních onemocnění.

List of abbreviations

AA	Antimycin A
AD	Alzheimer's disease
Anx1	Annexin A1
APP	Amyloid precursor protein
ATCC	American Tissue Culture Collection
ATP	Adenosine triphosphate
Aβ	Amyloid beta
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CNS	Central nervous system
2DG	2-Deoxygluocose
DAPI	4',6-Diamidino-2-Phenylindole
ddH2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOR	δ-Opioid Receptor
EC50	Half maximal effective concentration
ECAR	Extracellular acidification rate

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinase
ETC	Electron transport chain
Ex/Em	Excitation/Emission
FBS	Fetal Bovine Serum
FCCP	Carbonyl cyanide4(trifluoromethoxy)phenylhydrazone
FITC	Fluorescein isothiocyanate
G6P	Glucose 6-phosphate
GPCR	G-protein coupled receptor
H2O2	Hydrogen peroxide
HO-1	Hemeoxygenase-1
HRP	Horseradish peroxidase
IC50	Half maximal inhibitory concentration
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
kDa	Kilodalton
KOR	κ-Opioid Receptor
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

MOR	μ-Opioid Receptor
mTOR	Mammalian target of rapamycin
MTT	3(4,5dimethyl2thiazolyl)2,5diphenyl2Htetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NRF2	Nuclear factor erythroid 2–related factor 2
OCR	Oxygen consumption rate
ORL1	Opioid Receptor-Like Receptor 1
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PVDF	Polyvinylidene fluoride
RIPA	Buffer Radioimmunoprecipitation Assay Buffer
RLU	Relative luminescence units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate

STAT3	Signal transmitter and transcription activator 3
TBS	Tris-buffered saline
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2
TX-100	Triton X-100

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1. Introduction

Microglia, which are immune cells in the central nervous system (CNS), play a crucial role in various aspects of neuroinflammation, including cell death, regeneration, repair, and immunosuppression (Gao and Hong, 2008; Glass et al., 2010; Katzman, 1986). Their ability to adopt different phenotypes in response to specific signals is what enables them to carry out these functions (Colton, 2009; Haque et al., 2018). When faced with neuronal damage, activated microglia initiate an inflammatory response and remove damaged cells through phagocytosis. However, this microglial activation can also be a hallmark of brain pathology. Microglia express various G protein-coupled receptors (GPCRs) that, upon activation by modulators, mediate their activation and polarization. This GPCR-mediated microglial activation can have both beneficial and harmful effects, and it is involved in processes like amyloid precursor protein (APP) cleavage and amyloid β (Aβ) generation. Furthermore, microglial GPCRs also regulate Aβ degradation and clearance through phagocytosis and chemotaxis (Chen et al., 2008; Chhor et al., 2013; Chung et al., 2011). When Aβ binds to microglial Aβ receptors (TREM-2), it triggers multiple inflammatory pathways, leading to a phenomenon known as reactive microgliosis (Hemonnot et al., 2019; Serrano-Pozo et al., 2011). The roles of microglial cells in various neuropathological conditions are still debated, as chronic activation of microglia can result in neuronal damage through the release of harmful molecules like proinflammatory cytokines, reactive oxygen intermediates, nitric oxide, proteinases, and complement proteins (Dheen et al., 2007; Mika et al., 2014). Given the potential significance of microglia in neurodegenerative diseases, there has been interest in suppressing microglia-mediated inflammation as a potential strategy for treatment. Opioids, such as morphine, have been found to repress microglial activation and exert neuroprotective effects in the central nervous system (Hu et al., 2015; Mika et al., 2014; Peterson and Flood, 2012; Skrabalova et al., 2013). However, the exact molecular mechanisms underlying these effects are not fully understood. Some evidence suggests that opioids may influence Toll-like receptors (TLRs), which play a role in initiating immune responses, particularly TLR4, expressed on microglia and implicated in neuroinflammatory diseases (Kielian, 2006; Lehnardt et al., 2003). Microglia in an activated state have been extensively studied and found to perform multiple functions, including migration towards signals, proliferation, and engulfment of injured cells. Activated microglia can increase tau hyper-phosphorylation and secrete proinflammatory

cytokines (Askarova et al., 2011). The classical activated state (M1 phenotype) and the alternative active state (M2 phenotype) were previously categorized as old terminologies. The M1 phenotype involves the release of proinflammatory cytokines like TNF-α , IL-1β, IL-12, IL-17, IL-6, and IL-23, while the M2 phenotype is triggered by IL-4, IL-10, IL-13, CD86, CD206 or transforming growth factor-β, and promotes brain repair and regeneration by reducing neuroinflammation (Bohlson et al., 2014; Ferrante and Leibovich, 2012).

Several neurodegenerative diseases have been associated with oxidative stress and mitochondrial damage. Mitochondria, being producers of reactive oxygen species (ROS), are susceptible to oxidative stress, leading to mitochondrial DNA mutations, respiratory chain damage, altered membrane permeability, and disrupted calcium homeostasis and mitochondrial defense systems (Andersen, 2004; Sas et al., 2007). Microglia can produce nitric oxide (NO) in response to certain stimuli, which can damage neurons through various mechanisms (Nakajima and Kohsaka, 2001). Opioids have been studied for their potential antioxidant effects by enhancing antioxidant enzyme activity and inhibiting the release of cytochrome c, but they may also induce oxidative stress (Ke et al., 2009; Qian et al., 2007; Raina and Sen, 2018). The impact of opioids on mitochondrial function in microglial cells specifically is still under investigation. During CNS inflammation, activated microglia consume large amounts of glucose, which is essential for their functioning and for producing toxic inflammatory mediators. Studies have shown that inflammation induces a shift in microglia from mitochondrial oxidative phosphorylation to anaerobic glycolysis, emphasizing the significance of glucose metabolism in microglial activation (Orihuela et al., 2016). Activation of δ-opioid receptors (δ-ORs) has been associated with increased glucose uptake in skeletal muscles of diabetic mice, hinting at a potential role for δ-ORs in peripheral glucose transport. Mu-opioid receptors (mu-ORs) have been found to stimulate glucose transport by enhancing GLUT1 intrinsic activity through a signaling cascade, but their impact on glucose homeostasis in microglia remains unclear. Overall, understanding the intricate interactions between microglia and their responses in neuroinflammatory processes, as well as the effects of opioids on these mechanisms, holds significant promise in the field of neurodegenerative disease research.

2. Literature review

2.1. Microglia biology

2.1.1. Microglia origin

The CNS has historically been viewed as immune-privileged due to its lack of conventional lymphatic drainage and limited ability to present antigens (Galea et al., 2007). While the CNS does possess distinctive immunological characteristics compared to peripheral tissues, it is now recognized that it can activate robust immune responses when needed. These responses involve innate cells like macrophages, microglia, and dendritic cells (DCs). Notably, distinct areas in the CNS, including the meninges, perivascular spaces and choroid plexus, harbor specialized macrophages and DCs that act as resident innate immune defenders, capable of orchestrating potent inflammatory reactions (Nayak et al., 2012). On the other hand, the brain parenchyma contains microglia, the predominant innate immune cells residing in the brain. Constituting around 20% of glial cells, microglia are often referred to as the tissue-resident macrophages of the CNS. In contrast to the aforementioned macrophages and DCs, microglia originate from the yolk sac and colonize the CNS before its vasculature forms (Ginhoux et al., 2010; Greter and Merad, 2013). Mature microglia have small cell bodies and extensively branched structures, setting them apart from macrophages and DCs. They play roles in CNS development, maintenance, and responses to disturbances. While their cell bodies remain stationary, their processes continuously survey the surrounding extracellular space, establishing direct communication with neurons, astrocytes, and blood vessels. This constant mobility allows them to swiftly react to damage or infections by transitioning into an activated state and performing inflammatory actions. Microglia exhibit remarkable adaptability, responding effectively to diverse challenges. Upon detecting specific signals resulting from tissue injury, degeneration, or infection, microglia undergo shape changes and swiftly activate genetic programs designed to counteract and repair CNS damage. Alterations in microglial morphology, phenotype, and function are evident across various neuropathological conditions, such as degenerative disorders, infections, strokes, tumors, and brain injuries. The foundation of microglia research traces back to the pioneering contributions of Santiago Ramón y Cajal, Franz Nissl, and Pío del Río Hortega. The latter, of Ramón y Cajal's student, bestowed the name "microglia" upon these cells and is revered as the Father of Microglia (Kettenmann et al., 2011). Employing silver staining techniques and light microscopy, Río Hortega meticulously

documented microglial morphological features, predictions of their phagocytic role, and other aspects that remain valid today. His thorough histological examinations established the foundation for our present understanding of microglial biology. Subsequent to Río Hortega's investigations, the field has expanded dramatically. Microglia are now understood as pivotal intermediaries connecting neurological and immunological processes in the CNS (Kettenmann et al., 2011). Given their intricate structure and adaptability, microglia adapt to their ever-changing environment, functioning not only as brain-resident phagocytes but also serving roles beyond phagocytosis.

2.1.2. Physiological functions of microglia during brain development and homeostasis

Microglia play an essential role in the proper development of the brain. Because the generation of microglia and neurons happens simultaneously during brain development, it was anticipated that their destinies would be interconnected. Consistent with this assumption, research conducted in the last ten years has uncovered the fact that microglia engage with neurons both during developmental and also in mature stages of the CNS. Disturbing these interactions can significantly and adversely affect both CNS development and its functioning. During the initial stages of development, specific neuronal lineages and the establishment of neural circuits are influenced by a range of neurotrophic factors that promote differentiation and survival. Interestingly, in adulthood, some of these same neurotrophins continue to support neuron health and survival. Notably, microglia play a role in the local cellular environment by releasing trophic factors that aid in the formation of neural circuits and enhance neuron survival. To illustrate, microglia in the vicinity release insulin-like growth factor-1, which supports the survival of cortical neurons in layer V during postnatal development (Ueno et al., 2013). Immunohistochemical examinations have indicated that microglia congregate around callosal axon and sub-cerebral fibers shortly after birth (1 to 7 postnatal days), suggesting their interaction and support for these fibers (Ueno et al., 2013). This supposition gained support from evidence demonstrating that inhibiting or depleting microglia led to the demise of layer V cortical neurons. Moreover, the modulatory function of microglia is partly shaped by the action of fractalkine signaling (CX3CL1-CX3CR1) and the secretion of IGF-1 by microglia, as demonstrated by comparable results dealing with the viability of layer V cortical neurons when CX3CR1 is absent or IGF-1 is inhibited. While the precise connection between fractalkine and IGF-1 signaling remains unclear, the ability of microglia to generate IGF-1 in some manner is likely to impact the destiny of various cell lineages

within the nervous system. For instance, IGF-1 encourages multipotent neural progenitor cells derived from the adult rat hippocampus to differentiate into oligodendrocytes (Hsieh et al., 2004). Additionally, IGF-1 safeguards premature oligodendrocytes against apoptosis triggered by glutamate (Ness et al., 2004). In addition to IGF-1, microglia release various other trophic factors such as hepatocyte growth factor, basic fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, brain-derived neurotrophic factor and nerve growth factor, all of which have significant functions in neural development, preservation, and continuous operation. (Araujo and Cotman, 1992; Nakajima and Kohsaka, 2001; Trang et al., 2011). As such, microglia stand as vital agents that promote the well-being and longevity of neurons within the intricate framework of the nervous system.

Roughly half of the developing neurons undergo programmed cell death as a natural process during brain development. While the exact reasons behind this extensive programmed cell death remain somewhat unclear, it's theorized that the elimination of surplus defective neurons resulting from faulty differentiation and migration is necessary. Additionally, neurons that are unable to establish correct neural circuits or exhibit only transient functionality must undergo programmed cell death (apoptosis). This neuronal cell death process can be instigated by intrinsic factors intrinsic to the neurons themselves or by auxiliary cells like microglia, which possess the capability to induce programmed cell death and eliminate ensuing cellular remnants. This phenomenon was observed in the retinas of developing chick embryos, where microglia released nerve growth factor, instigating programmed cell death in retinal nerve cells, an essential component of typical developmental advancement (Frade and Barde, 1998). Similarly, microglia induce apoptosis in neurons within the developing murine hippocampus (Wakselman et al., 2008). In this specific region, microglia release ROS in a manner dependent on CD11b and DNAX activation protein of 12 kDa (DAP12), leading to neuronal cell death. Inhibition of CD11b shown to reduce neuronal cell death in the developing hippocampus. This comparable mechanism was witnessed in the developing mouse cerebellum, where targeted removal of microglia decreased the death of neuronal (Purkinje) cells. This process was partly attributed to the release of ROS by microglia, which contributed to the demise of Purkinje neurons (Marín-Teva et al., 2004). This underscores that microglia are not mere bystanders that respond to neuronal cell death; rather, they play an active role in promoting this process, particularly in certain brain regions. Given the significant level of cell demise that takes place during development, there is an urgent requirement for an

efficient mechanism to remove the resultant cellular remnants. As expected, microglia participate in this cleanup process by means of phagocytosis, and notably, they can engage in phagocytic activity without provoking an inflammatory response (Hristova et al., 2010; Takahashi et al., 2005). The phagocytic capability of microglia has been conserved throughout evolution. Investigations in the developing spinal cord of chick embryos, for instance, reveal a notable presence of microglia during early developmental stages actively involved in the phagocytosis of apoptotic motor neurons. (Calderó et al., 2009). Non-inflammatory phagocytosis by microglia is driven by signaling through the triggering receptor expressed on myeloid cells-2 (TREM2) (Takahashi et al., 2005). The activation of TREM2 signaling leads to the phosphorylation of the adaptor protein DAP12, subsequently initiating cytoskeletal reorganization and promoting phagocytosis. Importantly, proinflammatory cytokines such as TNF-α, IL-1β, and NOS2 are not produced following TREM2 signaling. In fact, an overexpression of TREM2 can even dampen microglial proinflammatory tendencies. Instead, this signaling pathway encourages the expression of CCR7 and the chemotaxis of microglia. Consequently, dying neurons release factors that draw in microglia, actively involving them in the phagocytic process. This phagocytic function is vital not only for the developing CNS but also for maintaining healthy neural networks in the mature brain. For instance, when damaged neurons release soluble fractalkine (CX3CL1), it enhances microglial phagocytosis by promoting the release of milk fat globule–epidermal growth factor 8 (MFG-E8) (Noda et al., 2011). Microglia express MFG-E8 and employ it to facilitate the phagocytosis of apoptotic cells via the CD47 signaling pathway associated with integrins (Li et al., 2012). Consequently, microglia are indispensable for the removal of deceased cells, both during development and in the mature CNS. In the absence of this critical function, the well-being of the CNS declines, potentially leading to neurological impairments.

In addition to their role in clearing deceased cells, the phagocytic activity of microglia holds a pivotal role in maintaining synaptic equilibrium (Paolicelli et al., 2011). Microglia actively engage in the process of neuronal pruning during developmental phases and exhibit responses to synaptic activity and plasticity. The proper functioning of synapses relies on a variety of trophic factors and signals that foster synapse formation, some of which are sourced from microglia. A study by (Roumier et al., 2004) emphasized the significance of DAP12 signaling in synaptic function and plasticity. DAP12, which is a transmembrane immune receptor protein, is expressed in myeloid and lymphoid cells and is thought to have a pivotal role in innate immune processes. In the mouse

brain, DAP12 expression is limited to microglia, and its absence results in a notable reduction in the synaptic expression of tyrosine kinase receptor B (TRK-B), the receptor responsible for brain-derived neurotrophic factor, ultimately leading to impaired synaptic function and plasticity. (Roumier et al., 2004). Recent investigations employing ex vivo studies on organotypic hippocampal brain slice cultures have shown that microglia can influence synaptic activity by regulating glutamatergic receptors, synapse densities, and the number of dendritic spines (Ji et al., 2013). Furthermore, microglia are also implicated in experience-dependent synaptic plasticity. Detailed imaging studies conducted on the developing mouse visual cortex demonstrated the active involvement of microglia in reshaping synaptic architecture (Tremblay et al., 2010). The exposure to sensory stimuli, like light, exerts an impact on the arrangement and structure of microglia concerning synapses. In the maturing visual cortex, microglia exhibit a close association with synaptic clefts, suggesting their involvement in the maintenance of these synapses. Notably, deprivation of light prompts microglia to develop phagocytic structures and establish closer connections with dendritic spines that appear to be undergoing disassembly. These changes are reversed upon reintroduction to light. These findings indicate that microglia share a close association with synaptic structures and are responsive to alterations in these structures triggered by environmental cues that modulate their activity.

Programmed axonal pruning and the specific removal of faulty synapses play a crucial role in establishing operational and mature neuronal circuits during the developmental stages of the brain. Microglia selectively eliminate excessive and redundant neuronal processes that could impede the formation of fully developed neuronal circuits. This function is of utmost importance for the typical progression of brain development (Paolicelli et al., 2011). In their study, Paolicelli and colleagues observed the destiny of typical pre- and postsynaptic proteins. They showed that, as synapses mature, these proteins are found within microglia, suggesting that microglia engulf synaptic structures in the intact brain. This process of synaptic pruning is, to some extent, regulated by fractalkine signaling. This is supported by findings that mice lacking CX3CR1 exhibited higher densities of dendritic spines and fewer mature synapses when evaluated 2–3 weeks after birth (Paolicelli et al., 2011). Additionally, the complement system seems to facilitate microglial synaptic pruning. During development, neurons generate the complement protein (C1q) after being exposed to immature astrocytes. (Stevens et al., 2007). Interestingly, C1q is found at synapses throughout the postnatal central nervous system and retina. It can trigger the activation of C3.

Given that microglia express C3 receptors (CR3), activation of the synaptic complement system can potentially initiate phagocytosis and the removal of synapses. This idea is supported by the fact that mice deficient in C1q or C3 demonstrated deficiencies in the elimination of synapses, leading to the retention of surplus synaptic connections (Stevens et al., 2007). Furthermore, recent research has shown that microglia modulate postnatal retinogeniculate synapses in response to neural activity through phagocytosis associated with CR3 signaling. (Schafer et al., 2012). Suppression of neural activity using tetrodotoxin heightened synaptic engulfment by microglia, while a lack of CR3 reduced microglial phagocytic activity and resulted in a persistent deficit in synaptic connectivity. Taken together, these findings suggest that microglia actively participate in both the development and maintenance of synapses. This understanding holds significant implications for our comprehension of regular brain stability, as any disruption in these normal processes has the potential to lead to neurological disorders.

2.1.3. Microglia phenotypes in classical nomenclature

The role of microglia in the central nervous system seems to encompass a range of functions, both protective and potentially harmful. In a normally functioning and healthy brain, microglia can become active due to various triggers, such as foreign pathogens, abnormal protein accumulations, and dying cells. This activation is characterized by a significant transformation from a complex branching structure to a more rounded, amoeba-like shape (Kreutzberg, 1996). accompanied by a sequence of cellular and molecular changes that result in the release of many inflammatory agents and molecules with harmful effects, including nitric oxide (NO), ROS, and prostaglandin (Colton and Gilbert, 1987; Hanisch and Kettenmann, 2007; Hurley et al., 1999). These immune responses within microglia are meticulously regulated to uphold tissue equilibrium. Traditionally, depending on the nature of the injury or stimulus that initiates the activation, microglia can adopt distinct polarized profiles known as classical activation, associated with the proinflammatory (M1) phenotype, or alternative activation/acquired deactivation, linked to the immunosuppressive (M2) phenotype. However, this simplistic classification tends to oversimplify the intricate process of microglial activation (Tang and Le, 2016). (see Figure 1)

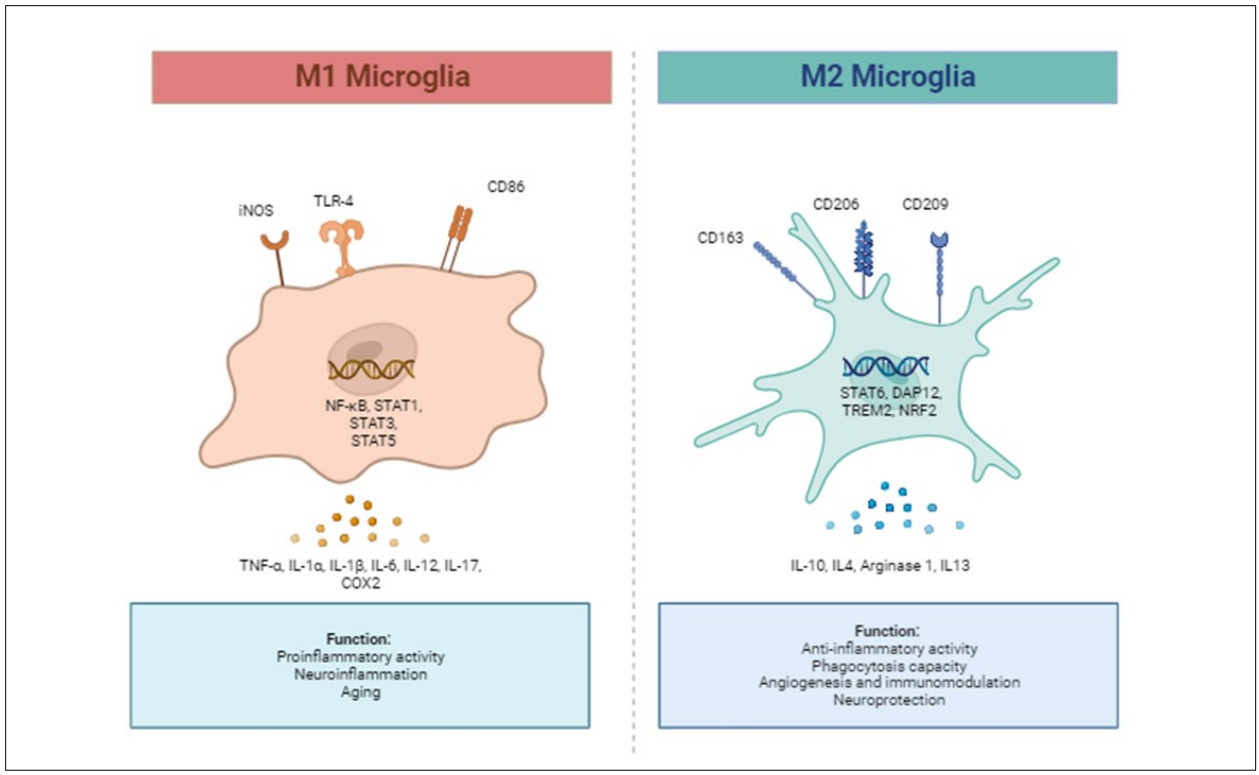

Figure 1. Classical Nomenclature of microglia activation and functions.

Microglial activation is a common event that occurs in any pathological brain lesion. Classical neuropathological studies have revealed that activated microglia undergo distinct morphological changes, and transition from their highly specialized forms to amoeboid forms. From the macrophage field, comes the proposition that there are two primary activation states: the classical M1 activation state, which promotes a pro-inflammatory response, and the alternative M2 activation state, which is associated with repair functions (Hanisch and Kettenmann, 2007). Under normal physiological circumstances, microglia remain in a quiescent state. Nevertheless, alterations in the external environment, such as injury or inflammation, can prompt trigger their activation. Activated microglia take on hypertrophic features, characterized by a bushy appearance with enlarged somas and extensions. These are often referred to as "activated" or "hyperreactive" microglia (Savage et al., 2019). Predominantly, M1 microglia are found at the site of injury in the late stages of a disease and interfere with the neuroprotective and reparative processes associated with M2 microglia (Tang and Le, 2016). Microglial cells can adopt different polarization states when stimulated by different substances. Under normal conditions, microglia exhibit a surveillance

phenotype to support all cell types in the CNS, including neurons. To sustain this state of vigilance, microglia they secrete an array of factors, including regulatory signaling proteins such as CD172, chemokines such as CX3CL1, colony-stimulating factor 1 receptor (CSF1R). Upon M1 activation induced by agents such ae lipopolysaccharide (LPS), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon-gamma (IFN-γ), microglia release multiple pro-inflammatory substances as part of the M1 phenotype, leading to neurotoxic effects. Conversely, upon alternative activation triggered by IL-4, IL-10, or IgG, microglia adopt an M2 phenotype that is neuroprotective through the release of several anti-inflammatory molecules. Recent studies show that LPS may attach to the cell surface receptor TLR4 in conjunction with MD2 (TLR/MD2), which then triggers interleukin-1 receptor-associated kinases via TRIF and MyD88. This, in turn, leads to the movement of transcription factors such as interferon regulatory factors, STAT5, activating protein-1 (AP1), and NF-κB. (Kawai and Akira, 2007). Furthermore, M1 activation triggered by IFN-γ occurs through IFN-γ receptors 1 and 2, leading to the recruitment of Janus kinase 1 and 2 (JAK1/2), which phosphorylate and transport IRFs and STAT1 to the nucleus. GM-CSF-induced M1 activation occurs when GM-CSF binds to its receptor GM-CSF-R. This initiates signaling pathways involving rat sarcoma oncoproteins (RAS), Src-family kinase (SFK), and Janus kinase 2, leading to the translocation of STAT5 into the nucleus. As a result, NF-κB, STAT1, STAT5, activating protein-1 (AP1), and interferon regulatory factors (IRFs) are activated, ultimately leading to an increase in pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α. M1 stimulation also prompts transcriptional upregulation of cell surface markers (CD86, CD16/32) and intracellular inducible nitric oxide synthase (iNOS). Essentially, microglial M1 activation is associated with pro-inflammatory functions and neurotoxicity. Conversely, M2 activation is linked to immune regulation, inflammation inhibition, and repair processes. This dichotomy mirrors the general role of M1 and M2 activations in macrophages. Additionally, miRNAs play a role in polarizing microglia toward M1 or M2 phenotypes.

