

REGULATION OF CALCIUM HOMEOSTASIS IN MICROGLIA: CURRENT VIEW ON THE PATHOGENESIS AND CORRECTION OF NEUROINFLAMMATION

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Abstract. The focus of this review is on evaluating the contribution of various regulatory mechanisms of calcium metabolism to the execution of key microglial functions such as patrolling, migration, proliferation, polarization, as well as mitochondrial plasticity and inflammasome assembly. We address current issues on the regulation of calcium homeostasis in microglial cells and microglia-like cells (MLCs). A concise historical overview of microglia and MLCs is provided, followed by an analysis of their functioning in both normal and pathological conditions. We refer to the functional classification of various calcium channels and transporters expressed in the plasma membrane and endoplasmic reticulum of microglia along with elucidation of the mechanisms leading to elevated cytosolic Ca²⁺ concentrations in microglial cell upon their activation. Then, we discuss the contribution of NAD⁺-glycohydrolase/CD38 to the regulation of calcium homeostasis in microglia. The review highlights contemporary approaches for manipulating microglial calcium metabolism with potential implications for the treatment of neurodegenerative diseases and neuroinflammation. Additionally, we briefly mention on modern imaging methods for studying calcium signaling in microglia. Thus, we summarize current data that shed the light on the intricate interplay between calcium regulation and microglial function in brain (patho)physiology. It also offers insights into potential therapeutic strategies and visualization techniques in the context of diagnostics and treatment of neurodegenerative disorders and neuroinflammation.

Keywords: microglia, microglia-like cells, calcium signaling, neuroinflammation, CD38.

List of Abbreviations

BBB – blood-brain barrier
CNS – central nervous system
ER – endoplasmic reticulum
ADPR – adenosine diphosphate ribose
AMPK – 5' adenosine monophosphate-activated protein kinase
cADPR – cyclic adenosine diphosphate ribose
CaM – calmodulin
CAMKII – Ca²⁺/calmodulin-dependent protein kinase II
CaMKKβ – Ca²⁺/calmodulin-dependent protein kinase kinase-β
Cav1.2 Calcium channel, voltage-dependent, L type, α1C subunit;
CRAC – calcium release-activated channels
DAG – diacylglycerol
DAMPS – damage-associated molecular patterns
CD – cluster of differentiation
GLUT-1 – glucose transporter type 1

GM-CSF – granulocyte-macrophage colony-stimulating factor
HMGB1 – high-mobility group box 1 protein
IFN-γ – interferon-gamma
IL – interleukin
iMG – monocyte-derived induced microglia
iNOS – Nitric oxide synthase, inducible
IP3 – inositol 1,4,5-trisphosphate
IP3R – inositol 1,4,5-trisphosphate receptor
LPS – lipopolysaccharide
L-VDCC – voltage-dependent calcium channels, type L
MHC-II – major histocompatibility complex, class II
MLC – microglia-like cells
MPP⁺ – 1-methyl-4-phenylpyridinium
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD⁺ – nicotinamide adenine dinucleotide
NADP⁺ – nicotinamide adenine dinucleotide phosphate

NCX – sodium-calcium exchanger
 NFATc2 (NFAT1) – nuclear factor of activated T-cells, cytoplasmic 2
 NO – nitric oxide
 NRF2 – nuclear factor erythroid 2-related factor 2
 N-VDCC – voltage-dependent calcium channels, type N
 ORA1 – calcium release-activated calcium channel protein 1
 P2X, P2Y – purinergic receptor
 PAMPS – pathogen-associated molecular patterns
 PLC γ 2 – phospholipase C gamma-2
 PLN – phospholamban
 PPAR- γ – peroxisome proliferator-activated receptor gamma
 ROCs – receptor-operated calcium channels
 RyR – ryanodine receptor
 sCD – cluster of differentiation, soluble form
 SERCA – sarco/endoplasmic reticulum Ca²⁺-ATPase
 siRNA – small interfering RNA
 SOCs store-operated calcium channels
 STIM1 – stromal interaction molecule 1
 TGF- β – transforming growth factor beta
 TLR – toll-like receptor
 TNF- α – tumor necrosis factor alpha
 TRPM, TRPV – transient receptor potential cation channel
 UDP – uridine diphosphate
 VOCs – voltage-operated calcium channels