The M2 activation state of microglia also participates in immune regulation, suppression of inflammation, and the processes of repair and recovery from damage. During M2 activation, a number of mediators are produced, including extracellular matrix proteins, glucocorticoids, anti-inflammatory cytokines, and other substances. In contrast to macrophages, the mechanisms behind microglial M2 activation are still relatively unclear. Microglia are thought to adopt various M2-like phenotypes that resemble to those of macrophages (Herber et al., 2006; Morgan et al., 2005;

Schwartz et al., 2006). The polarization features of M2 microglia closely parallel those of macrophages (Chhor et al., 2013; Freilich et al., 2013). IL-4 and IL-10 are produced by molecules such as Arg1 (Arginase 1), Fizz1 peroxisome proliferator-activated receptor, and Ym1 (Michelucci et al., 2009). M2 macrophage activation can be categorized into M2a, M2b, and M2c states, which can be considered analogous to microglia (Boche et al., 2013). The M2a activation state is triggered by IL-4 or IL-13 and is engaged in processes such as phagocytosis and tissue regeneration. IL-4 stimulates JAK1 or JAK3, subsequently activating STAT6, which contributes to the transcription of M2a-associated genes, including CD206, SRs, and the inhibitor of cytokine signal transduction factor 3. M2b activation is triggered by Toll-like receptors (TLRs) and IL-1 receptors, participating in the recruitment of regulatory T cells. This phenotype is achieved through the interaction of activated TLRs and Fcγ receptors with IgG. It is worth mentioning that in M2b macrophages, there is activation observed in the NF-κB, MAPK, IRF3, and PI3K/Akt pathways. The activation of M2b macrophages results in the increased expression of IL-10, CD86, and MHC-II. (L.-X. Wang et al., 2019). M2c activation is triggered by IL-10 and glucocorticoid hormones, and it is linked to anti-inflammatory and therapeutic roles. IL-10 binds to IL-10R1 and IL-10R2, initiating JAK1 and facilitating the movement of STAT3 into the nucleus. This STAT3 translocation actively suppresses most of the pro-inflammatory cytokines associated with the M1 phenotype (Franco and Fernández-Suárez, 2015; Michell-Robinson et al., 2015). In general, M2 activation is associated with the promotion of tissue repair processes, whereas M1 activation serves as the initial line of defense with pro-inflammatory functions. Additionally, various studies have employed microarray and RT-qPCR array technology to examine the expression profiles of miRNAs in polarized M1 and M2 macrophages from both mouse and human sources. Specifically, miR-9, miR-125b, miR-127, and miR-155, have been shown to drive microglial polarization toward the M1 phenotype. In contrast, miR-34a miR-124, miR-125a-5p miR-132, miR-146a miR-223, and let-7c promote M2 polarization of macrophages by targeting various adaptor proteins and transcription factors. (Essandoh et al., 2016). In a broader context, activation of the central nervous system immune response encompasses cells related to the blood and peripheral nervous system. Parenchymal microglia and astrocytes are activated by stimuli such as opioids and injury, resulting in the release of cytokines (Interleukins), chemokines, cellular adhesion molecules (CD86/16/206/32), and surface antigens that contribute to the cascade of CNS immune response (DeLeo and Yezierski, 2001).

2.1.4. Microglia phenotypes in the single-cell era

Over the decades, microglia have been classified and differentiated primarily by their cellular density, morphology, surface marker expression, and electrophysiological traits (De Biase et al., 2017). Consequently, they have been regarded as a diverse cell population within the central nervous system (Hanisch, 2013). Given the variety of functions that microglia perform during CNS development and homeostasis, including tasks such as forming and refining synapses, providing nutritive support to neurons and oligodendrocytes, and monitoring CNS functions, it has been hypothesized that specialized subsets of microglia may exist in situ to facilitate a wide range of responses required under various healthy and disturbed conditions. In pathological situations, microglia undergo rapid activation, modify their expression profiles, and enter various context-dependent reactive states due to their remarkable adaptability (Shemer et al., 2018). Until recently, there has been a lack of comprehensive investigation of microglial heterogeneity in different brain regions during prenatal and postnatal development, adult homeostasis, and perturbations at the single-cell level. Additionally, limited knowledge existed regarding microglia states specific to diseases. Technical limitations had hindered neuroimmunologists from thoroughly exploring the spatial and temporal diversity of microglia at the single-cell level. Previous single-cell analysis techniques for microglia, such as, in situ hybridization, standard immunohistochemistry, and flow cytometry, were confined to a small selection of antibodies or mRNAs, hampering a comprehensive overview of the microglial landscape under various conditions. The recent emergence of advanced single-cell technologies, including single-cell RNA sequencing and single-cell mass spectrometry (CyTOF), has revolutionized the profiling of individual cells by generating high-throughput datasets. These methods offer an unbiased alternative workflow that enables sequencing of complete transcriptomes without prior knowledge of genes. They also facilitate cell clustering based on transcriptional profiles. Likewise, CyTOF permits the simultaneous study of more than 50 distinct metal-conjugated antibodies, facilitating extensive exploration of cellular diversity. In principle, single-cell analysis holds the promise of unveiling a molecular blueprint of microglia, thereby enabling the discovery of novel markers, signaling pathways, regulatory elements, and even previously unknown microglial states crucial for their functions in maintaining homeostasis, responding to disease, and contributing to development. Although the discovery of potentially targetable subsets or types of disease-associated microglia (DAMs) by single-cell techniques has been met with great enthusiasm, cautious interpretation is necessary. Techniques

that involve cell isolation from tissue inherently carry the potential for artifacts, as recently highlighted by the application of approaches such as TRAP and Ribotag to microglia studies (Ayata et al., 2018; Haimon et al., 2018). Moreover, it is plausible that context-dependent subsets of microglia that emerge under certain conditions essentially represent activation states of existing microglia rather than distinct subpopulations.

2.2. Role of microglia in neurodegenerative diseases

The neuroimmune system plays critical roles in the development, normal functioning, aging, and response to injuries within the central nervous system. Microglia, initially characterized a hundred years ago, stand as the primary neuroimmune cells, performing three fundamental functions. Firstly, they act as sentinels, constantly detecting alterations in their environment. Secondly, they engage in housekeeping activities that contribute to the well-being and regular operations of neurons. Lastly, they assume a defensive role, responding to environmental changes and offering neuroprotection. Microglia employ a specific set of genes to execute these roles. When faced with particular stimuli or during neuroinflammatory conditions, microglia are also capable of causing harm and neuronal loss. In conditions such as Alzheimer's (Selkoe and Hardy, 2016), Parkinson's (Dickson, 2018), Huntington's (Dayalu and Albin, 2015), and prion diseases, as well as chronic traumatic encephalopathy, amyotrophic lateral sclerosis, neuronal injury and frontotemporal dementia emerges due to disruption of sentinel or housekeeping functions and the dysregulation of the defensive function, leading to neuroinflammation. These injuries are associated with various pathways, including those tied to sensing and housekeeping, such as the Trem2, Cx3cr1, and progranulin pathways. These pathways function as immune checkpoints, maintaining control over microglial inflammatory responses. Additionally, scavenger receptor pathways play a role in eliminating injurious agents. Moreover, external factors, like systemic inflammation or disturbances in the gut microbiome, can influence the progression of such injuries. Neurodegeneration's onset or worsening is a consequence of the imbalance in these microglial functions. Addressing this imbalance could potentially serve as a therapeutic approach for managing these conditions. Numerous research studies have indicated that the inflammatory response of reactive microglia can have either a protective or harmful effect on the nervous system, depending on how intensely the microglial cells are activated and on the extent of neuroinflammation. The excessive activation of microglia has been connected to the loss and death of neurons (Subhramanyam et al., 2019).

2.2.1. Protection against injurious self and non-self-stimuli

Microglia play a pivotal role in safeguarding the host against various challenges, including infectious agents, harmful self-proteins like Aβ, aggregated α-synuclein, mutant huntingtin, oxidized superoxide dismutase (SOD), and prions, as well as primary or metastatic CNS tumors. To fulfill these tasks, microglia express receptors such as Fc receptors, Toll-like receptors, viral receptors, and antimicrobial peptides (Hickman et al., 2008) When exposed to these stimuli, microglia have the capability to initiate a neuroinflammatory response, which, akin to inflammation in the peripheral system, encompasses the production of cytokines such as TNF and IL-1 (El Khoury et al., 2003; Hickman et al., 2008), as well as possibly chemokines like Ccl2 (El Khoury et al., 2007), which attract additional cells to clear damaging agents and maintain brain equilibrium. However, unlike peripheral inflammation, neuroinflammation can remain confined to microglia and not recruit circulating white blood cells. Prolonged neuroinflammation can result in neurotoxicity, leading to the degeneration of neurons. An important finding is that microglia are in a perpetual state of activity – there is no concept of "resting" microglia. Their continuous involvement in sensing, housekeeping, and protective functions keeps them constantly on the move. In healthy brains, most microglia are capable of performing these roles. Any disruption in these functions leads to an imbalance that can initiate or propagate neurodegeneration.

2.2.2. Alzheimer’s disease

Alzheimer's disease (AD) is marked by the presence of Aβ-containing plaques, neurofibrillary tangles made up of hyperphosphorylated tau protein, and the loss of neurons (Selkoe and Hardy, 2016). The generally accepted sequence of events suggests that the accumulation of Aβ triggers a response from microglia, which in turn promotes tau hyperphosphorylation and the formation of neurofibrillary tangles. This process eventually leads to neurodegeneration and cognitive decline. In both AD patients and animal models, microglia gather around senile plaques, showing two to five times higher density than in normal tissue (D’Andrea et al., 2004). They exhibit internalized Aβ, indicating phagocytosis, and undergo morphological changes associated with inflammation, such as somatic swelling and shortened processes. These activated microglia also display elevated levels of proinflammatory markers, including major histocompatibility complex II, IL-1, IL-6, CD36, and TNF (Martin et al., 2017; Tooyama et al., 1990). The role of microglia in AD pathogenesis becomes clearer when considering Aβ deposition. Microglia are involved in Aβ clearance through processes like phagocytosis and endocytosis, which are mediated by microglial

scavenger receptors (SRs) and Aβ-degrading enzymes (Frenkel et al., 2013). Proinflammatory cytokines induced by Aβ inhibit microglial Aβ clearance and the activation of NLRP3, which leads to the aggregation of microglial apoptosis-associated speck-like protein containing a CARD with Aβ. This aggregation contributes to further amyloid accumulation and spreading. Moreover, Aβ-induced cytokines foster tau hyperphosphorylation and pathology creating a self-sustaining loop that exacerbates the disease (Venegas et al., 2017). The "double-edged sword" metaphor aptly characterizes the multi-faceted role of microglia in AD (Oddo et al., 2003; Villemagne et al., 2017). Over the course of the disease progression, microglia shift from being beneficial to becoming dysfunctional and ultimately harmful. Recent transcriptomic studies distinguish a subset of microglia around Aβ plaques, known as disease-associated microglia (DAMs). These DAMs display altered expression of genes associated with sensing, housekeeping, and host-defense functions. It is not yet clear how DAMs differ from "dark microglia," which are also linked to Aβ deposits and exhibit distinct condensed cytoplasm and nucleoplasm, along with high expression of CD11b and Trem2 (Bisht et al., 2016). These findings underscore the direct connection between abnormal microglial behavior and AD and suggest that certain microglia transition from a homeostatic state to DAMs in AD.

2.2.3. Parkinson’s disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, impacting around 1.2% of individuals aged 65 and above (Deng et al., 2018). The majority of cases are sporadic, while a smaller proportion, approximately 5-10%, have a genetic basis. The hallmark of PD is the degeneration of neurons in the substantia nigra, leading to the loss of dopaminergic connections in the striatum and the accumulation of Lewy bodies containing aggregated α-synuclein (Dickson, 2018). Within the substantia nigra of PD patients, there is a notable presence of reactive microglia expressing HLA-DR, indicating an inflammatory response (Gerhard et al., 2006). Positron-emission tomography (PET) studies reveal a widespread activation of proinflammatory microglia; however, this activation does not directly correspond with the severity of clinical symptoms, suggesting that it occurs early in the disease process. The underlying mechanisms through which microglia contribute to PD might share similarities with those observed in AD. Microglia play a role in internalizing and breaking down α-synuclein, potentially as a means of clearance. When this process is impaired, extracellular α-synuclein accumulates in a manner reminiscent of Aβ in AD. Microglia tend to cluster near deposits of α-synuclein and

undergo a proinflammatory transformation, a response that appears to rely on receptors also involved in Aβ binding, such as CD36 and TLR2 (Croisier et al., 2005; Kim et al., 2013; Su et al., 2008). It's important to note that these findings require confirmation through experimentation in PD animal models. These observations suggest a potential convergence of pathogenic pathways between AD and PD, hinting at the possibility that microglia might play a similarly multifaceted role in both diseases.

2.2.4. Multiple sclerosis

Multiple sclerosis (MS) patients exhibit demyelinated plaques in both white and gray matter regions of the brain. The progressive nature of the disease leads to neurodegeneration over time, resulting in brain atrophy. Neuroinflammation is a constant presence across all stages of MS, and the categorization of MS lesions is partly dependent on the presence or absence of microglia within these lesions (Kuhlmann et al., 2017). The impact of microglia in MS lesions can be either detrimental or beneficial. In the context of experimental autoimmune encephalomyelitis (EAE), a mouse model simulating MS, microglia are found to release various substances like proteases, proinflammatory cytokines, reactive oxygen species, and reactive nitrogen species. They also attract reactive T lymphocytes, ultimately leading to harm to neurons and oligodendrocyte precursors. Studies involving the targeted removal of the transforming growth factor (TGF)-β-activated kinase 1 in microglia within EAE have demonstrated a reduction in central nervous system inflammation, as well as axonal and myelin damage. This inhibition occurs through cell-autonomous suppression of the NF-κB, JNK, and ERK1/2 pathways (Kierdorf et al., 2013). Such findings point towards microglia contributing to tissue damage in EAE. Interestingly, during the onset of the disease, microglia have been shown to support axonal regeneration, remyelination, and the removal of inhibitory myelin debris. They also release neurotrophic factors, indicating a potential beneficial role. These observations extend to different stages of MS, as microglia are closely associated with actively demyelinating lesions (Yamasaki et al., 2014; Zrzavy et al., 2017). It's plausible that microglia's dual roles, both harmful and helpful, in MS might depend on the specific disease stage or the nature of the lesions. What is evident is that the fundamental functions of microglia are altered in EAE, potentially extending to MS, affecting their ability to detect and clear debris and mount a protective response.

2.2.5. Huntington's disease

Huntington's disease (HD) is a condition inherited in an autosomal dominant manner, primarily characterized by the expansion of CAG repeats within the huntingtin (Htt) gene. This expansion results in the production of a mutant HTT protein (mHTT) with an elongated polyglutamine segment. Consequently, this leads to significant degradation of medium spiny neurons and a reduction in the size of the striatum. Patients with HD typically exhibit a combination of cognitive decline, psychiatric alterations, and movement disorders (Dayalu and Albin, 2015). Interestingly, microglial activation during the early stages of HD has been observed through PET imaging and has been found to correlate with impaired neuronal function (Tai et al., 2007). Post-mortem examinations of HD-affected brains have shown a notable increase in activated microglia and elevated levels of IL-1β (Ona et al., 1999; Singhrao et al., 1999). In the plasma of individuals with HD, higher concentrations of TNFα, elements of the complement cascade, IL-6, and clusterin, which is associated with cellular debris clearance, have been identified (Björkqvist et al., 2008; Dalrymple et al., 2007). These findings suggest that microglia-driven neuroinflammation could contribute to the pathological processes in HD. Indeed, there is evidence of rapidly proliferating microglia in close proximity to neurons expressing mHTT (Kraft et al., 2012). Additionally, the striatal region of an HD mouse model, the R6/2 mouse, shows the expression of proinflammatory factors like TNFα, IFN-γ, and TGFβ1 (Crocker et al., 2006). In the context of HD, as well as in mouse models, the enzyme kynurenine 3-monooxygenase (KMO) exhibits significant upregulation, with its expression primarily limited to microglia. Given that KMO promotes the production of neurotoxic substances derived from tryptophan, it's been proposed that mHTT might induce microglia to express KMO, leading to increased neurotoxin production. Furthermore, the activation of caspase 1 in the brain of R6/2 mice contributes to the disease's development by facilitating the conversion of biologically inactive pro-IL-1β into active IL-1β, thereby intensifying the inflammatory response (Tai et al., 2007) . In line with this, stimulating the cannabinoid receptor 2 (CB2R) suppresses microglia-mediated neuroinflammation and reduces neuronal loss in the striatum. Microglia might also have a neuroprotective role in HD. When neurons expressing mHTT are co-cultured with normal microglia, neuronal survival is actually enhanced (Kraft et al., 2012).

2.2.6. Amyotrophic lateral sclerosis

The majority of individuals suffering from amyotrophic lateral sclerosis (ALS), commonly referred to as Lou Gehrig's disease, experience sporadic ALS. However, approximately 10% of patients have genetic mutations in specific genes, including SOD1, C9orf72, TDP43, and FUS (Lall and Baloh, 2017). The progression of this disease is influenced by the presence of mSOD1 in microglia, as its expression accelerates disease onset and activation of microglia worsens motor neuron degeneration (Gurney et al., 1994). The characteristics of microglia transform as the disease advances, with proinflammatory microglia appearing in the spinal cord prior to clinical symptoms, increasing as the disease progresses, and persisting into the late stages of the disease. Microglia taken from mice expressing mSOD1 at the onset of the disease exhibited a protective effect on neurons, unlike microglia collected during the later stages of the disease. The harmful impact of mSOD1 microglia on neurons is dependent on NF-κB and partly mediated by IL-1β (Meissner et al., 2010). These findings establish a direct link between microglia carrying mSOD1 and the advancement of ALS. The pathways that lead to the activation of microglia and their subsequent neurotoxicity in ALS stem from both internal cellular changes and external stimuli. The presence of mSOD1 in microglia disrupts the regulation of NADPH oxidase, resulting in excessive production of neurotoxic superoxide (Liao et al., 2012) . Microglia sense misfolded SOD1 present inside or outside neurons in a manner similar to their recognition of Aβ or α-synuclein through Toll-like receptors and scavenger receptors (SRs), causing them to become proinflammatory. These findings highlight two primary functions of microglia altered in ALS: their perception of external stimuli and danger signals, and their role in the host's response (Meissner et al., 2010). This shift in microglial behavior from neuroprotective to neurotoxic during disease progression aligns with similar patterns seen in Alzheimer's disease and Parkinson's disease. (See Figure 2)

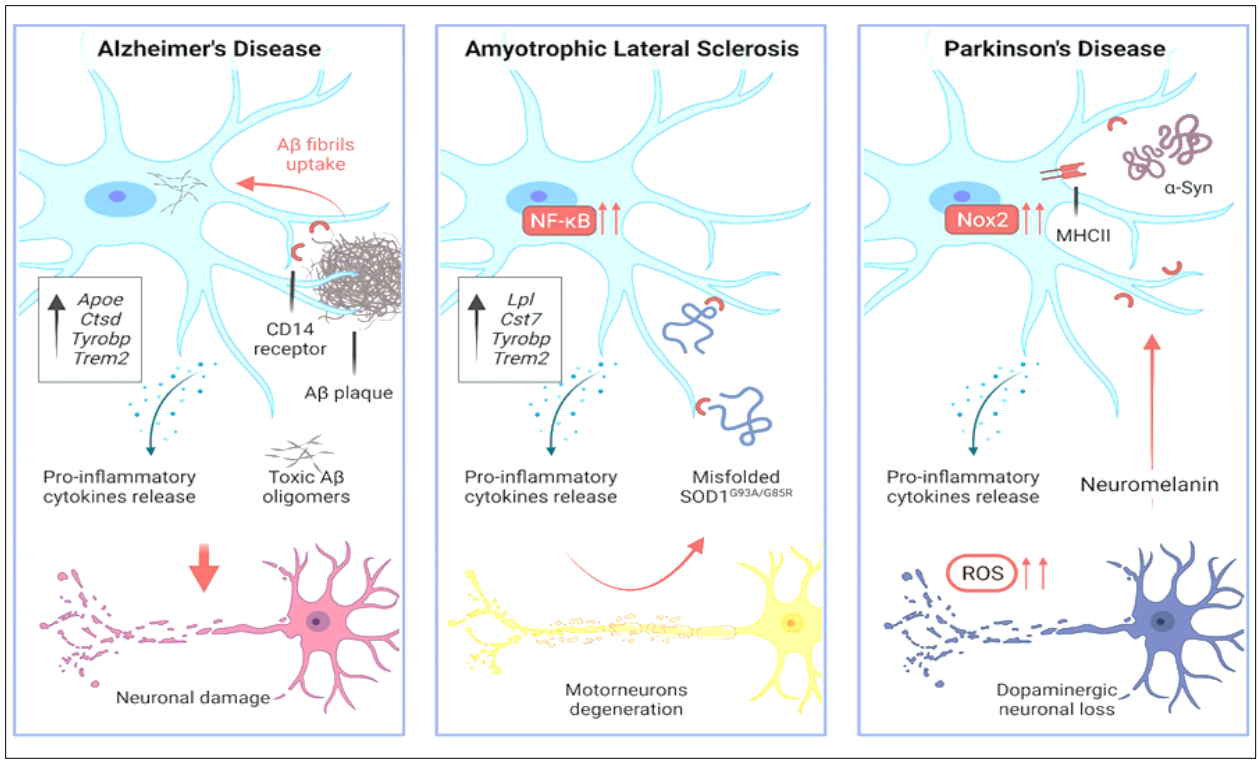

Figure 2. Microglial involvement in neuropathological process (Muzio et al., 2021).

2.2.7. Common pathways to neurodegeneration: microglial immune checkpoints

A recurring motif within neurodegenerative disorders involves microglia engaging in the harmful process of damaging and causing the demise of neurons, employing both direct and indirect mechanisms. Upon activation by various ligands such as infectious agents, PrP, LPS, Aβ, aggregated α-synuclein, or mSOD1, NADPH generates superoxide, which is then discharged and either converted into hydrogen peroxide by extracellular SOD or reacts with nitric oxide (NO) to form peroxynitrite (Simonian and Coyle, 1996). These molecules lead to either cellular necrosis or apoptosis (Brown and Vilalta, 2015). Furthermore, microglia induce neuronal death characterized by excitotoxicity, achieved either by heightened expression of inducible nitric oxide synthase (iNOS) or direct release of glutamate. Microglial enzymes like cathepsins are released in response to Aβ, instigating neuronal apoptosis, while matrix metalloproteases can inflict neuronal injury during hypoxia-ischemia (Maezawa and Jin, 2010). Indirectly, microglia can harm neurons through the release of TNF or by reducing the production of beneficial brain-derived neurotrophic factor and insulin-like growth factor, leading to increased cellular apoptosis (Brown and Vilalta, 2015). Evidently, the microglial defense function equips these cells with the means to transition

from safeguarding to becoming agents of neuronal destruction. This process isn't continuous due to various immunological checkpoints and pathways in place to prevent excessive responses to external triggers. These mechanisms encompass the Trem2, Cx3cr1–fractalkine sensing, and housekeeping pathways, along with progranulin pathways, maintain inflammatory responses within bounds, as well as scavenger receptor pathways that facilitate the removal of harmful stimuli. Disruption or mis regulation of any of these pathways, or impairment of their surveillance and maintenance functions, initiates or worsens the process of neurodegeneration.

2.3. Role of opioid receptors in neurodegenerative diseases

Opioid receptors are part of a larger family of receptors known as 7 transmembrane spanning G protein-coupled receptors (GPCRs). To date, nearly 370 non-olfactory GPCRs have been identified, with approximately 90% of them located in the brain. These receptors play pivotal roles in regulating mood, cognition, pain, appetite, and synaptic transmission (Vassilatis et al., 2003). Opioid receptors are also distributed in various other parts of the body, including the nervous system, lungs, heart, liver, gastrointestinal tract, and reproductive organs (Jutkiewicz, 2018). GPCRs are involved in several neurotransmitter systems associated with Alzheimer's disease (AD), including glutamatergic, serotonergic, adrenergic, and peptidergic pathways. Dysregulation of these systems is implicated in the development of AD and can be influenced by the activity of opioid receptors (Thathiah and De Strooper, 2011). Modulating these mechanisms has the potential to offer protection against disease progression by potentially influencing the formation of Aβ plaques or the aberrant signaling that occurs after plaque formation. (Thathiah and De Strooper, 2011). Activation of opioid receptors can occur through endogenous peptides as well as exogenously administered opioid drugs like morphine. While these drugs are effective for pain relief, they also carry the risk of addiction and are considered substances of abuse (Satoh et al., 2000). Furthermore, opioid receptors, specifically MOR, DOR, and KOR, share structural and functional similarities and can form complex combinations with non-opioid receptors, which can modify the response to opioid ligands (Pasternak, 2004) . The pharmacological response generated by opioid ligands can vary depending on the specific opioid receptor complexes involved. Synthetic opioid peptides and alkaloids, on the other hand, tend to be highly selective for MOR, DOR, and KOR, making them useful for studying the pharmacological properties of isolated opioid receptors. This complexity in opioid receptor function may have implications for their role in AD. GPCRs are attractive targets for drug discovery due to their versatility. Drugs that target

GPCRs can act as either agonists or antagonists for G protein signaling. Upon binding of an agonist to a GPCR, a conformational change occurs, activating receptor-associated G proteins and downstream signaling pathways. Additionally, a family of GPCR regulatory proteins known as β-arrestins can initiate independent signaling events, adding another layer of complexity (DeWire et al., 2007). In the context of AD pathogenesis, GPCRs are involved in various stages of amyloid precursor protein (APP) proteolysis, affecting APP processing by α-, β-, and γ-secretases and regulating Aβ degradation and toxicity (Wisely et al., 2014). GPCRs have been shown to interact with key enzymes such as β-secretase and γ-secretase, but the underlying signaling mechanisms are not fully understood, particularly with regard to the regulation of the γ-secretase complex by GPCRs. Opioid receptors play roles in learning and memory and exhibit dysregulation in specific regions of the AD brain (Mathieu-Kia et al., 2001). Recent studies indicate that these receptors, along with their ligands like enkephalin, play a role in regulating β-secretase activity and the consequent production of Aβ, indicating a direct link between dysfunctional opioid receptors and AD pathology (Thathiah and De Strooper, 2011). In contrast, a recent study presented differing roles for DOR and MOR in regulating BACE1 expression, highlighting the potential neuroprotective effects of DOR against AD-related damage. Activation of DOR with a specific agonist reduced BACE1 expression and activity in an AD-mimicking cell model, while DOR antagonism reversed these effects and increased Aβ42 production under normal conditions (Anthony et al., 2010). These findings suggest a possibility of developing a novel AD therapy by selectively targeting DOR and MOR. Moreover, genetic meta-analysis of AD patient cohorts revealed that a specific DOR mutation increased the risk of AD, particularly in late-stage AD patients with elevated BACE1 and γ-secretase activities (Sarajärvi et al., 2015). This suggests that DOR antagonism could be a promising therapeutic approach for AD treatment. Conversely, MOR activation has been reported to have potential benefits against AD through various mechanisms (Dhull and Kumar, 2018). These contrasting results underscore the complexity of opioid receptor activity in AD and the need for further research to understand these differences. Furthermore, interactions between MOR and DOR, both in terms of physical presence and functional effects, have been observed in the nervous system. Ligands with mixed interaction profiles at both receptors or specific dimer pairs may offer a novel therapeutic approach for AD treatment (Fan et al., 2005; Levac et al., 2002). The KOR also plays a significant role in cognitive functions and may influence AD pathology. KOR is widely distributed in the human brain and is implicated in

conditions such as depression, anxiety, and alcoholism. Activation of KOR has been associated with stress-related memory impairment and may regulate glutamate neurotransmission and synaptic plasticity involved in memory formation. Some studies have indicated increased KOR binding sites in AD brains, suggesting a potential involvement of KOR in AD and related disorders (Cai and Ratka, 2012; Loacker et al., 2007). In conclusion, opioid receptors and their interactions with other receptors and signaling pathways play intricate roles in the pathogenesis of Alzheimer's disease. Understanding these complexities could pave the way for novel therapeutic strategies with fewer side effects for the treatment of AD.

2.4. Microglia and opioids

Peterson and his colleagues documented that in human PBMCs, morphine hindered the generation of reactive oxygen intermediates, specifically superoxide and peroxide. These intermediates play a crucial role in the immune defense mechanism of phagocytes when responding to opsonized zymosan. Moreover, morphine also had a suppressive effect on interferon-γ and tumor necrosis factor-alpha (Peterson et al., 1987). It was suggested that the immunosuppressive cytokine transforming growth factor-beta (TGF-β), produced by lymphocytes, played a role in mediating the down-regulation of reactive oxygen intermediates induced by morphine (Chao et al., 1992). Additionally, cytokines produced by macrophages, such as IL-1β or IL-6, as well as the macrophage-activating cytokine IFN-γ, were able to restore antibody responses, suggesting that morphine either decreased macrophage numbers or their production of pro-inflammatory cytokines. Wang et al. reported that heroin, when added to human macrophage cultures, inhibited both IFN-α and IFN-β, which are molecules with antiviral properties (Wang et al., 2015).. Their conclusion was that this immunosuppression was due to the production of nitric oxide (NO). This result was somewhat unexpected, as nitric oxide is typically considered a product of activated macrophages. As previously mentioned, morphine pellets resulted in macrophages that were not activated but rather down-regulated, as indicated by reduced production of pro-inflammatory cytokines (Khabbazi et al., 2015) The study observed that morphine hindered the transformation of murine primary bone marrow macrophages and the RAW264.7 cell line into the alternatively activated M2 state, which is induced by IL-4. This inhibition was achieved by impeding the IL-4-induced expression of matrix metallopeptidase 9 (MMP-9) and arginase-1, both of which are indicative of M2 macrophages. Given that arginase-1 suppresses the production of nitric oxide associated with M1 macrophages, the impact of morphine would be to favor the activation pathway

associated with M1. Some reports supported the idea that morphine played a role in amplifying the LPS-induced activation of primary murine microglia (Gessi et al., 2016; Merighi et al., 2013). In vitro experiments showed that exposure to morphine after microglia activation with LPS increased the production of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, and nitric oxide. This effect was mediated through the activation of PKCε and the Akt pathway upstream of ERK1/2 and inducible nitric oxide synthase (Merighi et al., 2013). Furthermore, low doses of morphine activated NF-κB via PKCε (Gessi et al., 2016). However, it's important to note that morphine alone, without the presence of LPS, had no impact in either assay. Other researchers demonstrated that an incision in the paw of rats (Inan et al., 2018) or mice (Clark et al., 2007) induced the production of pro-inflammatory cytokines, and this production was either unaffected or reduced by morphine. Some study reported that acute doses of morphine in vivo suppressed the levels of both IL-12 and IL-10 in thioglycolate-elicited peritoneal macrophages when cultured and stimulated with LPS, with or without IFN-γ (Limiroli et al., 2002). Recent study reported that the addition of morphine to human monocytes in culture reduced TNF-α levels while increasing the anti-inflammatory cytokine IL-10 (Long et al., 2016). Additionally, Roy et al. noted that micromolar doses of morphine added to murine peritoneal macrophages inhibited IL-6 and TNF-α, while nanomolar doses of the opioid up-regulated these pro-inflammatory cytokines (Roy et al., 1998) . These opposing dose-dependent results correlated with the depressed and activated levels of NF-kB. In the context of infections, studies showed that mice implanted with slow-release morphine pellets exhibited decreased levels of pro-inflammatory cytokines IL-17 (T cells) and IL-23 (macrophages and dendritic cells). Furthermore, research explored the impact of morphine on the production of cytokines by T cells (Martucci et al., 2007). Most of these studies collectively pointed to morphine's ability to down-regulate cytokine production, affecting both macrophage-produced and T cell-produced cytokines (Breslow et al., 2010; Wang et al., 2011).

2.5. Microglial metabolic modulation

Growing evidence underscores the involvement of metabolic reprogramming in governing the innate inflammatory response. Alterations in metabolic functions, transitioning from growth-favoring attributes to cytotoxic/inhibitory traits, empower macrophages to execute context-specific functions adeptly (Mills, 2012; Odegaard and Chawla, 2011; Rodríguez-Prados et al., 2010). Under normal oxygen conditions, cellular energy is sourced through two distinct mechanisms. Firstly, glucose undergoes glycolysis, resulting in pyruvate, which enters the mitochondrial

tricarboxylic acid cycle (TCA) to engender ATP via oxidative phosphorylation (Dashty, 2013). Conversely, hypoxic conditions usher in anaerobic glycolysis, converting pyruvate to lactate. This metabolic shift is orchestrated by PI3K/Akt signaling, hampered by AMP-activated PK (AMPK) (Hardie, 2007) , and IL-10 (Murray et al., 2014). Recent findings indicate that immune cells possess the capacity to transition from oxidative phosphorylation to aerobic glycolysis—a phenomenon not unlike the Warburg effect observed in tumor cells (Warburg, 1956). In this transition, cells prioritize glycolysis over catabolic mitochondrial pathways, conserving and generating metabolic resources essential to meet the demands of cellular proliferation and activation, while maintaining an adequate ATP supply.

2.5.1. Role of nitric oxide and superoxide in microglial metabolism

Nitric oxide serves as a small signaling molecule synthesized by a group of enzymes referred to as nitric oxide synthases (NOS), consisting of four types: neuronal NOS (nNOS) and its mitochondrial isoform (mtNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Yuste et al., 2015). These enzymes catalyze the production of NO from L-arginine, in the presence of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), yielding citrulline as a co-product. Notably, nNOS and eNOS are constitutively expressed enzymes that rely on calcium-calmodulin for their activity, resulting in the brief release of picomoles of NO. Conversely, iNOS operates independently of calcium-calmodulin and generates a prolonged release of nanomoles of NO (Zhou et al., 2018). While iNOS is typically not expressed in the brain under normal conditions, it can be induced in microglia in response to immunological insults or inflammatory stimuli. NO plays various physiological roles, including diffusion, signaling, and cytotoxic effects. iNOS-mediated NO production can induce toxicity by inactivating mitochondrial respiratory complex I, ultimately leading to apoptosis in the target cell (Brown and Vilalta, 2015). NO has also been reported to stimulate cyclooxygenase-2 (COX-2), an inducible isoform of the cyclooxygenase enzyme believed to be implicated in the development of Alzheimer's disease. This stimulation results in an increase in the production of pro-inflammatory prostaglandins. However, it's important to note that the reported toxic effects of NO are not primarily attributed to NO itself but are more likely due to its oxidation products (Liang et al., 2013). The available evidence indicates that a substantial portion of cellular damage is attributed to the interaction between nitric oxide (NO) and another free radical, namely the superoxide anion (O2.-), resulting in the formation of peroxynitrite (ONOO-) (Zhou et al., 2018). Superoxide is a compound containing $O_2^{\cdot-}$, which is

one of the reactive oxygen species (ROS). $O_2^{\cdot -}$ is generated from molecular oxygen (O_2) in the presence of enzymes like NADPH oxidase, COX, xanthine oxidase (XO), and NOS, and it is a highly reactive species capable of causing cellular damage. In microglia, there are three isoforms of NADPH oxidase, namely Nox1, Nox2, and Nox4, which leads to the production of $O_2^{\cdot -}$, (Forrester et al., 2018). Counteracting these reactive species, there are also protective antioxidant mechanisms in place. One such mechanism involves superoxide dismutase (SOD), which facilitates the conversion of $O_2^{\cdot -}$ into O_2 and hydrogen peroxide (H_2O_2). Subsequently, catalase and glutathione further catalyze the conversion of H_2O_2 into water and oxygen (Allen and Bayraktutan, 2009; Starkov et al., 2004) (see Figure 3)

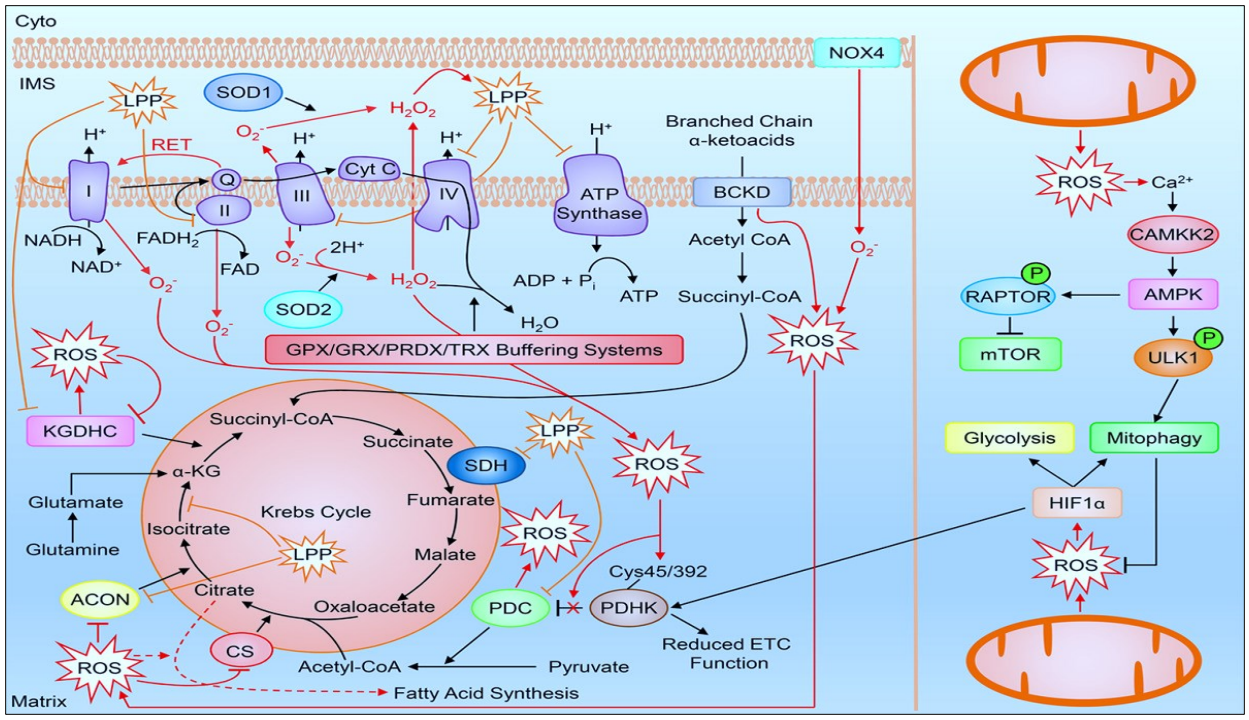

Figure 3. A schematic showing the production of reactive oxygen species in mitochondria (MitoROS). MitoROS are generated as a natural byproduct of mitochondrial respiration and the enzymatic activities involved in metabolism. In situations where there is an elevated production of ROS due to disrupted enzymatic functions and cellular stress, MitoROS can impact various metabolic pathways, including fatty acid synthesis, ATP generation, glycolysis, and mitophagy. α-KG indicates α-ketoglutarate; ACON, aconitase; AMPK, AMP-activated protein kinase; BCKD, branched chain keto acid dehydrogenase; CAMKK2, Ca2+/calmodulin-dependent protein kinase 2; ETC, electron transport chain; GPX, glutathione peroxidase; GRX, glutaredoxin; IMS, intermembrane space; KGDHC, α-ketoglutarate dehydrogenase; LPP, lipid peroxidation product; mTOR, mammalian target of rapamycin; NOX, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; PDC, pyruvate dehydrogenase; PRDX, peroxiredoxin; RET, reverse electron transfer; SOD, superoxide dismutase; TRX, thioredoxin; and ULK1, uncoordinated 51-like kinase 1 (Forrester et al., 2018)

2.5.2. Metabolism in different microglial subsets

2.5.2.1. Disease-associated microglia

In classically activated M1 microglia and dendritic cells, there is a metabolic shift towards glycolysis along with increased production of nitric oxide (NO) and citrulline. This shift triggers enhanced glucose uptake and lactate production (Krawczyk et al., 2010; Rodríguez-Prados et al., 2010) involving activation of the pentose phosphate pathway (PPP) and reduced mitochondrial oxygen consumption (Haschemi et al., 2012). Succinate, an intermediate in the Krebs cycle, assumes a pivotal role in M1 macrophages by regulating hypoxia-inducible factor 1α, thus facilitating sustained IL-1β production (Galván-Peña and O'Neill, 2014). This glycolytic increase enables rapid initiation of microbiocidal activity and allows cells to thrive in hypoxic environments. An essential feature of M1 macrophages is the generation of reactive oxygen species (ROS) to aid in the eradication of phagocytosed bacteria (West et al., 2011). The intracellular harm caused by ROS is mitigated through augmented NADPH generation, crucial for maintaining reduced glutathione (Salvemini et al., 1999) and NO production (Knowles and Moncada, 1994). NO, produced via oxidation of L-arginine by inducible NOS (iNOS), inhibits mitochondrial respiration at high concentrations by competing with O2 in cytochrome c oxidase. Consequently, reduced mitochondrial respiration leads to augmented ROS production. Prolonged NO production can result in the formation of peroxynitrite ($ONOO^-$) upon reacting with O_2^-, leading to irreversible electron transport chain inhibition (Bolanos et al., 2004). Functionally, NO synthesized by M1 macrophages serves as an effector molecule with microbiocidal activity and the ability to hinder cell proliferation (McPherson et al., 2014).

Recent investigations suggest that the specific modulation of glycolytic energy flux plays a critical role in macrophage activation, likely influencing cell polarization (Haschemi et al., 2012). Non-protein nutrient kinases such as CARKL are reported to contribute to this process (Haschemi et al., 2012). While most of the research on the bioenergetics of polarization states has been conducted in peripheral immune cells (Biswas and Mantovani, 2012; Yamasaki et al., 2014). Microglia are believed to rely on oxidative phosphorylation metabolism in the surveillance state. Upon stimulation with Toll-like receptor agonists like LPS, microglia transition from oxidative to glycolytic metabolism (Volobоueva et al., 2013). This glycolytic shift was observed in BV-2 cells upon LPS stimulation, with lactate production increasing and mitochondrial oxygen consumption and ATP production decreasing (Volobоueva et al., 2013). Another study demonstrated that LPS

stimulation augmented by IFN-γ led to increased glucose consumption, elevated glycolytic enzyme activities, and heightened lactate release, signifying potentiated (Gimeno-Bayón et al., 2014). In primary murine microglia, exposure to LPS replicated the mitochondrial bioenergetics effects observed in BV-2 cells, including loss of mitochondrial function, reduced basal respiration, and increased extracellular acidification rate (ECAR). Integrating data on peripheral immune cells with these findings, a two-stage process during activation following TLR signaling is proposed. Initially, cells utilize both oxidative and glycolytic metabolism while activating the PPP. Subsequently, microglia primarily rely on glycolytic metabolism for survival and PPP activation (Orihuela et al., 2016) (Figure 4).

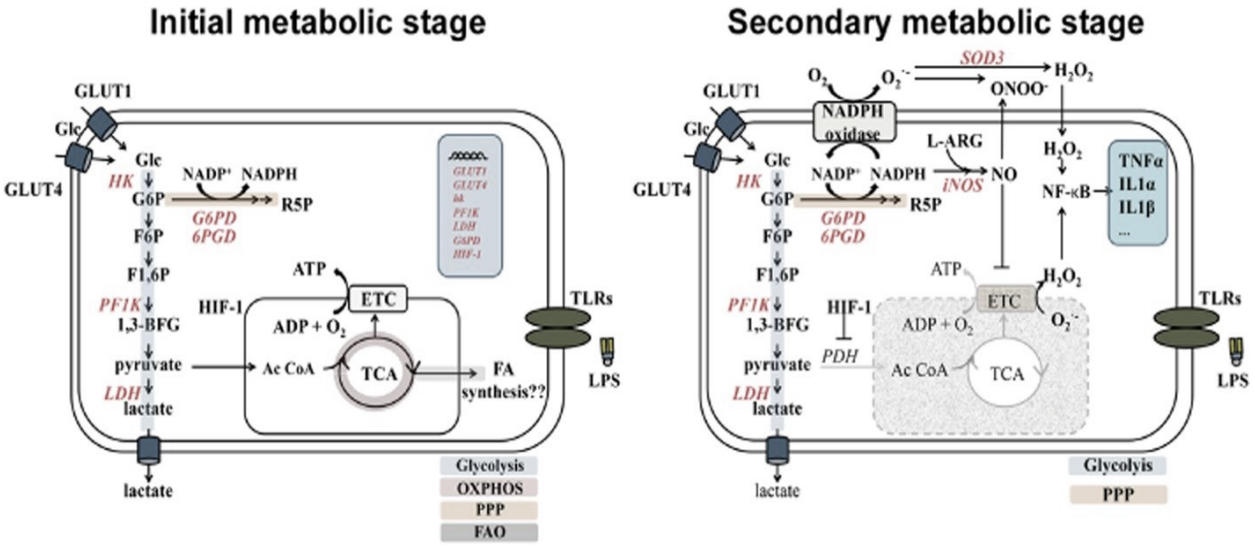

Figure 4. Microglial metabolism modulation after LPS stimulation. When LPS activates Toll-like receptors (TLRs) in microglia, it triggers significant metabolic changes. This activation leads to a shift towards glycolysis, reducing mitochondrial oxygen consumption and increasing extracellular acidification due to lactate production. Similar to other immune cells the process of microglial activation appears to involve two distinct metabolic stages. In the initial stage, immediately After LPS stimulation, there is an independent boost in glycolytic metabolism, which leads to an elevated uptake of intracellular glucose (Glc) through glucose transporters (GLUTS), even in the absence of nitric oxide production, as well as the production of various glycolytic enzymes. Importantly, the electron transport chain (ETC) remains operational during this phase, and cells continue to rely on both oxidative phosphorylation and glycolysis for energy generation. In the second stage, NADPH generated through the PPP is utilized to produce ROS, including hydrogen peroxide (H_2O_2). These ROS serve both as bactericidal agents and second messengers that modulate the nuclear factor kappa B (NF-κB) pathway. In the presence of NADPH, the enzyme inducible nitric oxide synthase oxidizes L-arginine (L-ARG) to produce nitric oxide, which inhibits Cytochrome c. This inhibition forces the cells to rely exclusively on glycolysis for their survival. Consequently, mitochondrial dysfunction leads to the production of additional mitochondrial ROS that are transported to the cytoplasm, where they activate NF-κB, intensifying the pro-inflammatory response (Orihuela et al., 2016).

2.5.2.2. Homeostatic microglia

M2 macrophages primarily rely on oxidative metabolism to fulfill their long-term functions related to tissue repair and wound healing (Biswas and Mantovani, 2012). This characteristic has been regarded as the inherent polarization state of resident macrophages, involved in functions such as promoting cell proliferation, repair, fibrosis, and tissue remodeling (Murray and Wynn, 2011). In

M2 macrophages, glucose consumption is notably lower compared to M1, and the sedoheptulose kinase CARKL plays a pivotal role in regulating the pentose phosphate pathway (Odegaard and Chawla, 2011). M2 macrophages induced by IL-4 rely on fatty acid oxidation and oxidative respiration as their primary energy production pathways, with a shift in arginine metabolism towards ornithine and polyamines to facilitate phagocytosis and regulate energy demands (Mills, 2012; Odegaard and Chawla, 2011). The role of mitochondria in M2 activation of microglia remains relatively unexplored. (Gimeno-Bayón et al., 2014) demonstrated that IL-4-stimulated BV-2 cells exhibited reduced glucose consumption and lactate production, suggesting a shift associated with the phagocytic activity and decreased need for anabolic reactions. These findings contrast with peripheral macrophages, where IL-4 induces both glucose uptake and fatty acid metabolism alongside mitochondrial biogenesis (Vats et al., 2006). Primary murine microglia also maintained an oxidative metabolic state when stimulated with IL-4/IL-13, with no alteration in basal respiration or OCR and ECAR similar to non-stimulated cells. Unlike peripheral macrophages, primary microglia showed no inhibition of IL-4-mediated reduction in LPS-induced IL-1β protein secretion (Ferger et al., 2010). In mixed glia cultures, IL-4 enhanced LPS-induced IL-1β production, suggesting the potential activation of the NLR inflammasome under these circumstances (Cao et al., 2007).

All these findings and aspects should be taken into account and the process of characterization of microglia should encompass evaluating their morphological phenotype, the distinction between resident and infiltrating microglia, the analysis of their metabolic behavior, and, when feasible, investigating their functional properties. Enhancing our comprehension of the connection between mitochondrial function and inflammation will provide valuable backing for forthcoming endeavors aimed at devising therapeutic strategies that foster the natural and precisely regulated performance of these adaptable cells.

3. Aim of the thesis

Microglial activation plays a pivotal role in neuroinflammation. Opioids appear to have diverse effects on microglial M1/M2 polarization, depending on the specific receptor type engaged. Moreover, some opioid ligands can activate the innate immune toll-like receptor 4 (TLR4), leading to distinct cellular responses. However, the precise impact of individual opioid receptors and TLR4 on modulating microglial function remains inadequately understood. Another aspect often overlooked in microglia physiology is metabolic regulation, where increasing evidence suggests that metabolic reprogramming influences microglial behavior. While there are indications that opioids might affect mitochondrial function and energy metabolism, these aspects have yet to be explored in microglia. A better understanding of the connection between opioid effects, metabolic states, and inflammatory responses of microglia, may pave the way for potential therapeutic approaches to improve microglial function in neurodegenerative diseases. Therefore, this project aimed to investigate how different opioid ligands modulate microglial polarization and elucidate the underlying molecular mechanisms. Additionally, it assessed the impact of opioid ligands on mitochondrial function, metabolic state, and oxidative balance in microglial cells.