Introduction

Microglia represents a subtype of glial cells that encompass a broad spectrum of functions within the central nervous system (CNS) (Muzio *et al.*, 2021; Shao *et al.*, 2022). These functions include phagocytosis of pathogens and products of cell destruction, release of cytokines and metabolites, involvement into inflammatory processes, patrolling of the brain tissue and completion of synaptic pruning (Morini *et al.*, 2021; Subramanian *et al.*, 2020). MLCs, or microglia-like cells, represent non-typically originated CNS macrophages. Unlike the conventional bona-fide microglia whose precursors migrate into the CNS during early

stages of embryonic development, MLCs differentiate during the post-embryonic phase as a consequence of events related to blood-brain barrier (BBB) disruption and various forms of neuroinflammation. A major question that relates to MLC is how identical are monocyte-derived macrophages to the “classical” microglia? There is an evidence suggesting that under pathological conditions associated with neuroinflammation and BBB breakdown, infiltrating MLCs may constitute a significant portion of brain macrophages (Chen *et al.*, 2022), and in certain cases, even entirely replace microglia (Ji *et al.*, 2007). According to some studies, bona fide microglia exhibits numerous phenotypes that do not conform to the classical macrophage dichotomy of activated and resting states (Savage *et al.*, 2019). However, for MLCs, researchers typically employ the classical classification into ramified and amoeboid forms (Cuadros *et al.*, 2022; Leone *et al.*, 2006).

In the context of seeking differences among bona fide microglia, MLCs, and blood monocytes, a particular interest arises in comparing the characteristics of calcium homeostasis across these cell types. It is known that fluctuations in intracellular calcium levels, particularly in the cytosol, in monocyte-macrophage lineage cells are intricately linked to cell functional activity and associated changes in gene expression, morphology, metabolism and secretory phenotype (Zumerle *et al.*, 2019). Therefore, regulation of calcium metabolism in microglial cells is tightly linked to the development of novel approaches for managing their functioning in the context of brain pathology. The assessment of the contribution of calcium signaling to microglial activation has been the focus of numerous insightful analytical studies in the past two decades (Brawek & Garaschuk, 2013; Kettenmann *et al.*, 2011; Kushnireva *et al.*, 2019; Parashchenko A.O. *et al.*, 2022; Peng & Pan, 2021; Umpierre & Wu, 2021). The aim of our review is to summarize recent data and to identify prospective approaches to cure neuroinflammation based on the target modulation of calcium homeostasis in activated microglia and MLCs.

Origin and Functions of Microglia and MLCs in Health and Disease

Microglial cells have a distinct origin compared to neurons, astrocytes, or oligodendrocytes. There are three main theories concerning the origin of microglia discussed elsewhere (Alekseeva *et al.*, 2019). The first theory is based on the mesodermal origin of microglia. It was proposed by Pio del Río-Hortega, a disciple of S. Ramón y Cajal, who is the pioneer in microglial studies. According to this concept, embryonic macrophages during fetal development, in stages preceding the maturation of the BBB, traverse various brain regions such as the corpus striatum, pallidum, internal capsule, cerebellum, and cranial nerve roots. As they progress, these embryonic macrophages transform into the ramified form of microglia (del Río-Hortega P., 1965). After the full establishment of the BBB which usually happens at the very early stages of neonatal brain development, the microglial population is maintained through in situ proliferation (Ginhoux *et al.*, 2013; Hashimoto *et al.*, 2013). The second theory relates to the idea on the derivation of microglia from neuroectoderm. According to this view, subependymal cells arising from neuroectoderm are the precursors of microglia. However, experiments validating the neuroectodermal origin of microglia are currently deemed, at the very least, controversial (Alekseeva *et al.*, 2019). The third theory states the potential origin of microglia from blood monocytes (Sántha & Juba, 1933; Shapiro *et al.*, 2009), and it might be an important component of immune cells recruitment in a case of neuroinflammation or excessive (neo)angiogenesis when the BBB is not well-sealed, thereby providing better access of blood monocytes into the brain tissue.