The specific objectives of the present dissertation were as follows:

1. To investigate the influence of various opioid ligands (DAMGO, DADLE, U50488) on microglial activation and unravel the molecular mechanisms driving their actions.
2. To examine mitochondrial function and metabolic state in microglial cells exposed to opioid ligands.

Anticipated outcomes and implications of this study include a deeper comprehension of how opioid receptor subtypes and signaling pathways contribute to microglial activation, which may facilitate the development of more effective therapeutic strategies to modulate neuroinflammation. The findings from this project may also provide critical insights into the potential benefits and limitations of opioid use in the treatment of neuroinflammatory diseases. Furthermore, the results may shed new light on the role of metabolic changes in influencing microglial activation and function.

4. Material and methods

4.1. Materials

The C8-B4 cell line, a mouse microglial cell line, was acquired from the American Type Culture Collection (ATCC®, CRL-2540™), located in Rockville, MD, USA. Specifically selected opioid receptor (OR) agonists (DAMGO, DADLE, and U-50488), lipopolysaccharide (LPS; Escherichia coli 055:B5), cytochalasin D, and interleukin 4 (IL4) were procured from Sigma-Aldrich, based in St. Louis, MO, USA. Essential supplies such as fetal bovine serum (FBS), HBBS, cell culture media, and laboratory plasticware were obtained from ThermoFisher Scientific in Waltham, MA, USA. Nitrocellulose membranes (Protran BA83, BA85) were purchased from Schleicher & Schuell BioScience, headquartered in Dassel, Germany. All remaining chemicals were sourced from Sigma-Aldrich (St. Louis, MO, USA) and were of the utmost available purity. To ensure the ongoing viability of C8-B4 cells, regular cryopreservation procedures were performed. These cryopreserved C8-B4 microglia, with a concentration of 1 x 10^6 cells/ml, were suspended in Sigma freezing medium containing DMEM, 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 10% DMSO (to prevent damage to cell membranes), and then stored at a temperature of -80°C. When required, a vial of frozen cells was thawed and introduced into pre-warmed C8-B4 medium within a T75 culture flask.

4.2. Methods

4.2.1. Cell culture and treatment

C8-B4 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin within a humidified incubator set at 37°C, maintaining an atmosphere composed of 5% CO2 in air. For all experiments, the cells were initially treated with various concentrations (ranging from 0.1 to 10 μM) of specific opioid ligands (DAMGO, DADLE, and U-50488) for 1, 24, 48, or 72 hours. Following this pretreatment, the cells were then subjected to incubation in the presence or absence of lipopolysaccharide (LPS; 1 μg/mL). In an alternative approach to stimulation, the cells were alternatively activated using recombinant mouse IL4 (20 ng/mL). Cells that were not stimulated served as controls and were treated with phosphate-buffered saline (PBS) buffer.

4.2.2. Cell viability assay

The MTT assay, a commonly employed colorimetric method, is widely utilized to assess cell viability following treatment with drugs or stimuli in cell culture settings. In this assay, the yellow MTT compound is enzymatically reduced to a purple formazan dye by mitochondrial dehydrogenase in viable cells. Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. C8-B4 microglial cells were introduced into 96-well plates at a density of 4×10^4 cells per well and allowed to adhere by being cultured in the medium. After a period of 24- or 72-hours following pretreatment with varying concentrations of opioid ligands (0.01, 0.1, 1.0, 10 μM), LPS (0.5,1 and 2 μg/mL), naloxone, or tert-butyl peroxide (50 μM), MTT solution was introduced into each well. Subsequently, the cells were incubated at 37°C for 4 hours. Following this incubation, the medium was carefully removed, and the generated insoluble purple formazan crystals were dissolved using 50 μL of DMSO. The plate was allowed to incubate at room temperature for ten minutes with periodic shaking to enhance crystal solubility. Subsequently, the absorbance was measured at 570 nm using a microplate reader (BioTek Synergy HT).

4.2.3. Cytotoxicity assay (LDH)

The assessment of cytotoxicity was conducted employing the Cytotoxicity Detection KitPLUS LDH (#04744926001; Merck) on C8-B4 cells. These cells were subjected to treatment with opioid ligands (DAMGO, DADLE, and U-50488) at concentrations of 0.01, 0.1, and 1 μM, both in the presence and absence of LPS (1 μg/mL) for a duration of 24 hours. The assay operates on the principle of quantifying lactate dehydrogenase (LDH) activity released from the cytosol of compromised cells. The degree of cytotoxicity was ascertained using the subsequent formula: Cytotoxicity (%) = (experimental value − low control) / (high control − low control) × 100. Each experiment was carried out a minimum of three times.

4.2.4. Process outgrowth analysis

C8-B4 cells were introduced into twelve-well plates and cultivated for durations of both 24 hours and 72 hours, both in the presence and absence of opioid ligands or LPS. The cellular morphology was periodically examined using an inverted microscope (Arsenal AIF 5013i-T, 20x objectives), and the observations were captured through a CCD digital camera (Tucsen TCC-5.0ICE). To quantify the extent of process outgrowth, the five longest processes from a minimum of five

random fields within each well were measured. This was accomplished using the NeuronGrowth plugin in ImageJ tracing (UNAM; http://www.ifc.unam.mx/ffm/), following a previously established protocol (Cockova et al., 2019). The length of each process was defined as the measurement between the center of the cell soma and the tip of the process. Data collected from a minimum of three independent experiments were pooled to calculate the average neurite length.

4.2.5. Nitric oxide release assay

Nitric oxide (NO) release from microglia has been identified as a crucial pro-inflammatory factor involved in the neuroinflammatory response and neurotoxicity mediated by microglia, particularly at elevated concentrations. Consequently, the Griess assay is employed to estimate the accumulated quantity of nitrite (NO2-) subsequent to treatment with pro-inflammatory ligands in cell cultures. Additionally, it serves as a means to assess the anti-inflammatory effects of compounds within cell cultures when exposed to pro-inflammatory ligands like LPS. It is noteworthy that NO2- is quantified as an indicative measure for NO production, as it serves as a stable end product of NO within the cell culture medium. Initially, sulfanilamide undergoes a quantitative transformation into a diazonium salt through reaction with NO2- within an acidic solution. Subsequently, this diazonium salt couples with N-[1-naphthyl]-ethylenediamine dihydrochloride (NED) to form an azo compound. In the context of the Griess reaction, sulfanilamide and NED compete for NO2-. (Dirsch et al., 1998). C8-B4 cells pretreated with selected opioid ligands for 1 h and then with LPS (1 μg/mL) for 24, 48, or 72 h, fifty μL of cell culture medium was collected and mixed with an equal volume of Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate, followed by incubation for 10 min at room temperature. Finally, the absorbance was measured at 540 nm using a microplate reader.

4.2.6. Scratch wound migration assay

In the scratch wound assay, 8 x 10^4 C8-B4 cells were initially placed into 12-well plates. An hour subsequent to cell plating, the regular medium (DMEM with 1% FBS) was introduced. One hour later, either LPS (1 μg/ml) or IL4 (20 ng/ml) was added to the culture. The cells were cultivated for durations of both 24 hours and 72 hours, during which time they reached an approximate confluence of 90%. At this juncture, the cell monolayer was gently scratched using a sterile 200 μl pipette tip, followed by a subsequent wash to eliminate cellular debris. To facilitate migration to

the denuded area, the cells were incubated for an additional 24 hours. Photographs of the scratched cell monolayers were captured at a 10× magnification at the baseline (0-hour time point) and after the 24-hour incubation period. The widths of the scratches were quantified utilizing ImageJ software (specifically the ImageJ WH_NJ macro; http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool). The velocity of cell migration was computed across five separate cultures for each distinct experimental condition.

4.2.7. Phagocytosis assay

Microglial cells phagocytic activity was assessed using a standard fluorometric assay (Phagocytosis Assay Kit; Red Zymosan; ab234054) following the manufacturer's instructions. Briefly, C8-B4 cells in 48-well plates were exposed to selected OR agonists, LPS, IL-4, or cytochalasin D (a potent phagocytosis inhibitor) for 24 hours at 37°C. Following this incubation, a zymosan slurry (5 µL) was added. After a 3-hour incubation, the cells were collected by centrifugation, washed with ice-cold phagocytosis assay buffer, and their fluorescence was immediately measured at 540/570 nm using a microplate reader (BioTek Synergy HT). The results were quantified in terms of relative fluorescence units (RFUs).

4.2.8. Intracellular ATP measurement

The intracellular ATP levels were assessed following the instructions of the ATP assay kit (ATP Bioluminescence Assay Kit CLS II) with slight modifications. Cells were collected through trypsinization and washed twice with PBS. Approximately 10^5 cells were subjected to centrifugation (800g, 10 minutes), and the resulting cell pellets were lysed by resuspending them in TE solution (100 mM Tris, 4 mM EDTA; pH 7.75) and then heating them at 95°C for 7 minutes. Subsequently, each sample was combined with luciferase reagent in a white 96-well plate and measured in luminescence mode using a BioTek Synergy HT microplate reader.

4.2.9. Glucose uptake assay

Glucose uptake was assessed using the Glucose Uptake-Glo (Promega) bioluminescence assay in accordance with the manufacturer's guidelines. Following a 24-hour treatment with OR agonists and LPS, cells were washed with warm PBS and transferred to white-bottomed 96-well plates containing 50 µL of PBS. Subsequently, 2-Deoxyglucose (1 mM) was introduced for a 20-minute uptake period. The process was halted and neutralized, followed by the addition of the 2-

deoxyglucose-6-phosphate detection reagent. Luminescence readings were recorded 30 minutes later using a BioTek Synergy HT microplate reader.

4.2.10. Reduced glutathione assay

The assessment of reduced glutathione levels was conducted using the Reduced Glutathione (GSH) Assay Kit (ab239709, Abcam, Cambridge, UK). C8-B4 cells were cultured in 12-well plates. These cells were exposed to opioid ligands (DAMGO, DADLE, and U-50488) at concentrations of 0.01, 0.1, and 1 μM, both in the presence and absence of LPS (1 μg/mL), for a 24-hour period. Following treatment, the cells were lysed by adding the provided lysis buffer from the kit and then subjected to centrifugation at 14,000× g for 10 minutes. In this experimental setup, GSH in the supernatant reacted with DTNB to form 2-nitro-5-thiobenzoic acid, which is a yellow compound. The absorbance of the samples was subsequently measured using a BioTek Synergy HT microplate reader.

4.2.11. NADPH assay

NADPH was measured using the NADPH assay kit (ab186031, Abcam, Cambridge, UK). C8-B4 cells were grown in 12-well plates. The cells were treated with opioid ligands (DAMGO, DADLE, and U-50488; 0.01, 0.1, and 1 μM concentration) with and without LPS (1 μg/mL) for 24 h. After treatment, the cells were lysed by addition of lysis buffer provided in the kit, sonicated and centrifuged at 14,000× g at 4 °C for 10 min. The NADPH in the supernatant was detected by adding the NADPH probe provided by supplier. The absorbance was measured by BioTek synergy HT microplate reader.

4.2.12. Mitochondrial superoxide assay

C8-B4 cells were stained with MitoSOX as described previously (Kauffman et al., 2016), followed by flow cytometric analysis. Cells were seeded in 24-well plates of 105 cells/well. Cells were treated with opioid ligands (DAMGO, DADLE, and U-50488; 0.01, 0.1, and 1μM concentration) with and without LPS (1 μg/mL) for 24 h, cells were incubated with MitoSOX (5 μg/mL) in the dark for 30 min, after the incubation period cells were washed twice with PBS and harvested by trypsinization. Finally, the fluorescence was estimated by BD LSR flow cytometer *BD Bioscience and data analysis was carried out with Kaluza 2.1 software.

4.2.13. Intracellular ROS measurement

The DCFH-DA fluorescent probe, which primarily detects superoxide and hydrogen peroxide, was used to measure the generation of reactive oxygen species (ROS). On 12 well plates, C8-B4 cells were seeded and given pharmacological treatments in accordance with the information in the Cell treatments section, the cells were pretreated with opioid ligands (DAMGO, DADLE, and U-50488; 1 μM concentration) or LPS (1 μg/mL). Following a 4-5 rinse in fresh growth media, cells were live stained at 37°C for 30 min in the dark with DCFH-DA (10 μM). Due to its capacity to induce ROS H_2O_2 (50 nM) was utilized as a positive control for ROS production. Cells were then rinsed three times with 37°C PBS and examined using a fluorescence microscope (Arsenal AIF 5013iT, 10x eyepieces and 20x objective lens). Average cellular DHE fluorescence intensity and percentage of DHE positive cells were quantified at 488 nm for the assessment of fluorescence microscopy images using ImageJ software.

4.2.14. Measurement of cellular oxygen consumption rate through high resolution respirometry

Cellular oxygen consumption rate was measured by high-resolution respirometry in Oroboros oxygraph-2k in standard configuration, with 2.1 ml final volume of treated and control cells on both chambers at 37ºC and 750 rpm. For all experiments 0.8 million cells were used per chamber and the data was acquired in pmol of O2/second/no. of cells. The software Oroboros DatLab 7.4.0.4 (Oroboros instruments, Innsbruck, Austria) was used for data acquisition. The protocol used consisted of application of oligomycin (Sigma), carbonyl cynide-4-trifluromethoxyphenylhydrazone (FCCP, Sigma), and Antimycin A.

4.2.15. Western blot analysis

Western blot experiments were performed as previously described (Ihnatovych et al., 2001). In brief, cells were lysed using RIPA lysis buffer supplemented with protease inhibitors (cOmplete protease inhibitor cocktail, Sigma-Aldrich). Equal amounts of protein lysates were mixed with Laemmli sample buffer and boiled for 2 minutes before loading onto a Bio-Rad gel (50 mm). Electrophoresis was conducted at a constant voltage of 200 V for approximately 60 minutes, or until the dye front reached the end of the gel. Subsequently, the separated proteins were transferred to nitrocellulose membranes with a pore size of 0.45 μm or 0.2 μM (depend on protein size). The membranes were blocked with 5% skim milk in TBS-T buffer (10 mM Tris, 150 mM NaCl, 1% Tween 20; pH 8.0) for 1 hour at room temperature and then incubated overnight at 4°C with

primary antibodies. The following primary antibodies were employed: anti-B7-2/CD86 (sc-28347), anti-CD206 (sc-70585), anti-Arg-1 (sc-271430), anti-COX2 (sc-19999), anti-DOR1 (sc-9111), anti-IL10 (sc-8438), anti-- bp40 (sc-365389), anti-IL1b (sc-52012), anti-IL-4 (sc-28361), anti-IL-6 (sc-32296), anti-CD11b/integrin alpha M (sc-1186), anti-IL-13 (sc-134363) anti-KOR1 (sc-374479), anti-NF-κB p65 (sc-514451), anti-NF-κB p65 (p49.Ser 311; sc-135769), anti-TNF-α (sc-52746), anti-TREM2 (B3; 373828), and anti-TLR4 (sc-293072), : anti-catalase (sc-271803), anti-GPx-1/2 (sc-133160), anti-GPx-3(sc-58361), anti-GPx-6 (sc-55102), anti-HO-1(sc-136960), anti-Nrf2 (sc-722), anti-SOD2 (sc-30080), anti-SOD3 (sc-32222), anti-mTOR (sc-8319), and anti-mTOR-p (sc-101738). All of the above antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-GLUT 1 (ab-652), anti-GLUT2 (Bs-0351), anti-GLUT3 (ab-15311), anti-GLUT 4 (ab-654), All of the above antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-MOR1 antibody was purchased from Origene (AP53000PU-N; OriGene Technologies GmbH, Germany). Following a rinse with TBS-T buffer, the blots were exposed to suitable horseradish peroxidase-linked secondary antibodies (anti-mouse/anti-rabbit/anti-goat immunoglobulin G) for 1 hour at room temperature. Following the removal of unbound antibodies by washing, the membrane was treated with SuperSignal chemiluminescent substrates (Pierce Biotechnology, Rockford, IL, USA) for 1 minute. Blots were then scanned and subjected to quantitative analysis using ImageJ software. Signal intensities from immunochemical reactions were standardized relative to total protein content as determined by Ponceau S staining, and results were reported as fold changes relative to control.

4.2.16. Statistical analysis

At least three independent biological replicates were performed for all experiments. All plots, calculations and statistical analyzes were conducted using GraphPad Prism software version 8.0 (GraphPad Software, San Diego, CA, USA). All data are expressed as mean ± SEM (standard error of the mean). Differences between groups were analyzed by one-way analysis ANOVA followed by Tukey's multiple comparison post hoc test. P values of less than 0.05 were considered statistically significant.

5. Results

5.1. *LPS and opioid agonists influence microglia viability and cytotoxicity*

To begin with, we examined the presence of μ-, δ-, and κ-opioid receptors (ORs) in C8-B4 microglia both under normal resting conditions and after exposing the cells to LPS or opioid agonists for a 24-hour period. In order to determine the effective concentration range of opioid ligands, we evaluated their impact on the viability of C8-B4 cells using an MTT cell proliferation assay. We found that the selected opioid ligands, even at concentrations of up to 10 μM, did not exhibit any noticeable cytotoxicity in C8-B4 cells, both in the presence and absence of LPS stimulation (1 μg/mL) for either 24 hours (Figure 5) Consequently, we chose to use opioid agonists at concentrations of 0.1 μM or higher and 1 μg/mL LPS for all subsequent experiments. Following the treatment of C8-B4 cells with increasing concentrations (ranging from 0.01 to 1 μM) of the chosen opioid ligands (DAMGO, DADLE, and U-50488) for 24 hours, the cells were further incubated for an additional 24 hours in the presence or absence of LPS (1 μg/mL). We then assessed cell viability and drug toxicity through MTT assays and LDH release assays, respectively. In stark contrast, exposure to LPS resulted in a significant reduction in cell viability by approximately 50% after 24 hours. However, pre-treatment with DAMGO, DADLE, or U-50488 displayed a dose-dependent ability to mitigate the cytotoxicity induced by LPS (as depicted in Figure 5A, B, and C) and enhanced overall cell viability (as shown in Figure 5D, E, and F). This suggests that the cytotoxic effects of LPS can be effectively attenuated by pre-treating cells with increasing concentrations of opioid agonists (H,I)

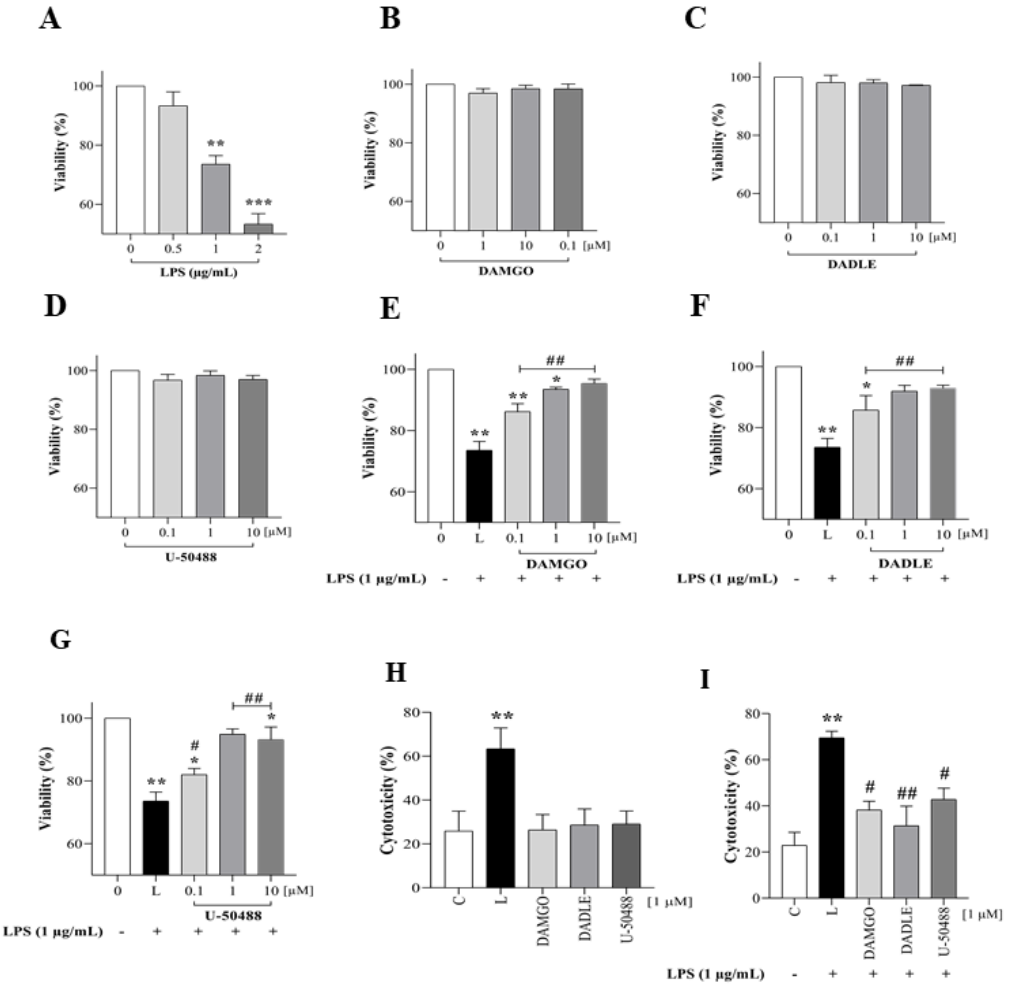

Figure 5. Effects of LPS and opioid agonists on viability of C8-B4 microglial cells. C8-B4 cells were treated with different concentrations of LPS (A) or DAMGO (B), DADLE (C) and U-50488 (D) for 24 hours, and cell viability determined after 24 hours by the MTT assay. C8-B4 cells were pretreated with different concentrations of DAMGO (E), DADLE (F) and U-50488 (G) and then incubated in the presence of LPS (1 μg/mL) for 24 hours before cell viability was determined. The cytotoxicity of LPS and opioid agonists (all at a concentration of 1 μg/mL) in C8-B4 cells was determined by measuring LDH release after 24 h of incubation (H). The effect of opioid agonists on LPS-induced cytotoxicity was determined in cells pretreated with DAMGO, DADLE or U-50488 (I). Data are presented as mean ± SEM of three independent experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with LPS-treated group).

5.2. LPS and opioid agonists influence microglia cell morphology

In addition, the morphological changes of C8-B4 cells were observed after treatment with DAMGO, DADLE, U50 488, LPS or IL-4. The typical morphology of resting microglia, characterized by small somas and extensive arborization of dynamic processes, changed after 24 h activation with LPS. The processes became shorter and cell bodies enlarged, which is characteristic of activated microglia. Notably, treatment of the cells with IL-4 or opioid ligands significantly increased the length of the processes (Figure 6).

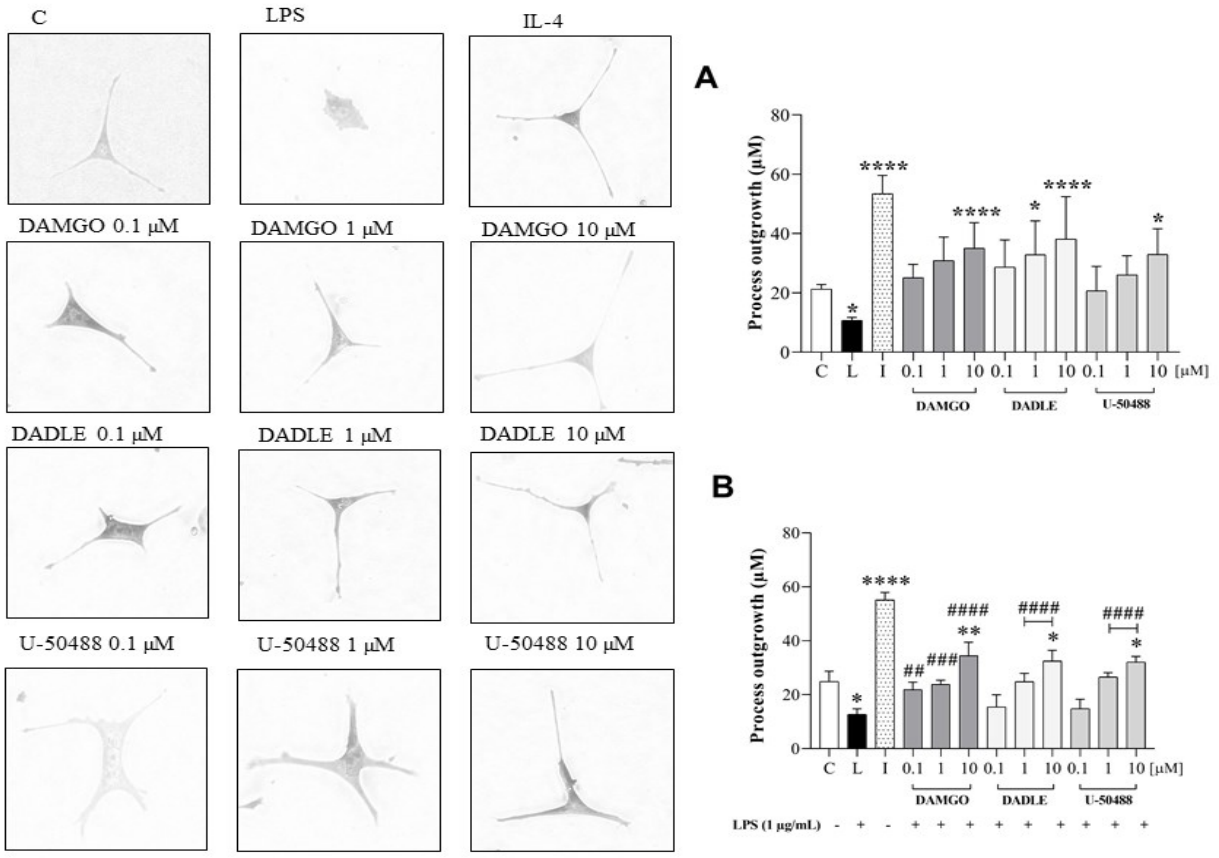

Figure 6. Effect of microglial polarization on outgrowth of cellular processes. C8-B4 microglial cells were either untreated (control, C) or treated with LPS (1 μg/mL, L) or IL-4 (20 nM, I) or opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 24 h and the length of microglial processes was determined as described in Methods. Typical photographs of microglia (A) and graphs showing the changes in microglial process length when treated with different compounds alone (B) and the changes in cells pretreated with opioids before the addition of LPS. Data are representative of three independent experiments. Values are the mean ± SEM (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ compared with control; $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ compared with LPS-treated group).

5.3. The speed of microglial migration depends on their activation state

The alterations in microglial morphology induced by opioids prompted us to formulate the hypothesis that the migratory capability of microglia could be contingent on their activation status. Enhanced migration towards areas of damage is a characteristic associated with both classically and alternatively activated microglia.

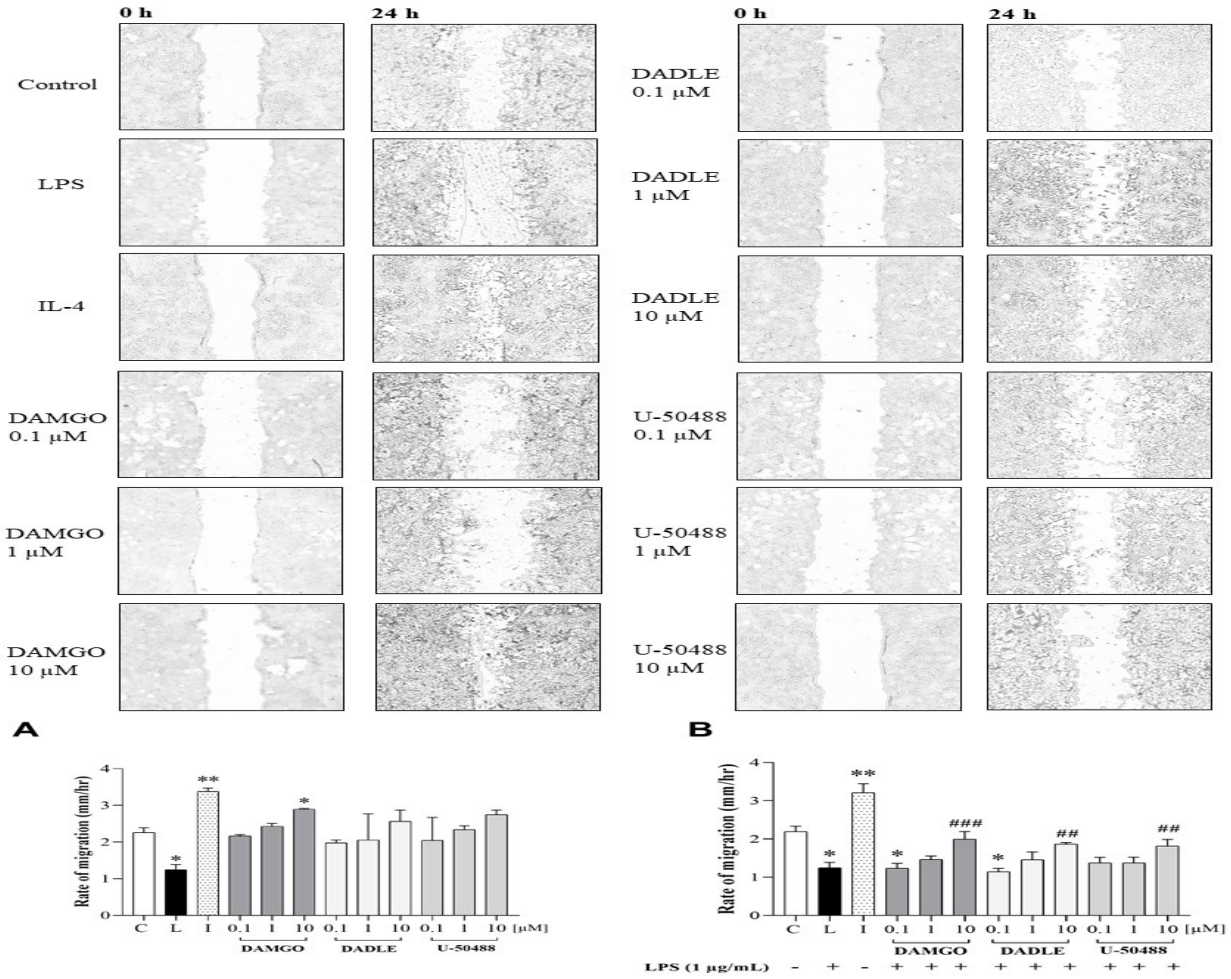

Figure 7. Effect of microglial activation on cell migration. C8-B4 microglial cells were untreated (control, C) or treated with LPS (1 μg/mL, L) or IL-4 (20 nM, I) or opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 24 h and migration into a scratch wound in a cell monolayer was determined after the next 24 h. Typical photographs of scratch wounds (A) and graphs showing the changes in migration rate when treated with different compounds alone (B) and the changes in cells pretreated with opioids before the addition of LPS. Scale bars, 10 mm. For each cell culture, images were taken of three random fields along the edge of the scratch, and the distance between the scratches was measured. Data are representative of three independent experiments and are reported as mean ± SEM (*$p < 0.05$, **$p < 0.01$ compared with control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS-treated group).

To explore the potential impact of opioid ligands on the movement of microglial cells, we conducted a scratch wound migration assay using C8-B4 cells treated with DAMGO, DADLE, and U-50488. Additionally, we carried out experiments to assess the effects of LPS and IL-4, and the results are depicted in Figure 7. Notably, the presence of LPS led to a notable reduction in the migratory ability of C8-B4 cells, resulting in approximately a 50% decrease. In contrast, IL-4 had a strikingly opposite effect, increasing migration nearly threefold. Interestingly, when the highest concentration of DAMGO (10 μM) was applied, it significantly boosted the rate of microglial migration. Conversely, the other two opioid agonists, DADLE and U-50488, did not induce any observable changes in microglial migration. Intriguingly, when all three opioid agonists were simultaneously present at the highest concentration (10 μM), they effectively counteracted the negative impact of LPS on the rate of microglial migration.

5.4. Opioid agonists inhibit nitric oxide production in microglial cells

Inflammation occurring within the central nervous system (CNS) often coincides with an increased generation of nitric oxide (NO) by microglia, a phenomenon that can contribute to oxidative damage and ultimately lead to cell death. In this study, we utilized the Griess reagent to measure the levels of nitrate, a stable byproduct of NO degradation. NO production was evaluated in two scenarios: first, in C8-B4 cells treated separately with LPS and opioid agonists, and second, in cells exposed to LPS for 72 hours. Surprisingly, our observations indicated that DAMGO, DADLE, and U-50488, across all tested concentrations, had no detectable impact on NO production. Upon stimulating C8-B4 cells with LPS, we noted a substantial and approximately tenfold increase in NO production. Interestingly, this LPS-induced rise in NO levels was dose-dependently suppressed by all three opioid agonists (see Figure 8). No significant NO production was observed after 24 hours. Collectively, these findings suggest that the activation of opioid receptors has the potential to modulate NO production.

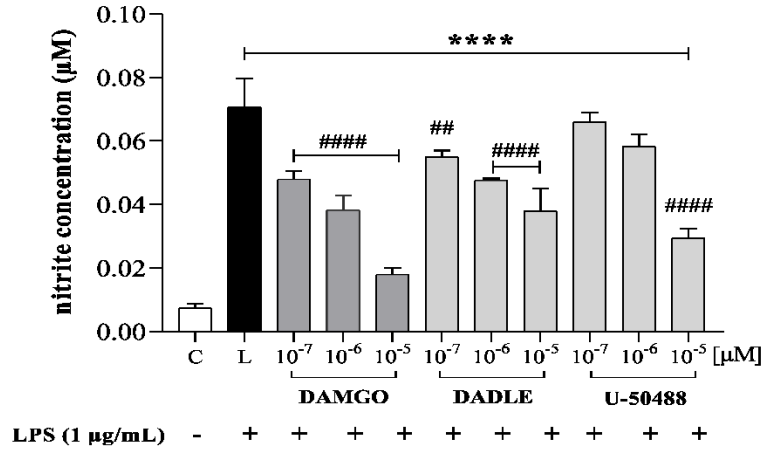

Figure 8. Effect of microglial activation on nitric oxide production. C8-B4 microglial cells were incubated with opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 72 hours in both the absence (A) and presence (B) of LPS (1 µg/mL, L) before determining the nitrite concentration in the cell culture supernatant with the Griess reagent. Cells were pretreated with opioids before addition of LPS. Data are presented as mean ± SEM of three independent experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared with LPS-treated group).

5.5. Opioid receptor agonists influence phagocytic activity of microglial cells

The ongoing debate revolves around the connection between the phagocytic capability of microglial cells and their polarization into M1 or M2 states. To investigate this question, we exposed microglial cells to treatments involving DAMGO, DADLE, or U-50488, both in the presence and absence of LPS. In certain cases, cells were also concurrently exposed to IL-4, a known promoter of the M2 state, or cytochalasin D, a potent inhibitor of phagocytosis. Naloxone, acting as an opioid receptor antagonist, and norepinephrine, employed as a positive control, were also incorporated into the treatments over a 24-hour period. To visualize the process of phagocytosis, we provided C8-B4 cells with zymosan particles pre-labeled with a red dye. The assessment of microglial phagocytic activity was not limited to spectrophotometric measurements. While the activation of C8-B4 microglia with LPS significantly enhanced phagocytosis, increasing it by approximately 40%, treatment with IL-4 reduced their phagocytic activity by roughly 50%. It is important to note that DAMGO, DADLE, U-50488, and naloxone did not manifest any discernible effect on phagocytosis (see Figure 9A). Interestingly, opioid agonists displayed a dose-dependent inhibition of LPS-induced microglial phagocytosis (see Figure 9B). It's worth

mentioning that no significant alterations in phagocytosis were observed after a 72-hour treatment compared to the 24-hour treatment duration. Consequently, these results suggest that the opioid receptor-dependent modulation of microglia has the capacity to redirect activated cells towards an anti-inflammatory polarization state.

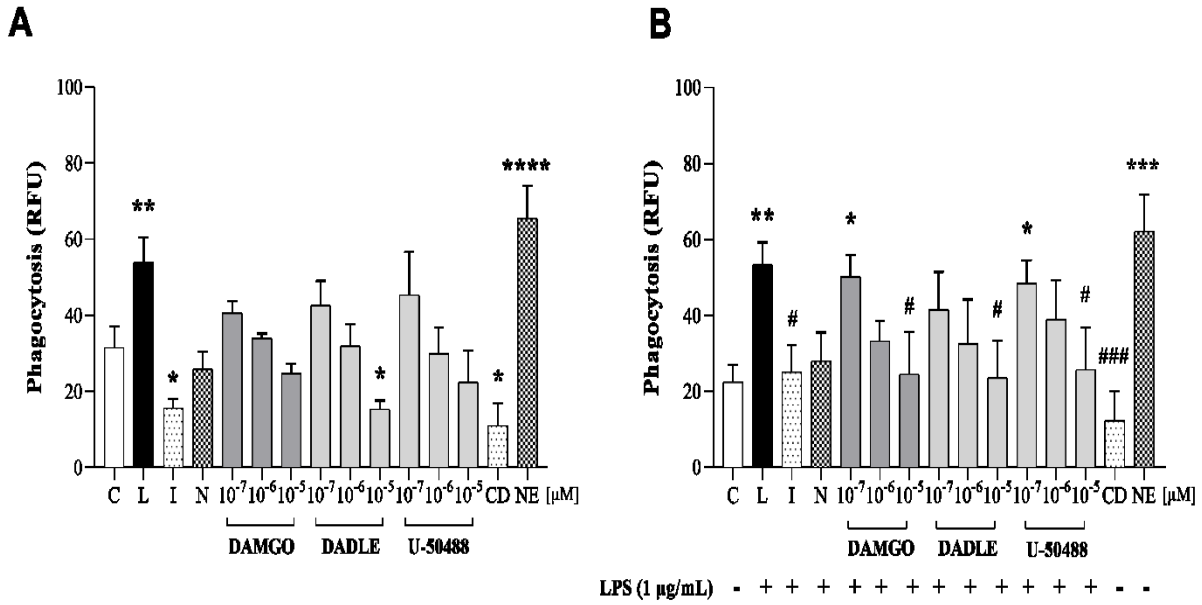

Figure 9. Effect of microglial activation on phagocytosis. C8-B4 microglial cells were incubated with opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 24 hours both in the absence (A) or presence (B) of LPS (1 µg/mL, L). C8-B4 microglial cells were treated with LPS (1 µg/ mL, L) or IL-4 (20 nM, I) or naloxone (1 µM, N) or cytochalasin D (10 µM; CD) or norepinephrine (1 µM, NE) or opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 24 h before determination of phagocytosis. Data are presented as mean ± SEM of three independent experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared with LPS-treated group).

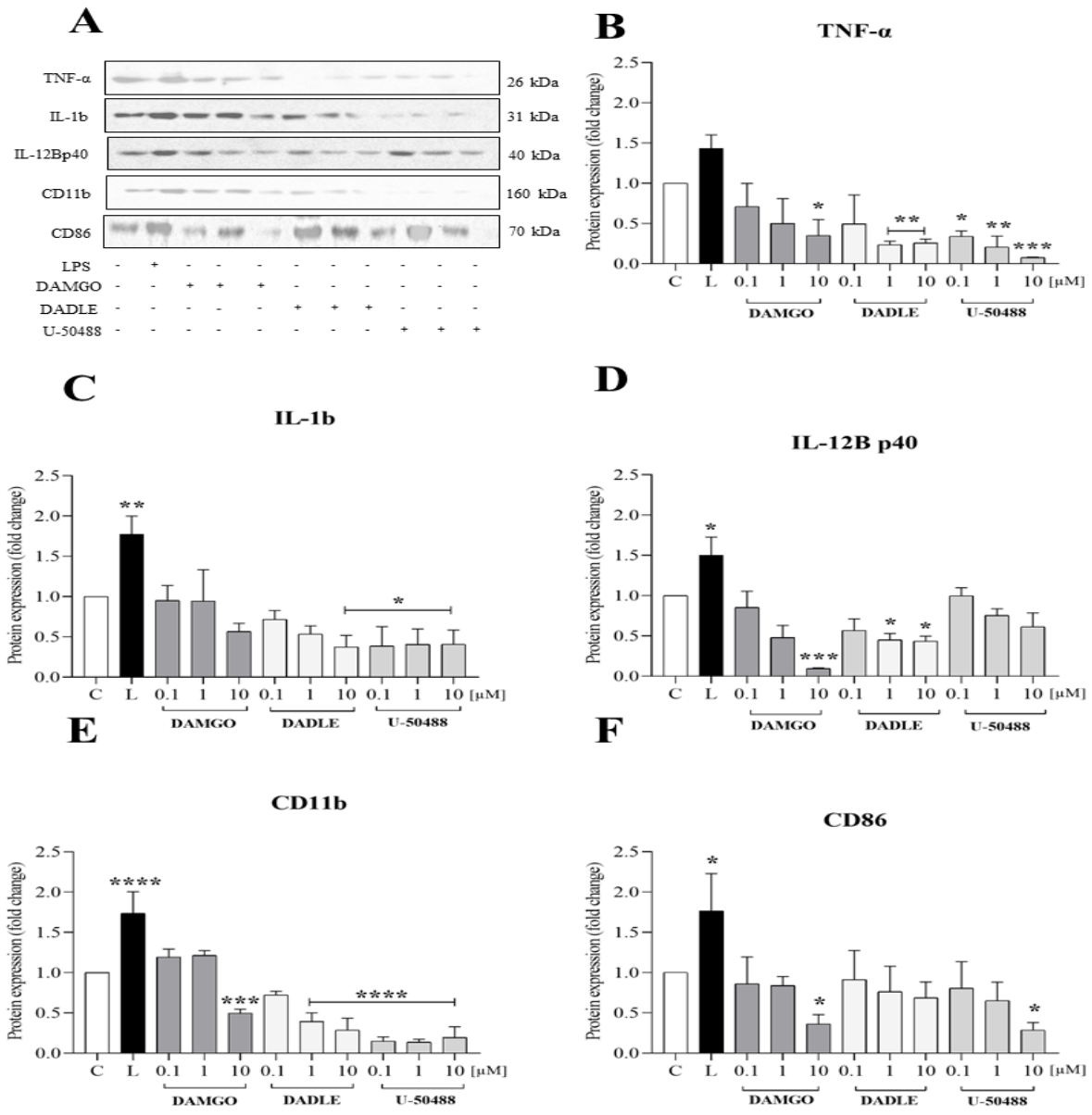

Figure 10. Effect of LPS and opioid agonists on the expression of selected pro-inflammatory markers. C8-B4 microglia were treated for 24 hours with LPS (1 μg/mL, L) or opioid ligands (DAMGO, DADLE, U-50488) at various concentrations. The levels of TNF-α, IL-1b, IL-12b, CD-11b, and CD-86 were determined by Western blot analysis (A) and the relative levels of TNF-α (B), IL-1b (C), IL-12b p40 (D), Cd-11b (E) and CD-86 (F) were expressed as fold changes compared with control. Data are reported as means ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with control).

5.6. Opioid agonists strongly affect expression of pro- and anti-inflammatory markers

When C8-B4 cells were stimulated with LPS, there was an increase in the expression of pro-inflammatory cytokines such as IL-1B and IL-6, along with pro-inflammatory markers CD11b and CD86 (as shown in Fig. 10). TNF-α exhibited a tendency to increase but was not as pronounced. In contrast, the levels of anti-inflammatory markers in LPS-treated cells remained mostly unchanged, except for IL-4. Subsequently, we examined the impact of opioid agonists. Opioid agonists DAMGO, DADLE, and U-50488 had minimal influence on microglial polarization but significantly suppressed the expression of all tested markers in a dose-dependent manner (Figure 10). Treatment of C8-B4 cells resulted in a notable increase in arginase 1 (Arg1) and the mannose receptor CD206. Additionally, the levels of most tested anti-inflammatory cytokines (IL-4, IL-10, and IL-13) were elevated when C8-B4 cells were treated with opioid agonists (as shown in Figure 12). Consequently, opioids demonstrated the ability to inhibit the production of pro-inflammatory cytokines and appeared to stabilize the M2 phenotype of microglia. In the subsequent series of experiments, we aimed to determine whether opioid agonists could mitigate the effects of LPS. We assessed the levels of the inflammatory interleukin IL-6 and COX-2 in C8-B4 cells pre-treated with DAMGO, DADLE, or U-50488 at various concentrations one hour prior to the addition of LPS. None of these ligands had an impact on the levels of IL-6 and COX-2 under normal conditions. Importantly, we observed that DADLE or U-50488 could suppress the LPS-induced upregulation of IL-6 and COX-2 (Figure 11).

5.7. Inhibition of TLR4/NF-κB and promoting TREM2 activation is required for the switching M2 microglial polarization by opioid receptors

To elucidate molecular mechanism driving the activation of microglia by opioid receptors, we investigated the levels of key regulatory elements, including toll-like receptor 4 (TLR4), NF-κB, and TREM2, in C8-B4 cells treated with LPS and specific OR agonists. Our findings point towards the potential involvement of these signaling molecules in the modulation of microglial M1/M2 polarization. Through Western blot analysis, we established that treating C8-B4 cells with either LPS or opioid agonists did not exert a significant impact on the expression of TLR4 and NF-κB. Conversely, LPS notably enhanced the phosphorylation of NF-κB and concurrently suppressed the expression of TREM2. Importantly, blocking TLR4 signaling with TAK242, a TLR4 antagonist, did not yield a substantial effect on microglial activation induced by OR ligands. Pre-treatment of

microglia with opioid agonists effectively counteracted both the LPS-induced upregulation of NF-κB and the downregulation of TREM2 (Figure 13). Furthermore, we observed that the downregulation of TREM2 was mediated by the TRAM/Stat6 signaling pathway. To unravel the underlying molecular mechanism of microglial activation by opioid receptors, we assessed the levels of key regulators such as toll-like receptor 4 (TLR4), NF-κB, and TREM2 in C8-B4 cells treated with LPS and specific opioid receptor agonists. Our findings suggest that these signaling molecules might play a role in modulating microglial M1/M2 polarization. Western blot analysis revealed that the treatment of C8-B4 cells with either LPS or opioid agonists did not significantly alter the expression of TLR4 and NF-κB (as shown in Figure 13). Conversely, LPS notably increased the phosphorylation of NF-κB and suppressed the expression of TREM2. Blocking TLR4 signaling by TAK242, TLR4 antagonist had no significant effect on microglia activation through OR ligands. Pretreatment of microglia with opioid agonists prevented both LPS-induced upregulation of NF-κB and downregulation of TREM2 (Figure 13A, B, C, D). We observed downregulation of TREM2 mediated by TRAM/Stat6 signaling pathway.

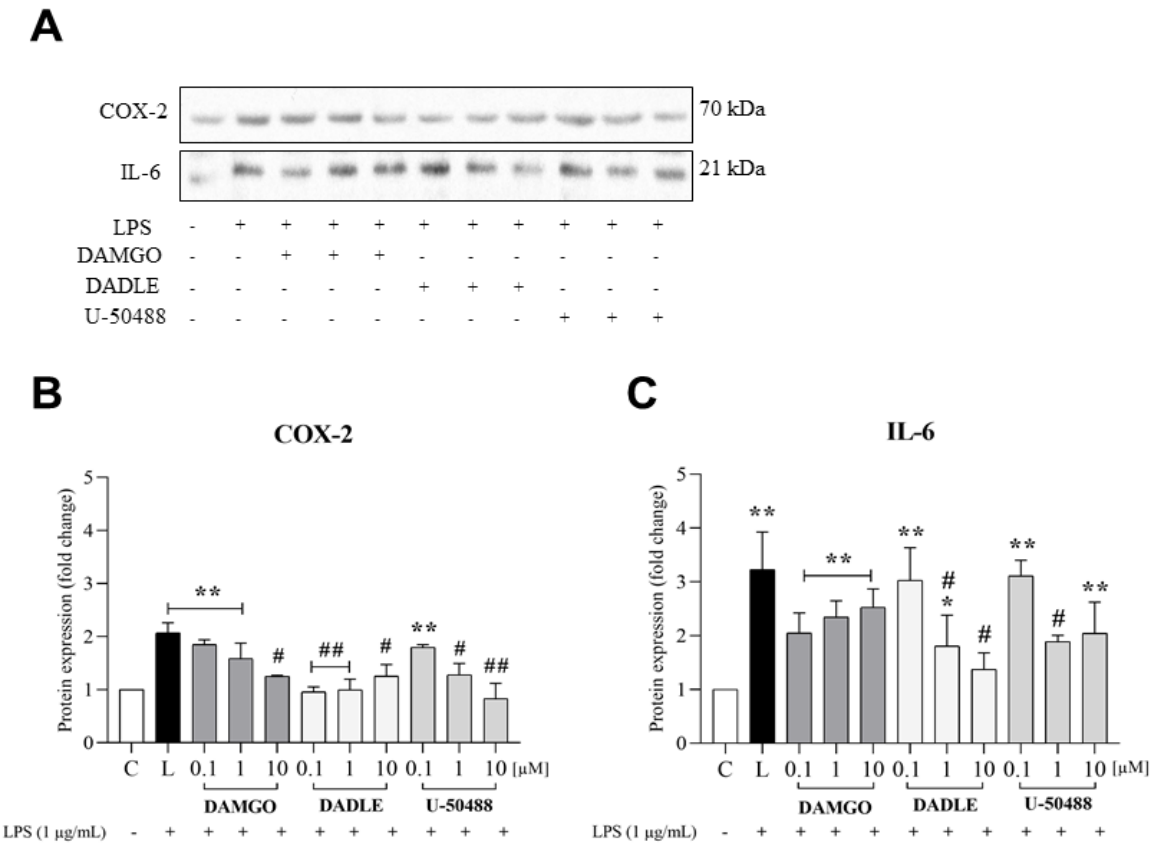

Figure 11. Effect of opioid agonists on LPS-induced activation of microglia. C8-B4 microglia were treated for 24 hours with LPS (1 μg/mL, L) in the absence or presence of opioid ligands (DAMGO, DADLE, U-50488) at various concentrations. The levels of IL-6 and COX2 were determined by Western blot analysis (A), and the relative levels of IL-6 (B) and COX2 (C) were expressed as fold changes compared with control. Data are reported as means ± SEM of three independent experiments (**$p < 0.01$ compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with LPS-treated group).