Despite contemporary evidence indicating that the majority of microglia have an embryonic origin (Hashimoto *et al.*, 2013) this hypothesis provided an explanation for the presence of MLCs within the CNS. These cells that are tissue macrophages of the CNS, exhibit similar morphology to microglia but originate from blood monocytes. During the postnatal period of development, the population of microglial cells in brain tissue undergoes dynamic

changes: under physiological conditions, microglia perform surveillance functions and participates in the regulation of synaptic plasticity, particularly through the mechanism of complement-dependent synapse elimination. In pathological conditions, the activation of microglial cells is accompanied by significant changes in morphology (retraction of processes), motility (directed migration), metabolism (switching between glycolytic and mitochondrial ATP production), and secretory activity (production and secretion of cytokines, reactive oxygen species, and metabolites with regulatory functions, e.g. lactate) (Maguire *et al.*, 2022). All these events are linked to the so-called microglia polarization from M1 (cytotoxic) to M2 (cytoprotective) phenotypes. Factors determining microglial polarization are classified as PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns). The former includes molecules such as LPS, which are components of the cell wall of Gram-negative bacteria. Examples of the latter include high mobility group box 1 protein (HMGB1), NAD^+ and nucleotides appearing in the extracellular milieu due to tissue damage (Ortiz *et al.*, 2017).

Thus, the key component of microglial activation upon the presence of extracellular PAMPs and DAMPs is its polarization. This process involves microglia adopting a specific functional phenotype in response to signals from the brain microenvironment. There are several approaches to classifying microglial phenotypes, primarily divided into two groups: those that consider extrapolating M1/M2 polarization of peripheral macrophages to microglial cells applicable, and proponents of the existence of distinct, microglia-specific states (Ransohoff, 2016; J. Wang *et al.*, 2023). Overall, there are two main phenotypic types of activated microglia: M1 and M2. M1-polarized microglia possesses pro-inflammatory properties and are capable of eliminating microorganisms as well as participating in the destroy of neurons and other brain cells. M1-polarized microglia secretes a range of cytokines such as IL-1 β (interleukin-1 beta), IL-6 (interleukin-6), IL-12 (interleukin-12), and TNF- α (tumor necrosis

factor- α) that collectively contribute to the initiation and progression of inflammation in the brain (Colonna & Butovsky, 2017; Orihuela *et al.*, 2016). In contrast, M2-polarized microglial cells exhibit anti-inflammatory and immunomodulatory effects that are associated with tissue remodeling and regeneration. The M2 phenotype of microglia is attributed to the secretion of cytokines such as IL-4 (interleukin-4), IL-10 (interleukin-10), and TGF- β (transforming growth factor-beta). These signaling molecules participate in the restoration and remodeling of damaged tissue (Colonna & Butovsky, 2017; Orihuela *et al.*, 2016).