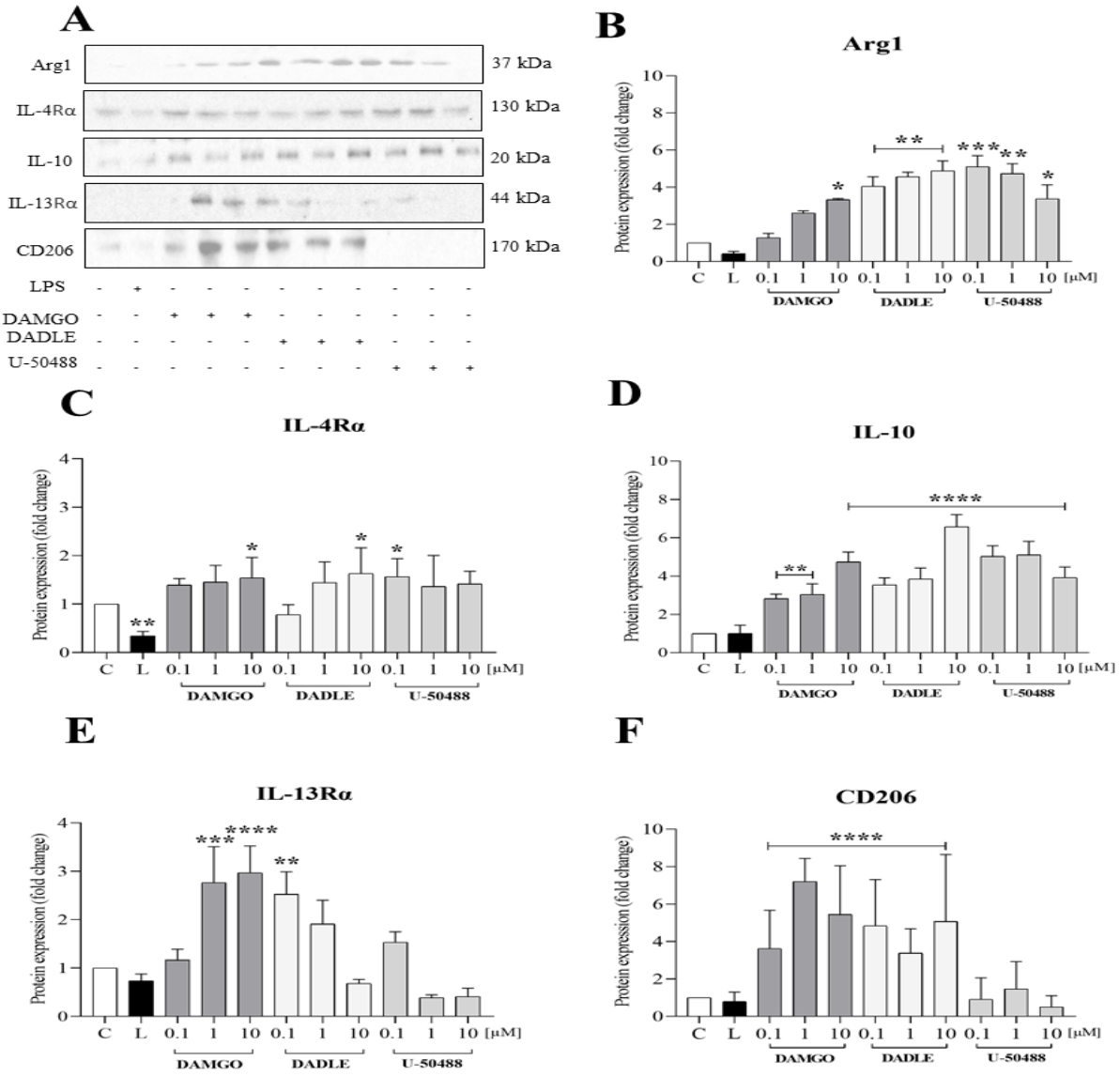

Figure 12. Effect of LPS and opioid agonists on the expression of selected anti-inflammatory markers. C8-B4 microglia were treated with LPS (1 μg/mL, L) or opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 24 hours. The levels of Arg1, IL-4Ra, IL-10, Il-13Ra2 and CD-206 were determined by Western blot analysis (A) and the relative levels of Arg1 (B), IL-4Ra (C), IL-10 (D), Il-13Ra2 (E) and CD-206 (F) were expressed as fold changes compared with control. Data are reported as mean ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with control).

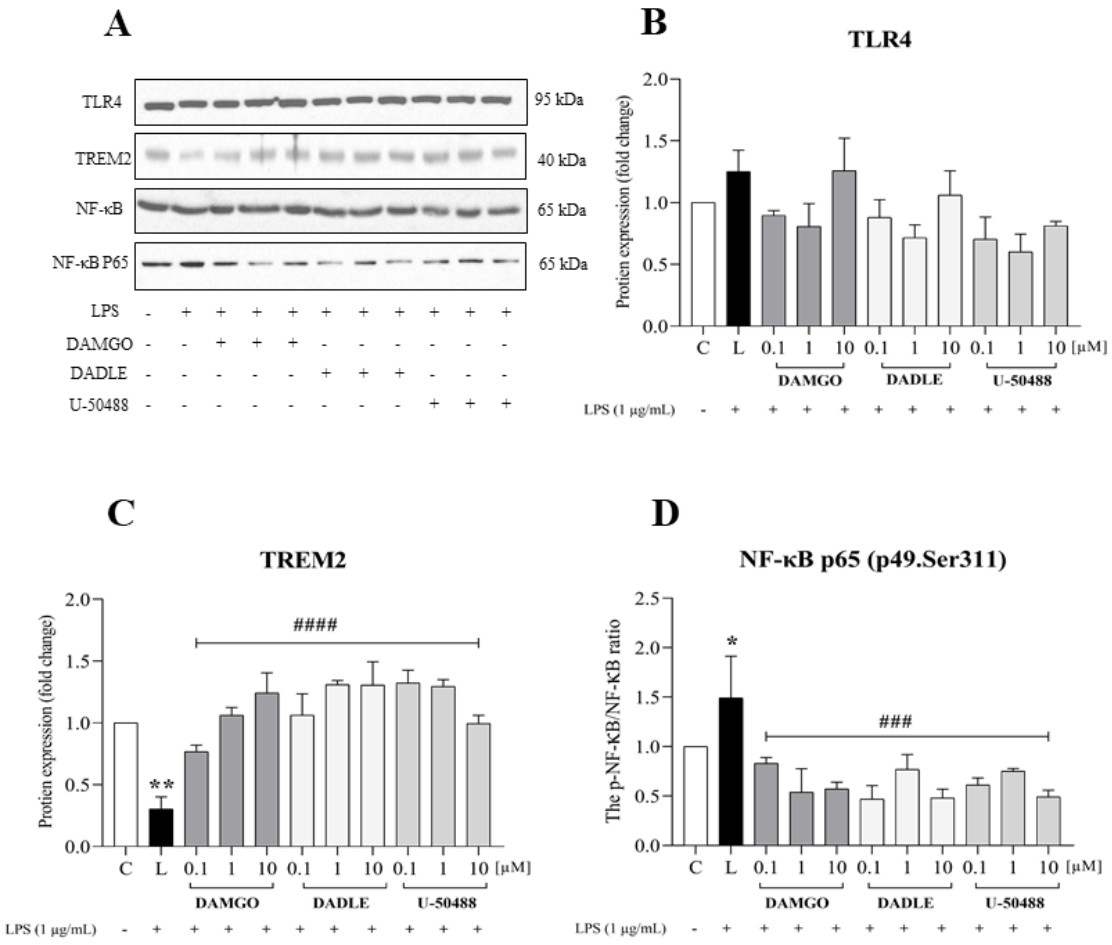

Figure 13. Effects of LPS and opioid agonists on activation of TLR4, NF-κB and TREM2 in microglia. C8-B4 microglia were treated for 24 hours with LPS (1 μg/mL, L) in the absence or presence of opioid ligands (DAMGO, DADLE, U-50488) at various concentrations. The levels of TLR4, TREM2, NF-κB p-65 and p-NF-κB p-65 were determined by Western blot analysis (A) and the relative levels of TLR4 (B), TREM2 (C) and p-NF-κB p-65 (D) were expressed as fold changes compared with control. Data are reported as means ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$ compared with control; $^{###}p < 0.001$, $^{####}p < 0.0001$ compared with LPS-treated group).

5.8. Opioid agonists prevent the LPS-induced increase in glucose uptake and downregulate GLUTs

Some previous studies suggest that glucose may contribute to inflammatory events in microglial cells (Wang et al., 2015; Xiang et al., 2021) Here, we investigated whether DAMGO, DADLE and U-50488 selected opioid ligands can affect the rate of glucose transport in LPS-stimulated microglial cells. To this end, we measured the uptake of 2-deoxyglucose (2-DG). We observed that treatment of cells with LPS led to a 2-fold increase in uptake of 2-DG (Figure 14C). Notably, pretreatment of cells with opioids suppressed the uptake of 2-DG in a concentration-dependent manner. Specifically, LPS-stimulated uptake of 2-DG decreased by approximately 40% in cells pretreated with 1 μM DAMGO, DADLE, or U-50488 (Figure 14A). We also wanted to determine which glucose transporters (GLUTs) are involved in glucose uptake in C8-B4 microglia cells. Therefore, we examined the expression of different GLUTs in C8-B4 microglia by Western blot analysis (Figure 14A), which revealed the presence of GLUT-1, 2, and 4 in these cells. To investigate the relationship between glucose metabolism and microglial activation, we stimulated microglial cells with LPS and OR agonists and LPS and then determined the expression levels of GLUTs. We found that GLUT-1 increased significantly in LPS-treated cells compared with control cells, whereas GLUT-2 and GLUT-4 were unaffected by LPS or opioid treatment (Figure 14B). We also found that DAMGO (0.1 and 1 μM) and DADLE (0.1 μM) downregulated LPS-induced GLUT-1 expression, whereas GLUT-1 was unaffected by U-50488 treatment (Figure 14C).

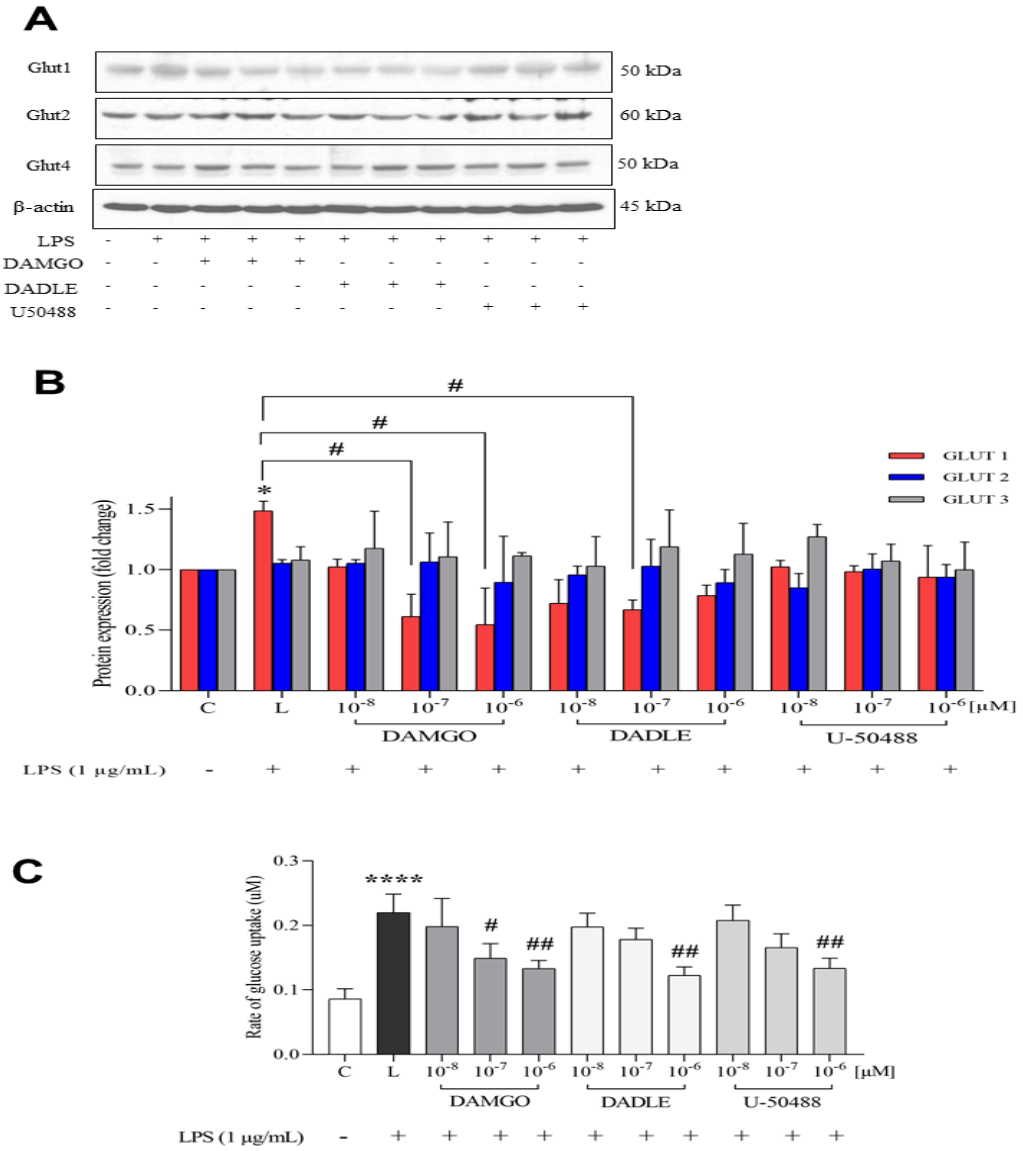

Figure 14. Effect of LPS and opioid ligands on glucose uptake and GLUTs expression. C8-B4 cells were either untreated (control, C) or pretreated with increasing concentrations (0.01–1 μM) of DAMGO, DADLE, and U-50488 for 1 hour and then incubated in the presence of LPS (1 μg/mL, L) for 24 hours. Glucose uptake (**A**) was measured using the Glucose Uptake-Glo Assay (Promega). Expression of GLUTs was determined by Western blot analysis (**B**), and relative GLUT1 levels were expressed as fold changes compared with control (**C**). Values represent the mean ± SEM of three independent experiments (* $p < 0.05$, **** $p < 0.0001$, compared with the control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with LPS-treated group).

5.9. Opioid agonists inhibit the LPS-induced inflammatory response by increasing antioxidant capacity, preventing the increase in NADPH production, and promoting GSH upregulation

Because chronic inflammation may be associated with oxidative stress, we examine the antioxidant activity of opioid ligands and monitored protein expression of antioxidant enzymes such as superoxide dismutase (SOD2, SOD3), catalase (CAT), and glutathione peroxidase (GPX 1/2) by western blot analysis (Figure 15A). We observed that selected opioids attenuated the accumulation of intracellular ROS and superoxide anions (O_2^-) associated with inflammation. Pretreatment with opioid ligands at concentrations of 0.1 and 1 μM significantly increased the level of glutathione peroxidase, catalase, and SOD (Figure 15B, C, D and E). As NADPH is essential for generating ROS through NADPH oxidase, and some glucose undergoes metabolism via the pentose monophosphate pathway to generate molecules that help regenerate NADPH from NADP+, we investigated whether the intracellular levels of NADPH were influenced by LPS and OR agonists. Our findings revealed that the treatment of microglial cells with LPS led to an increase of more than 40% in NADPH synthesis (as shown in Figure 16A), indicating that LPS promoted the production of NADPH. Conversely, the presence of all three opioids tested reduced NADPH production to control levels and even further (Figure 16A).

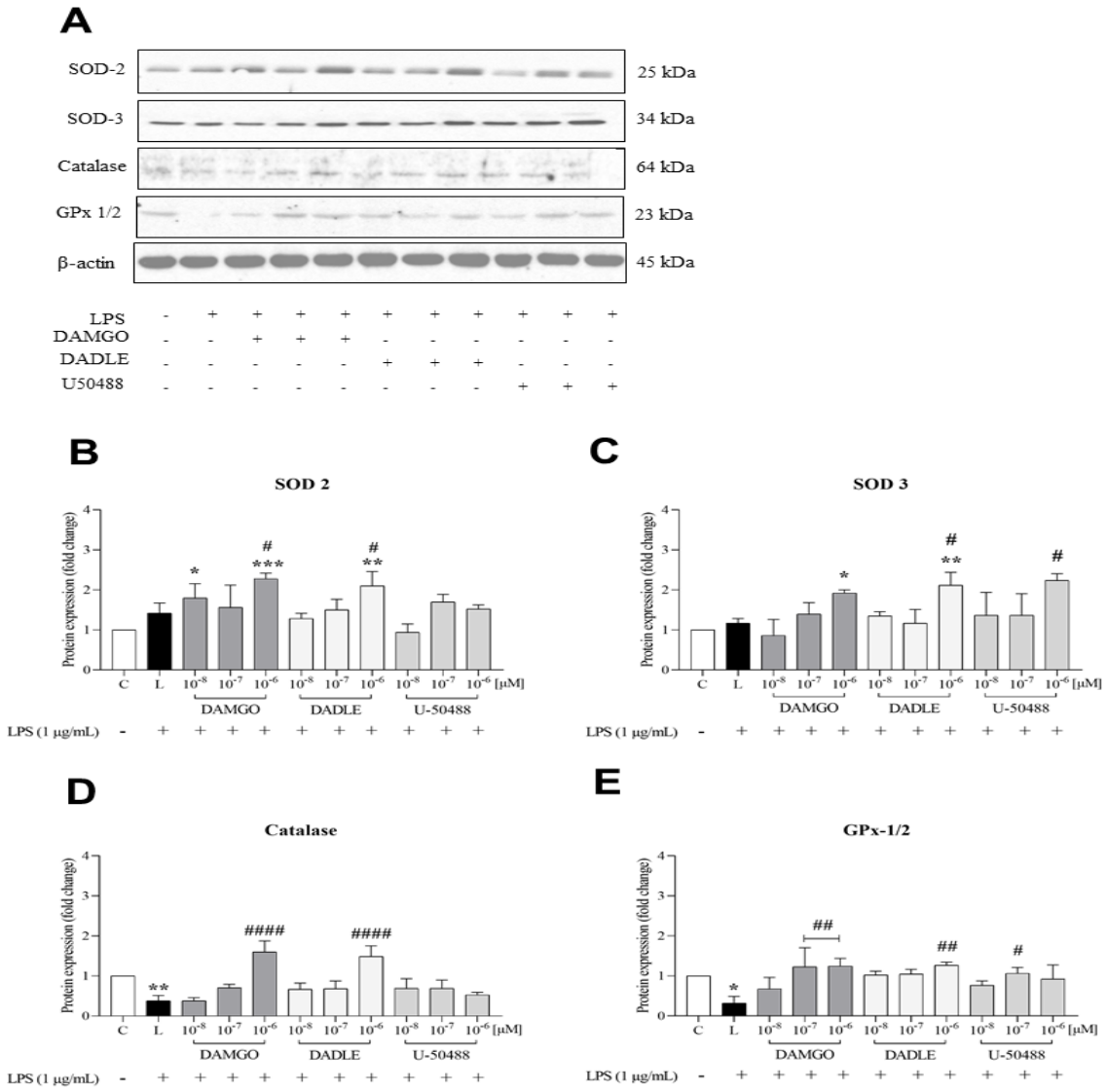

Figure 15. Effect of LPS and opioid receptor agonists on the expression of selected antioxidant markers. C8-B4 cells were either untreated (control, C) or pretreated with opioid ligands (DAMGO, DADLE, U-50488) at a concentration of 0.01–1 μM for 1 h and then incubated in the presence of LPS (1 μg/mL, L) for 24 h. The levels of selected antioxidant enzymes were determined by Western blot analysis (**A**), and the relative levels of SOD2 (**B**), SOD3 (**C**), catalase (**E**), and GPx 1/2 (**D**) were expressed as a fold change compared with the control. Values represent the mean ± SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ compared with LPS-treated group).

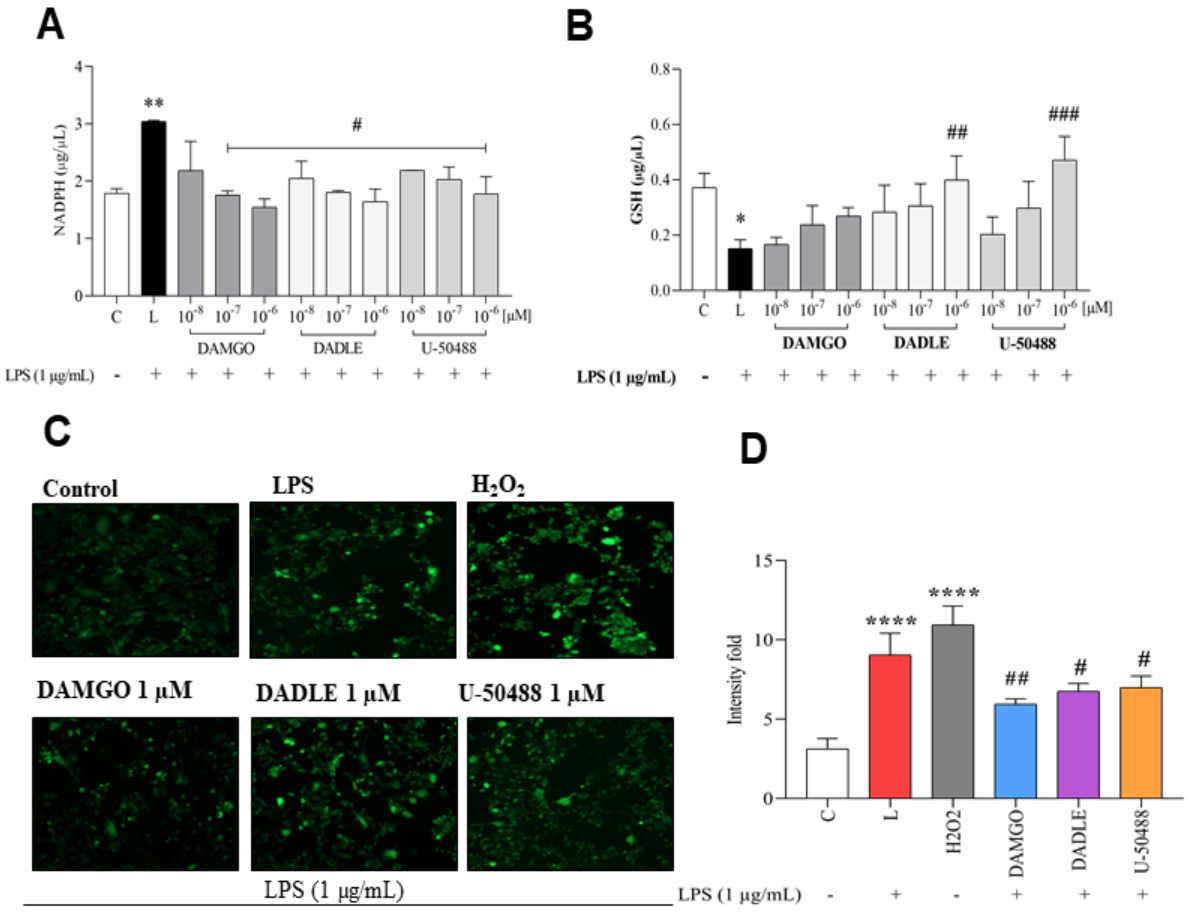

Figure 16. Effect of LPS and opioid ligands and LPS on NADPH and GSH levels and intracellular ROS production. C8-B4 cells were either untreated (control, C) or pretreated with opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 1 hour and then incubated in the presence of LPS (1 µg/mL, L) for 24 hours. When evaluating ROS, cells were treated with H_2O_2 (50 µM) as a positive control. The levels of NADPH (**A**) and GSH (**B**) were determined with appropriate kits as described in Methods. DCFDA staining was used to estimate intracellular ROS (**C**) and fluorescence intensity of each preparation was quantitatively evaluated using ImageJ (**D**). Values represent the mean ± SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$ compared with control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ compared with LPS-treated group).

It is well known that opioids have antioxidant capacity (Olga et al., 2015, Fu et al., 2017). Therefore, we decided to investigate the effects of opioid ligands on the level of the antioxidant

glutathione (GSH) in LPS-stimulated C8-B4 microglia. As shown in Figure 16B, GSH levels decreased by approximately 60% in PLS-treated cells and in contrast to DAMGO, DADLE and U-50488 were able to reverse this effect at the highest concentrations (1 μM).

5.10. Opioid agonists reverse LPS-induced dysregulation of cellular redox balance and bioenergetics

We examined the effect of LPS and opioid ligands on cellular redox balance. H2DCF-DA, a cell-permeable fluorescent probe, was used to measure intracellular ROS in C8-B4 cells (Figure 16C). As expected, the baseline value of intracellular reactive oxygen species was low in control (unstimulated) cells, and there was a significant increase after stimulation of the cells with 1 μg/mL LPS or 50 nM hydrogen peroxide (Figure 16D). Pretreatment of cells with selected opioid ligands significantly suppressed LPS-induced ROS production (Figure 16C). DCF fluorescence is capable of detecting total intracellular ROS levels without distinguishing their origin. To specifically assess mitochondrial ROS, we utilized MitoSOX Red, a fluorescence probe designed to target mitochondria, where it undergoes oxidation by mitochondrial ROS. Flow cytometry analysis with MitoSOX Red staining revealed that the treatment of cells with LPS led to a significant increase in MitoSOX Red fluorescence intensity, indicating a substantial production of ROS, approximately 3.5-fold higher. Significantly, the detrimental impact of LPS was mitigated in a dose-dependent manner by pre-treating cells with DAMGO, DADLE, or U-50488 (Figure 17A, B and C). Specifically, LPS-induced ROS production was reduced 1- and 2-fold in the presence of 0.01-1 μM DAMGO (Figure 17D) or DADLE (Figure 17E), respectively, and 2-fold in the presence of 0.1 and 1 μM U-50488 (Figure 17F).

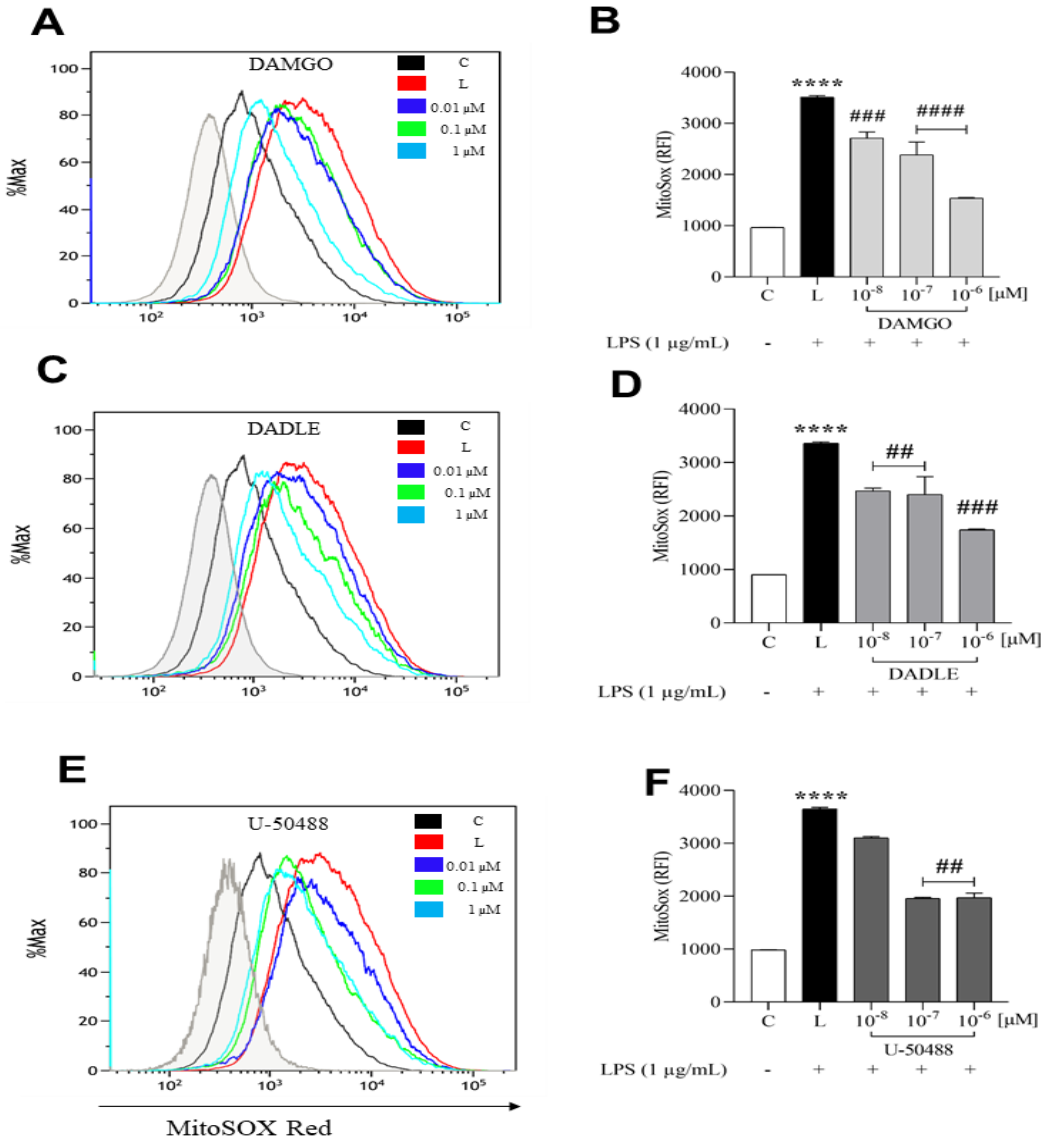

Figure 17. Effect of LPS and opioid ligands on mitochondrial ROS. C8-B4 cells were either untreated (control, C) or pretreated with opioid ligands (DAMGO, DADLE, U-50488) at various concentrations for 1 hour and then incubated in the presence of LPS (1 µg/mL, L) for 24 hours. Mitochondria ROS were assessed by flow cytometric analysis using the MitoSOX red probe. Representative flow cytometric histograms (**left panels**) and analyzes (**right panels**) of each experimental group: DAMGO (**A**,**B**), DADLE (**C**,**D**), and U-50488 (**E**,**F**). Values represent the median ± SEM of 3 independent experiments (**** $p <$ 0.0001 compared with control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ compared with LPS-treated group).

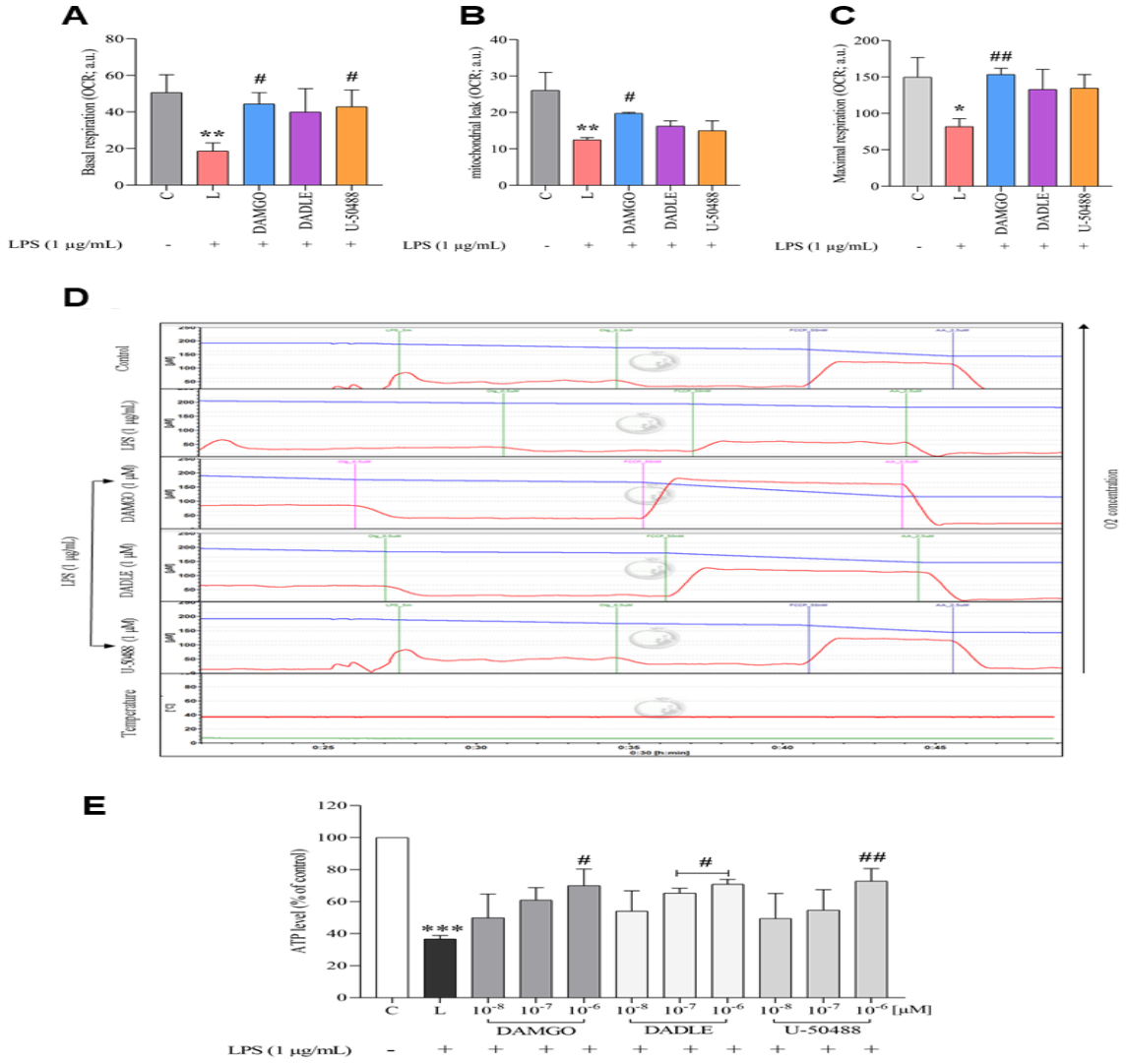

Figure 18. Effect of LPS and opioid receptor ligands on mitochondrial respiration and ATP content. C8-B4 cells were either untreated (control, C) or pretreated with opioid ligands (DAMGO, DADLE, U-50488) at a concentration of 1 µM for 1 h and then incubated in the presence of LPS (1 µg/mL, L) for 24 h. Mitochondrial respiration was assessed as extracellular oxygen consumption rate (OCR). OCR was measured under basal conditions (**A**) followed by sequential addition of oligomycin (0.5 µM) to determine mitochondrial leak (**B**), and FCCP (50 nM) and antimycin A (2.5 µM) to determine maximal respiration (**C**). oxygraphs (**D**) OCR values were normalized to a number of cells. Relative changes in ATP content (**E**) were determined using a bioluminescence kit. Values are reported as means ± SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared with LPS-treated group).

Subsequently, we explored the impact of LPS and opioids on the functional status of mitochondrial respiration in C8-B4 cells, employing an OROBOROS Oxygraph-2k apparatus (Figure 18D). This entailed real-time monitoring of extracellular oxygen consumption under various experimental conditions to assess mitochondrial respiration. The administration of LPS led to a significant reduction in both basal and maximal respiration (Figure 18A, B). Specifically, the oxygen consumption rate (OCR) related to leak-driven respiration notably declined upon exposure to 1 μg/mL LPS (Figure 18B), signifying a substantial impairment in the electron transport chain (ETC) functionality. However, when cells were pre-treated with DAMGO, DADLE, and U-50488 at a concentration of 1 μM, the ETC capacity remained intact, and basal mitochondrial respiration was almost completely restored. Additionally, we evaluated the effects of LPS and OR agonists on intracellular ATP levels (Figure 18E). The bioluminescence assay utilized for detecting intracellular ATP content revealed a decrease in ATP levels in C8-B4 cells following LPS treatment. Nevertheless, the decline in intracellular ATP induced by LPS was significantly ameliorated when the cells were pre-treated with opioid ligands.

5.11. Opioid agonists activate the Nrf2/HO-1 pathway in LPS-treated C8-B4 cells

Recent studies have shown that opioids can activate Nrf2, which in turn leads to increased transcriptional activation of HO-1 expression (Reymond et al., 2022). Previous research also suggested that morphine-induced anti-inflammatory effects can occur through Nrf2 and HO-1 activation (Matsuo et al., 2020). Here, we examined whether opioid receptors activation by DAMGO, DADLE, and U-50488 may increase the expression of Nrf2, and HO-1. Western blot analysis showed that after treatment with LPS, the expression of Nrf2 and HO-1 was downregulated, whereas pretreatment with DAMGO and U50488 upregulated Nrf2 and HO-1 (Figure 19). Interestingly, opioid treatment resulted in a dose-dependent upregulation of HO-1 expression, suggesting that OR agonists activate Nrf2 via HO-1 induction in LPS-stimulated C8-B4 cells.

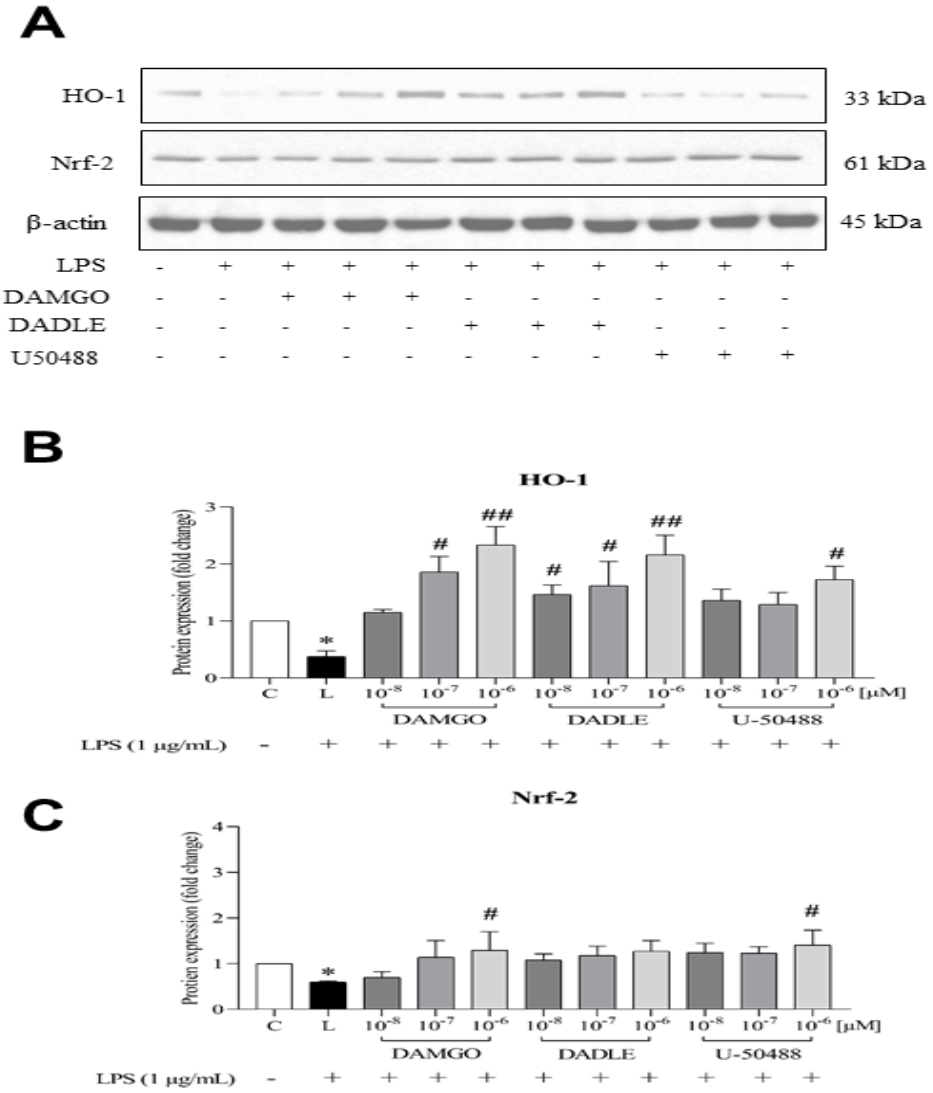

Figure 19. Effect of LPS and opioid receptor agonists on the expression of Nrf2 and HO-1. C8-B4 cells were either untreated (control, C) or pretreated for 1 hour with opioid ligands (DAMGO, DADLE, U-50488) at various concentrations and then incubated for 24 hours in the presence of LPS (1 μg/mL, L). Selected components of the Nrf2 pathway were determined by Western blot analysis (**A**). The relative levels of HO-1 (**B**) and Nrf2 (**C**) were expressed as fold changes compared with control. Values are reported means ± SEM of three independent experiments (* $p < 0.05$ compared with control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ compared with LPS-treated group).

6. Discussion

Microglia, the immune system's effector cells in the brain, play a critical role in scavenging dead cells under normal and pathological conditions, including neurodegenerative diseases. They influence brain regeneration and remodeling by releasing both neurotrophic and cytotoxic molecules (Nakajima and Kohsaka, 2001). Conversely, microglia-mediated neuroinflammation is associated with various brain disorders (Bachiller et al., 2018). Recent research has highlighted significant differences in the baseline expression levels of proinflammatory and other genes in microglial cell lines compared to in vivo studies. Furthermore, microglial cell lines derived from neonatal or embryonic central nervous system sources may not accurately reflect the phenotype of adult or elderly microglia. Despite these limitations, microglial cell lines are valuable for molecular, biochemical approaches, and high-throughput screening assays (Butovsky et al., 2014; Melief et al., 2016). Switching the polarization of microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype holds promise as a potential therapeutic strategy for mitigating certain CNS pathological conditions (Zhang et al., 2018). In our current study, we aimed to explore the potential role of μ-, δ-, and κ-opioid receptors in microglial polarization. Using the established LPS in vitro model, we discovered that opioid receptor agonists can exert potent anti-inflammatory and cytoprotective effects. These effects are mediated by the suppression of microglial M1 polarization and the promotion of M2 polarization. We also investigated the NF-κB signaling pathway as the mechanism through which opioids appear to regulate microglial activation. To characterize the states of microglial activation, we monitored the levels of specific molecular markers that reflect the pathophysiological state of microglia. These changes in microglial activation were accompanied by observable morphological alterations. Our experiments revealed that LPS treatment resulted in fewer process outgrowths in C8-B4 microglial cells, consistent with previous studies on LPS-activated microglia (Toulme et al., 2010). Conversely, treatment with IL-4 and μ-, δ-, and κ-opioid receptor agonists increased process outgrowths. Furthermore, our study clearly demonstrated that IL-4 and μ-, δ-, and κ-opioid receptor agonists directed microglial cells towards M1 and M2 polarization, respectively. LPS, a key component of the outer membrane of Gram-negative bacteria, serves as an inflammatory stimulus and can induce microglia to transition into the classical M1 activation state. In our study, we observed a substantial increase in nitrite production when C8-B4 microglial cells were

stimulated with LPS, an effect that was dose-dependently suppressed by opioid receptor agonists. Intriguingly, even at a concentration of 1 μM, μ- and κ-opioid receptor agonists were sufficient to inhibit LPS-induced NO formation. Nitric oxide (NO) is a highly reactive signaling molecule that plays a crucial role in mediating interactions between neurons and glial cells in the CNS (Contestabile, 2012). Excessive NO production can trigger an inflammatory response and contribute to neurodegenerative disorders characterized by neuronal cell death and glutamate-induced neurotoxicity (Yuste et al., 2015). Importantly, we observed that pretreatment of microglial cells with opioid agonists ameliorated LPS-induced cell injury. Additionally, our study unveiled, for the first time, the involvement of μ-, δ-, and κ-opioid receptors in modulating microglial phagocytosis. We noticed that the heightened microglial engulfment capacity induced by LPS was diminished in the presence of opioids.

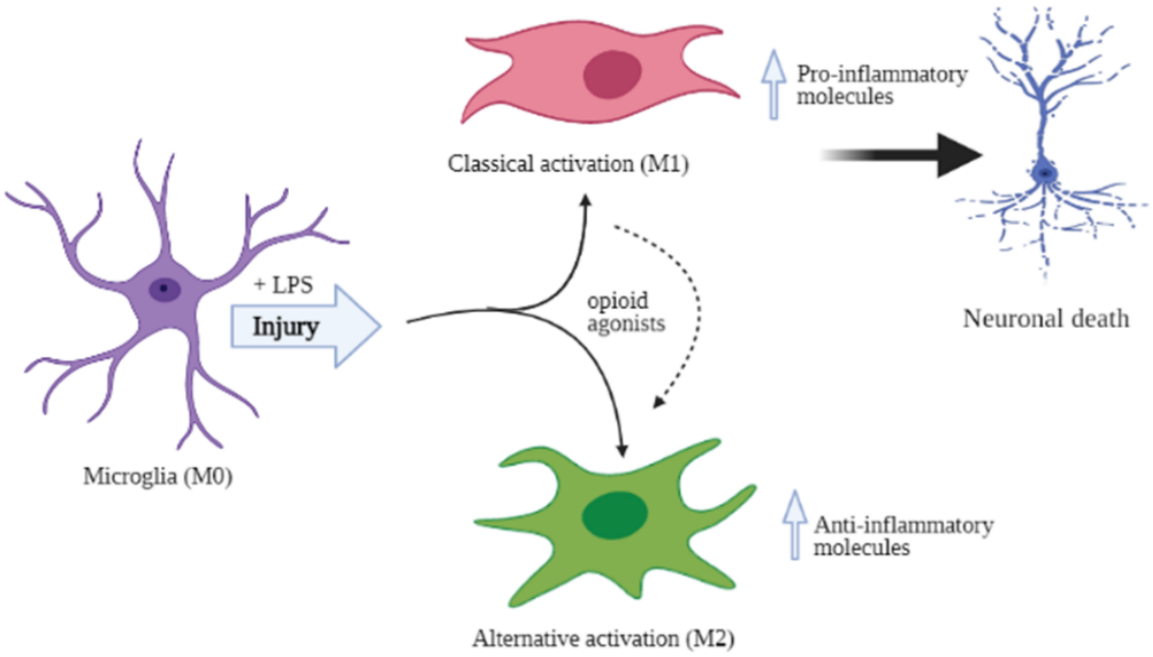

Figure 20. LPS induced M1 activation suppress by opioids agonists and promote M2 activation. Schematic representation of how opioid receptor agonists can reduce LPS-induced microglial M1 activation and inhibit neuronal death and promoting alternative activation.

These findings align with prior research examining the impact of morphine and DAMGO on phagocytosis by immune cells (Sowa et al., 1997). Other studies have also proposed that opioids might influence macrophage functions and modulate the expression of chemokine and cytokine receptors (Happel et al., 2008; Zeng et al., 2020). Regarding our scratch wound migration assay results, opioid agonists did not have significant effects on microglial migration. However, when applied at higher concentrations (10 μM), all tested ligands reversed the inhibitory influence of LPS on migration rates. Earlier investigations into the effect of LPS on cell migration have yielded conflicting results, suggesting that migration rates may be contingent on factors such as cell type, age, and species. For instance, (De Simone et al., 2010) observed impaired migration in newborn rats, while (Broderick et al., 2000) reported a similar effect in human microglia. On the contrary, (Maa et al., 2010; Tajima et al., 2008) found that LPS could enhance migration in rat macrophages and mouse peritoneal macrophages, respectively. In general, it is widely accepted that the activation of microglia is closely associated with cell migration and the transition to an M1 polarization state.

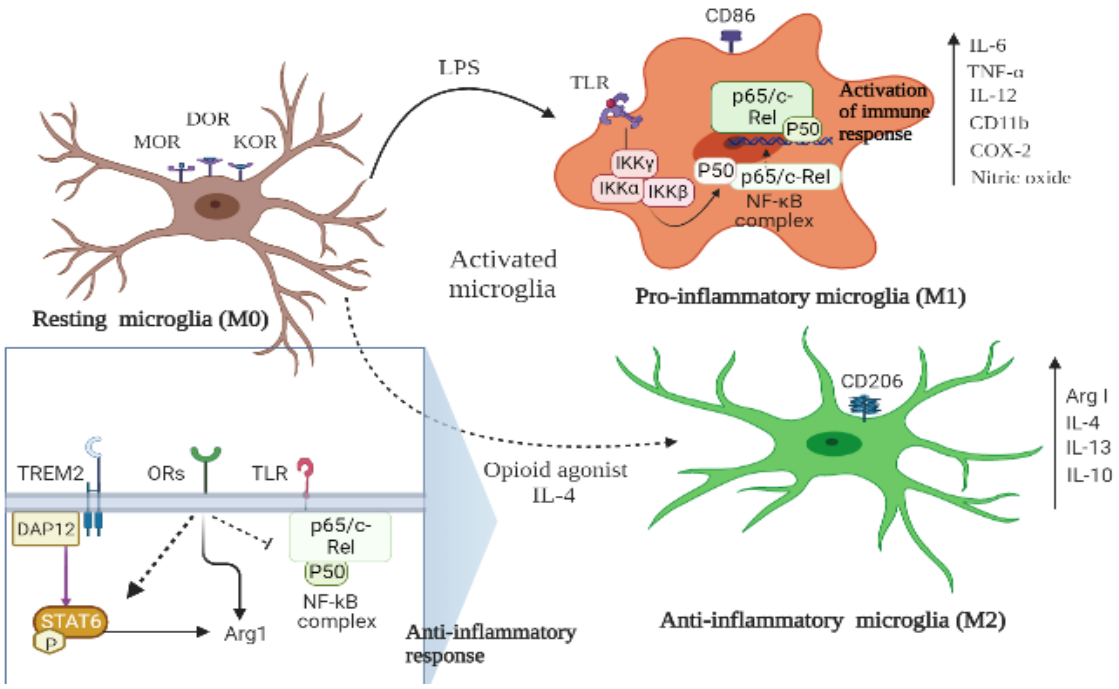

Figure 21. TREM2/STAT6 activation by opioid agonists. Illustration suggest activation of opioid receptors suppress pro-inflammatory state microglia and promote M2 polarization through NF-κB /TREM2 activation.