Microglial polarization depends on various signals coming from the tissue microenvironment, including cytokines, neurotransmitters, pathogens, and other factors. Microglial polarization plays a crucial role both in maintaining normal brain function and in the pathogenesis of various neurodegenerative diseases (Wendimu & Hooks, 2022; Woodburn *et al.*, 2021). The M1 phenotype, also known as the cytotoxic or classical pathway of microglial polarization, is associated with increased expression of CD38 (Akimoto *et al.*, 2013), NADPH oxidase, iNOS (inducible nitric oxide synthase), Fc receptors, MHC-II molecules, integrins CD11b, and CD11c (Guo *et al.*, 2022). It involves elevated secretion of pro-inflammatory cytokines as well as molecules participating in the autocrine regulation of microglia (Kuno *et al.*, 2005; Ryu *et al.*, 2012). Interestingly, during M1 and M2 polarization, microglia undergo specific metabolic changes: resting microglia predominantly utilizes energy generation through oxidative phosphorylation, while activated M1 microglial cells rely on glycolysis. This shift is accompanied by mitochondrial fragmentation (Katoh *et al.*, 2017; Montilla *et al.*, 2021). In M1 microglia, the tricarboxylic acid cycle is suppressed, while the expression of membrane glucose transporters GLUT-1 is up-regulated. The glycolytic shift leads to an elevation in acetate levels (Gimeno-Bayón *et al.*, 2014; Vologoueva *et al.*, 2013), inducing alterations in epigenetic mechanisms governing gene expression (D. Zhang *et al.*, 2019). Release of lactate into extracellular space via monocarboxylate

transporters results in the modulation of glial functional activity either due to the uptake of lactate by neighboring cells or the action of lactate at plasma membrane GPR81 receptors. On the contrary, the M2 phenotype of microglia is distinguished by a prevalence of aerobic metabolism, reduced glucose consumption, predominant secretion of the anti-inflammatory cytokine IL-10, and ornithine production (Orihuela *et al.*, 2016). In sum, M2-polarized microglia has lower migratory activity, extensive phagocytosis, and may efficiently suppress excessive inflammation or tissue damage.

Key Molecular Mechanisms Regulating Calcium Homeostasis in Microglial Cells

Alterations of calcium levels in the cytosol and intracellular calcium stores constitute an essential component of microglial polarization. The cytoplasmic calcium levels can be elevated through two primary mechanisms: the first pathway involves calcium transport through calcium channels from the extracellular space, and the second one entails the release of calcium from intracellular calcium stores into the cytosol.

It is worth noting that the analysis of calcium oscillation patterns in the cytosol of microglial cells serves not only as a means of registering the microglial response to external stimuli, such as inflammatory inducers, but also as a means to indirectly assess the extent of damage to brain cells. Microglial cells intensify calcium signaling in response to any neuronal tissue damage, thereby allowing for the evaluation of the degree of the tissue injury (Eichhoff *et al.*, 2011). To assess calcium oscillations or, more broadly, changes in calcium levels in vitro, protocols employing fluorescent probes are widely used. These probes exhibit altered fluorescence in response to even minimal fluctuations in cytosolic calcium concentrations (such as Fura-2, Indo-1, Fluo-3, etc). Genetically encoded indicators responsive to calcium levels in activated cells are employed mainly in vivo conditions, and this methodology requires the expression of calcium-sensitive fluorescent molecules (e.g., GCaMP5G, GCaMP6s, RCEPIAer, Twitch-2B, etc.) within relevant cellular compartments

under the control of microglia-specific promoters (Brawek *et al.*, 2017; Perry *et al.*, 2015). Detection of fluorescence in brain cells in vitro is possible with fluorescent or laser confocal microscopy as well as with spectrofluorimetry, while in vivo conditions it requires two-photon confocal microscopy, fiber optic photometry, or protocols based on the usage of miniscopes (Pozner *et al.*, 2015; Supekar *et al.*, 2022).

The release of calcium from intracellular stores into the cytosol of microglial cells facilitates a rapid increase in calcium concentrations crucial for activating calcium-dependent enzymes and inducing cascades of signal transduction. Control of the activity of channels through which calcium is released from the endoplasmic reticulum (ER) is carried out via channels regulated by inositol trisphosphate receptors (IP3R) or ryanodine receptors (RyR). The ligand of the first type of receptors (IP3) is generated by the phospholipase C (PLC)-mediated hydrolysis of membrane phospholipids upon activation of G-protein coupled receptors resulting in the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Ryanodine receptors (RyRs) are activated by cyclic ADP-ribose (cADPR) synthesized from NAD⁺ by NAD⁺-glycohydrolase/CD38. In mature microglia, ryanodine receptors are present in two isoforms RyR1 and RyR2, while RyR3 is only detected during embryonic development (Klegeris *et al.*, 2007).

NAD⁺-glycohydrolase/CD38 is a NAD⁺-converting enzyme with significant expression in neuronal, astroglial and microglial cells whose enzymatic ligand is NAD⁺, whereas non-enzymatic ligand is CD31 (Ma *et al.*, 2014; Noda *et al.*, 2014; Roboon *et al.*, 2021; Y.-M. Wang *et al.*, 2017). CD38 can be expressed not only on the plasma membrane, serving as an ectoenzyme, but also on the surface of intracellular vesicles, as well as in its soluble form (sCD38). Moreover, the molecule is capable of altering its compartmentalization depending on the prevailing conditions (Fang *et al.*, 2018). The enzymatic activity of CD38 involves the cleavage of the NAD⁺ molecule into cADPR and ADPR, both of which are pivotal secondary messengers affecting cell calcium homeostasis,

and post-translational modification of cell proteins, respectively. Despite the fact that the enzymatic activity of cell membrane-bound CD38 is oriented towards the breakdown of extracellular NAD⁺, there is a mechanism for the internalization of ADPR and cADPR, presumably, due to activity of connexin 43 close to the CD38 in the plasma membrane (Franco *et al.*, 1998). Thus, intracellular action of cADPR on RyR within the ER results in the fast release of calcium into the cytosol.

Overall, it is generally accepted that microglial activation is accompanied by an increase in CD38 expression and activity. Particularly, under conditions of neuroinflammation, neurodegeneration, and aging, the expression of CD38 in microglial cells is significantly up-regulated (Guerreiro *et al.*, 2020). It is commonly accepted that such elevated expression of CD38 in microglia underlies the rapid rise in intracellular calcium levels followed by the activation of Ca²⁺-dependent regulatory mechanisms responsible for cell motility, secretion, and corresponding metabolic changes. In a recent study (K. Banerjee *et al.*, 2023), a difference in CD38 expression on the surface of microglial cell subpopulations with various levels of CD38 expression – CD45^{low-intermediate}/CD11b^{intermediate} and CD45^{high}/CD11b^{high} – was clearly demonstrated. The authors interpret the elevated CD45 expression in the second subpopulation as a marker of microglial activation. However, this observation could also be construed as a characteristic feature of monocyte-derived microglia (MLCs) (Amici *et al.*, 2017).

In some cells, generation of cADPR as a calcium mobilizer from intracellular stores could be provided by another NAD⁺-converting enzyme – CD157 (BST1). It is an ortholog of CD38 whose possible contribution to the signal-driven calcium response in microglial cells is not clarified yet, although it is highly probable (Lopatina *et al.*, 2020). Similar to CD38, the substrate for the catalytic activity of this enzyme is NAD⁺ and NADP⁺. CD157 converts these molecules into cADPR and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively (Czura & Czura, 2006). CD157 is expressed in immune cells and takes part in the

regulation of their migration and activation. It's expression was also found in the brain tissue, particularly, within the subventricular neurogenic niche, as well as in microglial/macrophage-like cells (Komleva *et al.*, 2015). In monocytes, CD157 could be co-expressed with CD11b and CD18, and such co-expression (CD157/Mac-1) is also seen in microglia cells in neuroinflammation, particularly, in experimental Parkinson's disease (Malinovskaya *et al.*, 2015). Furthermore, it is interesting to note that polymorphisms in the CD157/BST-1 gene, specifically rs11931532 and rs4698412, have been identified as risk factors for the development of Parkinson's disease (J. Li *et al.*, 2019). Considering the fact that the Mac-1 receptor expressed in microglial cells acts as a sensor for extracellular double-stranded RNA, it is tempting to speculate that co-expression of Mac-1 and CD157 might play a role in the microglial response to viral infection associated with TLR3 activation (Tatematsu *et al.*, 2014), or tissue damage accompanied by the release of mitochondrial double-stranded RNA (Dela Justina *et al.*, 2020). However, these hypotheses need in experimental confirmation.