During the process of neuroinflammation, excessive phagocytic activity exhibited by microglia can potentially lead to the demise of viable neurons (Butler et al., 2021; Neher et al., 2011). Since that neuroinflammation is considered a central mechanism for neurodegenerative diseases, reducing phagocytosis as an anti-inflammatory response could offer potential benefits in such situations. However, it is worth noting that reduced phagocytosis could also represent immunosuppression. Interestingly, previous reports have indicated that morphine is capable of inducing immunosuppression within in the central nervous system (Ninković and Roy, 2013) At the center of the regulation of phagocytosis is TREM2, a crucial molecule in this process (Kobayashi et al., 2017). Therefore, we were interested to investigate the role of TREM2 in opioid-induced microglial M2 polarization. Our investigation revealed that opioid ligands suppressed LPS-induced phagocytosis in a dose-dependent manner. Additionally, we observed that TREM2 expression was downregulated in the pro-inflammatory state induced by LPS, whereas it was upregulated in C8-B4 cells treated with μ-, δ-, and κ-opioid receptor (OR) agonists. These findings suggest that activating the TREM2 pathway might serve as a promising strategy for alleviating neuroinflammatory diseases. The processes of phagocytosis and microglial engulfment are intricate, and opioids seem to play a role in modulating microglial phagocytosis, possibly through activation of TREM2. In parallel with the observed upregulation of TREM2, we detcted an increase in the levels of p-STAT6 following opioid treatment. This finding aligns with a recent study (Liu et al., 2020), which demonstrated that TREM2 can facilitate M2 polarization in microglia through the STAT6 pathway. Furthermore, previous studies have found that the expression of anti-inflammatory genes, including Arg1, critically depends on the phosphorylation of STAT6 (Gray et al., 2005). Intriguingly, the role of STAT6 in mediating the effects of the opioid agonist tramadol has already been demonstrated in macrophages (Zhang et al., 2018). However, it remains unclear whether opioid-induced TREM2 activation depends on ORs or operates independently. Additional studies are needed to further elucidate this aspect.

The M1/M2 model represents two distinct ends of the immune response spectrum, each representing a pro-inflammatory and an anti-inflammatory state (Orihuela et al., 2016; Ransohoff, 2016). This model is commonly employed to categorize macrophage responses and may also shed light on the function of microglia in the context of neuroinflammation. Activated microglia exhibit a dual nature (Konishi and Kiyama, 2018). The classically activated or M1 phenotype can be induced in vitro by exposure to substances such as LPS or lipoteichoic acid (LTA) and is

characterized by elevated expression of pro-inflammatory molecules such as IL-1β, IL-6, CD 86, COX-2, and iNOS. In contrast, IL-4 and betulinic acid have been shown to induce the M2 or alternative phenotype, which is characterized by increased expression of IL-4, IL-10, IL-13, and CD206 (Zhang et al., 2018). Notably, (Mizobuchi et al., 2020) recently reported that C8-B4 microglia can exhibit a mixed profile of pro- and anti-inflammatory molecules following repeated low-dose LPS stimulation. These microglia can dynamically switch between M1 and M2 polarizations, associated with neurotoxic and neurotrophic responses, respectively (Zhang et al., 2018). Consequently, a well-timed transition of microglia phenotype from M1 to M2 holds promise as a potential therapeutic approach for the treatment of neurodegenerative diseases. Recent studies have highlighted the beneficial effects of modulating the M1/M2 microglia states for central nervous system recovery by suppressing the detrimental consequences of inflammation while enhancing neuroprotective potential. For instance, betulinic acid treatment has been demonstrated to inhibit M1 microglia polarization and promote M2 polarization through CaMKKβ-dependent AMPK activation (Li et al., 2018). Other studies have shown that promoting M2 microglia polarization is associated with clearance of Aβ plaques in Alzheimer's disease and reduction of neurodegeneration in Parkinson's disease (Ma et al., 2020; Yang et al., 2017; Zhou et al., 2017).

In our current study, we observed that LPS-induced M1 polarization led to substantially elevated release of nitric oxide as well as the expression of proteins such as IL-1β, TNF-α, IL-6, CD11β, COX-2, and CD86. Conversely, treatment with DAMGO, DADLE, and U-50488 effectively reversed M1 polarization and markedly elevated the expression of microglial M2 markers, including IL-4, IL-10, IL-13, CD206, and Arg-1. It is worth noting that the microglial M2 activation state can be further categorized into four distinct subtypes based on their specific functions, namely M2a, M2b, M2c, and M2d/M2x. (Orihuela et al., 2016; Rőszer, 2015). In this context, we observed that exposure of cells to DADLE and U-50488 resulted in significant upregulation of Arg-1, IL-4, and IL-13, consistent with the features of M2a activation associated with suppression of nitric oxide (NO) production in neurodegenerative processes. Contrarily, DAMGO triggered the expression of IL-1β, CD11b, and CD206, indicative of an M2b, or type 2 M(Ic) activation state that plays a role in pathogen recognition and internalization of antigens. Additionally, U-50488 partially stimulated the expression of IL-10, suggesting involvement in the anti-inflammatory M2c and M2d activation states, which contribute to the reduction of the

inflammatory response and are associated with the repair mechanism. These findings indicate that different receptors may be involved in the fine regulation of microglial activation. Concurrently, opioid agonists mitigated the LPS-induced increase in the expression of COX-2, a crucial enzyme responsible for converting arachidonic acid into prostaglandins, which are implicated in numerous diseases, including inflammation and cancer. It is noteworthy that excessive COX-2 activation can potentially result in cognitive impairment and neuronal apoptosis (Rockwell et al., 2004). It is likely that inhibition of NO production and COX-2 expression by opioids can attenuate both neuroinflammation and neurodegeneration.

Here, we observed that OR agonists have the capacity to shift the polarization of LPS-activated C8-B4 microglia from an M1 phenotype to a predominant M2 phenotype, characterized by increased production of anti-inflammatory cytokines. Nevertheless, the precise molecular mechanism by which opioids induce alterations in microglial polarization is not yet entirely clear. Our findings suggest that the TL4/NF-κB/TREM-2 pathway may be implicated in LPS-induced M1 polarization of microglia and hat opioids may counteract activation of this pathway, facilitating the transition to the M2 phenotype. TLR4 receptors can be triggered by various stimuli, including LPS, LTA, and oxidative stress factors, eliciting specific cellular responses by initiating pro-inflammatory signaling pathways. TLR4 signaling plays a pivotal role in orchestrating the inflammatory response. The M1 phenotype of microglia induced by LPS was closely associated with activation of the TLR4/NF-κB pathway, and inhibition of this pathway by opioids resulted in M2 polarization. Numerous studies have investigated microglia activation and their polarization states, but the results are conflicting and information regarding the possible involvement of opioid receptors remains limited. It has been established that LPS can activate TLR4 receptors expressed on the surface of microglia. (Kielian, 2006; Lehnardt et al., 2003; Pan et al., 2017; Yao et al., 2013). Activation of TLR4 initiates a pro-inflammatory immune response by triggering the MAPK and NF-κB signaling pathways. Consequently, inhibition of TLR4/NF-κB signaling may be useful in mitigating inflammatory reactions (Gessi et al., 2016). In this context, it is important to highlight that β-arrestin2 can have a significant role in the potential beneficial effects of opioids. Feng and colleagues observed that activation of microglial β-arrestin2 by KOR effectively blocked LPS-induced phosphorylation of TGF-β-activated kinase 1, thereby preventing the expression of pro-inflammatory genes (Feng et al., 2014). Nevertheless, the cellular effects induced by opioids are likely to be multifaceted. In this study, we demonstrated that opioid agonists can exert robust anti-

inflammatory effects by inhibiting LPS-induced inflammation, nitric oxide production, enhancement of cell migration response, and upregulation of anti-inflammatory molecules in C8-B4 microglial cells, with minimal cell toxicity. These findings also suggest that the TREM2 pathway and STAT6 may be implicated in the anti-inflammatory response and phagocytic ability of microglia under these particular conditions. Next, our findings demonstrate that opioids can effectively inhibit inflammatory responses induced by LPS, by inhibiting, oxidative stress together with increased antioxidant enzymes expression via regulating Nrf2/Ho-1 pathway in C8-B4 microglia. We demonstrated under inflammatory conditions. GLUT 1 controls microglial states together with increased glucose uptake, NADPH content and reducing GSH level, these detrimental effects reversed by selected opioid ligands in dose dependent manner. These suggest that opioid ligands at lower concentration have ability to reduce cytotoxic effect during neuroinflammation.

Subsequently, we investigated glucose uptake in C8-B4 cells under different pathophysiological conditions. We observed that LPS treatment significantly increased glucose consumption, as indicated by the accumulation of 2-DG. In contrast, opioid ligands reduced the 2-DG uptake in LPS-treated cells, indicating that these ligands mitigated the inflammatory response by decreasing glucose uptake. Additionally, our investigations revealed that GLUT1-mediated glucose metabolism played a pivotal role in mitochondrial respiration in C8-B4 microglia. We identified three GLUTs in C8-B4 microglia cells, and intriguingly, GLUT1 exhibited the highest expression levels, which were further elevated following LPS stimulation. Preconditioning with opioids significantly reduced the heightened glucose uptake, suggesting a metabolic reprogramming of microglia towards anaerobic glycolysis (Sasaki et al., 2004; L. Wang et al., 2019; Xiang et al., 2021). Notably, the expression of other GLUTs remained unaffected by LPS and opioid treatments. Consequently, our findings suggest that GLUT1 plays a crucial role in regulating glucose uptake in microglial cells, particularly under inflammatory conditions. These data propose that opioids may offer neuroprotection by targeting GLUT1 in neuroinflammatory conditions. Our results indicate that during inflammation, microglia increase GLUT1 expression to enhance glucose uptake. Opioid treatment, by blocking GLUT1-mediated glucose uptake in inflammatory microglia, led to further metabolic reprogramming towards oxidative phosphorylation (OXPHOS). It's worth mentioning that opioids did not entirely inhibit glucose uptake, even at higher concentrations. For instance, while 1 μM DAMGO, DADLE, and U-50488 reduced glucose uptake

by 1.5-fold in C8-B4 microglia, 0.1 μM DAMGO reduced it by one-fold. These outcomes suggest that aside from GLUT1, other GLUTs also contribute to glucose uptake by microglia. This might explain why microglia did not undergo cell death following opioid treatment, as observed in certain tumor cells (Xintaropoulou et al., 2015). Our results indicate that opioids reduce LPS-induced NADPH production as a mechanism to exert their anti-inflammatory effects. Prior research employing the BV-2 microglial cell line has indicated that the transition from oxidative phosphorylation to aerobic glycolysis is associated with microglial polarization towards the activated state (Orihuela et al., 2016). In accordance with our oxygen consumption rate (OCR) data, we observed a substantial reduction in intracellular ATP levels in LPS-activated microglial cells. Notably, opioids alone had no impact on ATP production, suggesting their operation through interference with a glucose-independent mechanism. Glucose-6-phosphate can be channeled into the pentose phosphate pathway, generating nucleotide precursors and replenishing NADPH, a vital substrate for NADPH oxidase (Huang et al., 2022, 2014; Pavlou et al., 2017). We noted an increase in NADPH synthesis in LPS-treated microglial cells, signifying that this pathway was activated under inflammatory conditions at the expense of ATP production through aerobic glycolysis. Intriguingly, opioids significantly thwarted the rise in NADPH synthesis induced by LPS treatment, suggesting that opioids' anti-inflammatory action might also rely on this suppressive effect. Other treatments that counteract LPS-induced inflammation in microglia also efficiently reduce NADPH synthesis, indicating that opioids' inhibitory effects on glucose consumption and NADPH synthesis reinforce its intrinsic antioxidant potential in a mutually reinforcing process. This reinforcing effect may become particularly pronounced at low micromolar concentrations of opioid ligands, which possess moderate intrinsic antioxidant properties but robust anti-inflammatory effects.

In numerous neurodegenerative diseases, chronic inflammation is a typical feature that is commonly believed to be initiated by oxidative stress. Research indicates that the persistent activation of opioid receptors can mitigate the accumulation of intracellular ROS, ultimately averting oxidative stress (Mittal et al., 2014). Our study revealed protective effect of selected opioid ligands by inhibiting ROS production in microglia stimulated with LPS, through SOD-2. However, LPS can also increase reactive oxygen species production, which can cause cell death by inhibiting ATP generation from mitochondria (Fu et al., 2021). Our findings from the present study demonstrate that selected ORs agonist effectively reduce the production of intracellular and

mitochondrial ROS in activated C8-B4 cells. Specifically, exposing C8-B4 microglial cells to LPS (1 μg/mL) for 24 hours resulted in cell activation and a subsequent pro-inflammatory response with increased ROS production.

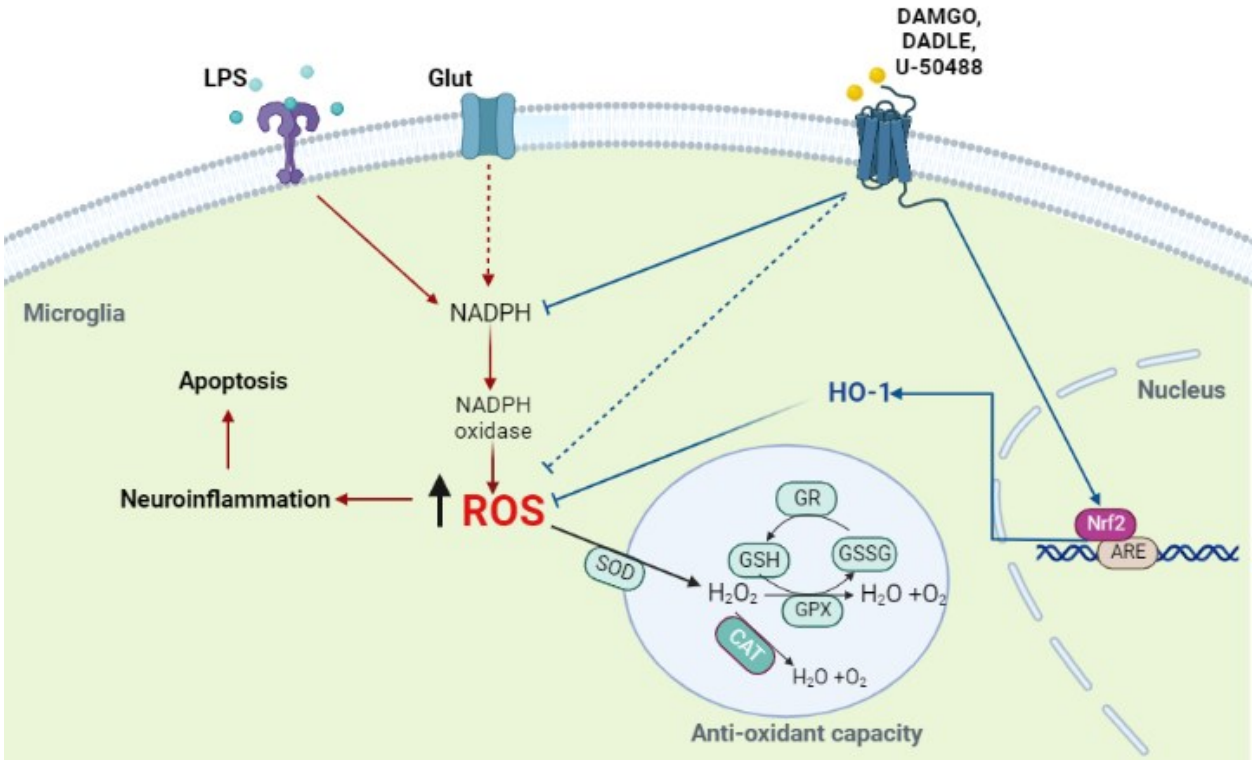

Figure 22. Opioid receptor agonists attenuate LPS-induced neuroinflammation by preventing the production of ROS via the Nrf2/HO-1 pathway—schematic representation of how opioid receptor agonists can reduce LPS-induced microglial inflammation. After entering the nucleus, Nrf2 binds to the antioxidant response element (ARE) and promotes the transcription of a number of antioxidants ARE–dependent genes, including HO-1. As a result, antioxidant capacity increases, intracellular ROS are reduced, and the microglia-mediated inflammatory response could be attenuated. Opioid receptor agonists could prevent microglial apoptosis by maintaining mitochondrial function while blocking excessive glucose uptake, NADPH loss, and ATP depletion.

Treatment of LPS-activated cells with opioids significantly diminished the production of superoxide, a potent mediator of brain injury during inflammation, and reduced oxidative damage to cell membranes. Excessive production of reactive oxygen species (ROS) can lead to lipid peroxidation, which is particularly detrimental to cells in the central nervous system (CNS) due to

their high content of polyunsaturated fatty acids (Halliwell, 2006). In comparison to control cells, activated microglial cells generated markedly higher levels of superoxide (3.5-fold increase). However, when LPS-activated cells were treated with opioid ligands, there was a substantial reduction (2-fold decrease) in superoxide production in a dose-dependent manner. Specifically, most tested concentrations of DAMGO (0.01 and 0.1 μM, $p < 0.01$; 1 μM, $p < 0.001$), DADLE (0.01 and 0.1 μM, $p < 0.05$; 1 μM, $p < 0.01$), and U-50488 (0.1 μM and 1 μM, $p < 0.01$) exhibited these significant effects. The overproduction of ROS from infiltrating immune cells can be highly detrimental and toxic to cells, as ROS can lead to the generation of peroxynitrite (formed from the combination of superoxide and nitric oxide) and hydroxyl radicals (from hydrogen peroxide), both of which serve as potent mediators of brain injury in cases of brain inflammation (Zhou et al., 2022). Furthermore, we observed that opioid ligands also elevated the cellular concentration of glutathione (GSH), a major cellular antioxidant present in both neuronal and non-neuronal cells. GSH interacts with ROS to reduce their levels and helps maintain a healthy balance of oxidative stress (Chinta et al., 2006; Mendiola et al., 2020). GSH acts either independently or in cooperation with enzymes to mitigate superoxide radicals, hydroxyl radicals, and peroxynitrites that are generated during regular cellular metabolism. It is a favored substrate for several enzymes involved in xenobiotic metabolism and antioxidant defense (Huang et al., 2022; Liu et al., 2022; Yang et al., 2022). GSH deficiency has been observed in aging and various pathologies, including neurodegenerative disorders.

Moreover, studies indicate that intracellular GSH levels play a crucial role in regulating the susceptibility of cells to NO and its derivatives (Gusarov et al., 2021). If oxidative stress conditions compromise GSH levels, neurons become particularly vulnerable to NO and $ONOO^-$ (Ghashghaeinia et al., 2019) and its supplementation decreased inflammation (Sivandzade et al., 2019). The pretreatment of DAMGO, DADLE and U-50488 increased GSH levels in activated microglia, helping to maintain low levels of oxidative stress in cells. Under inflammatory conditions, mitochondrial basal respiration, ATP production and maximal respiration were all significantly reduced, whereas mitochondrial superoxide is increased in C8-B4 microglia. (Huang et al., 2022) shows fatty acids were shown to support mitochondrial oxidative metabolism in macrophages. ATP is known to induce chemotaxis in microglia but not macrophage through Gi/o-coupled P2Y receptors (Honda et al., 2001). Our results suggest that the selected opioid ligands block the effect of LPS and promote mitochondrial respiration and ATP generation. Furthermore,

our western blot analysis found that the activities of superoxide dismutase 2 and 3 (SOD2 and SOD3), (GPX; antioxidant enzymes, which oxidize GSH) and catalase (CAT) were increasing in comparison to the untreated cells when assessing the effect of opioid ligands on LPS-activated C8-B4 cells. Further analysis showed that opioid increased the content of intracellular GSH, a non-enzymatic antioxidant, GPX might oxidize GSH and cause GSSG as a by-product of H2O2 to water, whereas GR converted GSSG back to GSH (Bhargava and Schnellmann, 2017). Opioid ligands also increased the activity of the antioxidant enzymes GPX in the context of inflammation (Bhargava and Schnellmann, 2017). SOD catalyzes the dismutation of superoxide anion radical into molecular oxygen or hydrogen peroxide, while catalase catalyzes the decomposition of hydrogen peroxide into water and oxygen. Both enzymes work together to reduce ROS levels and keep cells at healthy levels of oxidative stress when superoxide and hydrogen peroxide levels are excessively high. We observed SOD 2, SOD 3 GPx1/2 and catalase significantly inhibiting ROS production by blocking LPS stimulated inflammation. Our study suggests that opioids protect microglial cells by enhancing the activity of antioxidant enzymes.

Nrf2 is a pivotal transcription factor known for its role in mitigating oxidative stress by regulating the expression of antioxidant genes (Kansanen et al., 2013; Khan et al., 2017; Lu et al., 2016; Mitsuishi et al., 2012). Typically, Nrf2 remains bound to Keap1 in the cytoplasm, rendering it inactive. However, when oxidative stress occurs, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it activates the antioxidant response element (ARE) and upregulates the expression of Nrf2-controlled genes, including HO-1. This ultimately leads to a reduction in intracellular ROS levels, highlighting the Keap1/Nrf2/ARE signaling pathway as a crucial target for combating oxidative stress (Chen and Maltagliati, 2018; Loboda et al., 2016). To explore whether opioid receptor agonists can lower intracellular ROS levels in C8-B4 cells through the Nrf2/HO-1 pathway, we investigated the effects of DAMGO, DADLE, and U-50488 treatment on C8-B4 cells exposed to LPS. Oue findings indicated that LPS reduced the expression levels of HO-1 in cells, likely due to the early surge of ROS consumption, depleting the existing HO-1 within the cells. Additionally, the reduction in intracellular Nrf2 expression levels did not reach statistical significance, suggesting that the pathway might have just initiated, and new HO-1 had not yet been synthesized. However, when opioids were administered in combination with LPS, these opioid treatments facilitated the translocation of Nrf2 into the nucleus, resulting in increased HO-1 expression and the activation of downstream pathways in C8-B4 cells exposed to LPS. In

summary, the mechanism by which opioids suppressed LPS-induced oxidative stress in microglia involves the Nrf2/HO-1 pathway, as depicted in Figure 22. While our study offers valuable insights, it is important to acknowledge its limitations. First, we lack comprehensive information about the activation patterns of different types of opioid receptors. Second, it is essential to investigate energy metabolism in primary microglia and in vivo settings to gain a complete understanding of the role of opioid receptors in microglia. Lastly, we cannot rule out the possibility that changes in microglial activation may have influenced the correlation between the bioenergetic profile and microglial substates. Nevertheless, the data from our study strongly suggest that opioid receptors in microglial cells may play an important role in attenuating adverse changes under injurious conditions, such as neurodegeneration. The selected opioid ligands show therapeutic potential in reducing oxidative stress. This study represents the first investigation to demonstrate a significant effect of opioid receptor activation on microglial metabolism.

7. Conclusion

Our research findings have demonstrated that opioid receptor ligands, specifically DAMGO, DADLE, and U-50488, exhibit the ability to induce the polarization of LPS-activated microglial cells towards the M2 phenotype. This polarization is achieved through the inhibition of the NF-κB signaling pathway and downregulate TREM2 pathway. We observed selected opioid ligands exerts cytoprotective effect by inhibiting LDH release, nitric oxide production, and promoting process outgrowth, migration capacity. We observed that opioid ligands increase anti-inflammatory cytokines including ARG-1, CD206, IL4, IL13 and suppressing pro-inflammatory molecules such as CD86, COX-2, TNF-alpha, IL beta 1 and IL 12. We found that DAMGO, DADLE, and U-50488 exert antioxidant and anti-inflammatory effects by modulating the Nrf2/HO-1 signaling pathway in LPS-stimulated C8-B4 cells. These findings not only contribute to our understanding of the molecular mechanisms behind opioid actions in microglial cells but also provide valuable insights into their potential neuroprotective efficacy against microglial inflammation. This, in turn, adds weight to the idea of utilizing selected opioid receptor agonists as a potential therapeutic approach for the treatment of neurodegenerative diseases. However, it is essential to conduct further research and clinical studies to fully explore the viability of this approach and its implications for neurodegenerative disease management.

8. Acknowledgements

My final task is to express my gratitude to all those who have contributed to the work presented in this thesis. This thesis not only fulfills my personal aspirations but also those of my parents, who have supported me greatly throughout my academic journey. It has taken me five years to complete this dissertation, and I was fortunate to have the support and assistance of numerous individuals during this time. I want to extend my thanks to everyone who made it possible for me to finish this thesis. If your name is not mentioned here, please know that I am no less grateful for your contribution. I am deeply grateful to my esteemed guide, Assoc. Prof. Jiri Novotny, who provided me with a rich source of deep inspiration. His words of advice and guidance have left an indelible mark on me, and I will always strive to uphold his ideas. His simplicity, unwavering support, meticulous guidance, and nurturing work environment will be cherished throughout my life. I am also indebted to Dr. Lucie Hejnova for her timely help, encouragement, and for boosting my confidence in my research progress. Special thanks go to my beloved family, close friends Shiv, and amazing collogues cytometrist Denise, astrocologist Drx. Deb, all-rounder Michal, Lilla, Radka, Vendy, Ondrej, and Zuzana, for their unwavering support and encouragement.

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