In the context of regulating calcium exchange in activated microglia, CD38 and CD157 might be also important because their activity can directly link the characteristics of cell metabolism and functional polarization. The bioavailability of NAD^+ is largely determined by its synthesis, or by its regeneration in glycolysis (conversion of pyruvate into lactate) and in the electron-transport chain in mitochondria (NADH oxidation). Thus, the metabolic shift seen in activated microglia (from mitochondrial oxidative phosphorylation to glycolysis) should be considered an obligatory mechanism that controls the activity of CD38 and/or CD157 and prevents depletion of intracellular NAD^+ pool (Salmina *et al.*, 2021).

Replenishing calcium in intracellular calcium stores is a critical mechanism for microglial cell recovery following activation, ensuring their sensitivity to the action of subsequent stimuli. The primary role in calcium accumulation within the ER of microglia is attributed to the active calcium transporter SERCA2b. In

other cells, a regulatory mechanism is known involving allosteric modulation through the peptide phospholamban (PLN) (Gustavsson *et al.*, 2013). In its dephosphorylated state, PLN inhibits SERCA, however, a signaling pathway is known wherein SERCA is activated through the CALM-CAMKII-PLN phosphorylation cascade in response to elevated cytoplasmic calcium levels. However, molecular mechanisms of SERCA regulation in microglia remains unclear. According to www.proteinatlas.org, the expression of PLN in microglia is relatively low. Consequently, it can be speculated that the regulation of SERCA in microglia is minimally determined by a Ca^{2+} -PLN-dependent mechanism. Recent finding suggest that the activity of microglial SERCA is up-regulated in neurodegeneration and neuroinflammation (Morales-Ropero *et al.*, 2021), and astroglial cells take part in the control of SERCA activity in stimulated microglia (Kim *et al.*, 2022).

Like in other cell types, significant contribution to the control of basal and induced cytosolic calcium levels in resting and activated microglial cells is provided by numerous plasma membrane calcium channels and transporters:

1) ROCs (receptor-operated channels) include P2RX family of purinergic calcium channels, the transient receptor potential cation channels, subfamily M, member 2 (TRPM2), and some other ion channels. Stimulation of purinergic P2X receptors by extracellular molecules such as ATP or adenosine (whose concentrations substantially increase during tissue damage or neuroinflammation) typically leads to an elevation in intracellular calcium levels due to calcium influx via calcium channels, and enhanced production of intracellular reactive oxygen species. It was demonstrated that the activation of intracellular calcium signaling in response to an increase in extracellular ATP concentration in monocyte-derived microglia induced by M-CSF is mediated by P2X4 and P2Y11 purinoreceptors (Layhadi & Fountain, 2019). Activation of purinergic P2Y receptors, on the other hand, results in increased intracellular calcium levels due to calcium release from intracellular stores into the cytosol. Notably,

the repertoire of purinergic receptors expressed on microglial cells is quite extensive (Calovi *et al.*, 2019). Stimulation of P2X7 receptors by high concentrations of extracellular ATP leads to a sustained increase in cytosolic calcium levels in microglia, while lower ATP concentrations activate P2X4 purinergic receptors inducing oscillations in the cytosolic calcium levels. Notably, the former mechanism is sensitive to the action of AZ106, a selective antagonist of P2X7 receptors, which inhibits ATP-induced calcium signaling in target cells (Gilbert *et al.*, 2016). During embryonic and early postnatal development, microglia undergoes changes in the expression profile of P2RX receptors: on E12 in rats, the expression of P2X1 is observed in the forebrain, but it becomes completely negligible by the P13. In contrast, the expression of P2X7 in microglia of the rat forebrain increases during embryogenesis (Xiang & Burnstock, 2005). The finding that immature microglia in neonatal P1 mice are susceptible to activation by nucleotides (2MeSADP, 2-methylthioladenosine-5'-diphosphate) (Sunkaria *et al.*, 2016), along with data on the expression of P2X12 in human fetal microglial cells (Moore *et al.*, 2015), indicate the relatively early establishment of the P2X- and P2Y-mediated response system in microglial cells. Another type of ROC, TRPM2 is allosterically regulated by two structurally related molecules – ADPR (adenosine diphosphate ribose) and cADPR (cyclic adenosine diphosphate ribose) – which are the products of NAD⁺ conversion catalyzed by CD38. TRPM2 is expressed both in microglia and monocyte-derived macrophages. Furthermore, there is an increasing body of evidence indicating that TRPM2 serves as a pivotal molecule in monocytes and neutrophils infiltration into the neural tissue (Caravagna, 2019; Eder, 2010; Isami *et al.*, 2013; Tsutsui *et al.*, 2018). In sum, stimulation of two crucial types of ROCs - purinergic receptors and TRPM2 - by extracellular ligands that function as paracrine or autocrine signaling molecules (ATP and its derivative - adenosine, NAD⁺ and its derivatives - ADPR, cADPR), provides a basis to consider this calcium signaling pathway as pivotal for microglial cell activation during brain injury

and neuroinflammation. Furthermore, it is evident that this mode of activation must be tightly linked to metabolic changes in neuronal, endothelial, and glial cells, and thus likely serves as an indicator of the metabolic coupling within the neurovascular unit.

2) SOC (store-operated calcium channels) in microglia are represented by the membrane channel Orai1 and CRAC channels, as well as some purinergic receptors. Under cell culture in a calcium-free medium with depleted intracellular stores, contribution of SOC to calcium signaling activation is evident in microglial cells. Subsequent addition of extracellular calcium and monitoring of the calcium levels within intracellular stores reveals the activity of SOC. In this context, the cells remain devoid of calcium against the backdrop of increased intracellular oscillations of this cation (Gilbert *et al.*, 2016). Orai1 becomes activated in response to a decrease in intracellular calcium concentrations within the intracellular stores. This activation is achieved through the allosteric regulation by the protein STIM1 (Stromal interaction molecule 1) located in the ER (Prakriya *et al.*, 2006). A decrease in the calcium concentration within the ER leads to the oligomerization of STIM1, enabling the direct interaction between the cytoplasmic domains of STIM1 and Orai1. This interaction results in the opening of the calcium channel (Hartmann *et al.*, 2014). SKF96365 suppresses the activity of STIM1, thereby highlighting the essential role of SOC in the cell activation (Kim *et al.*, 2022). CRAC channels (calcium release-activated channels) in microglial cells display sensitivity to the inhibitor CM-EX-137. This sensitivity is accompanied by the suppression of nitric oxide production and calcium accumulation in microglia, alongside with the down-expression of pro-inflammatory NF- κ B transcription factor. As a result, an anti-inflammatory effect is observed (Mizuma *et al.*, 2019). Microglial metabotropic purinergic receptors of subtypes P2RY2, P2RY6, P2RY12, and P2RY13 are involved in the mechanisms of storage-regulated calcium entry into activated cells (Caruso *et al.*, 2023; Ohsawa *et al.*, 2010).

3) VOC (voltage-operated calcium channels) in microglia are represented by L-type

(L-VDCC – voltage-dependent calcium channels, type L) channels that are sensitive to the suppressive action of dihydropyridines or to the stimulatory action of BAY-K8644, as well as by N-type (N-VDCC – voltage-dependent calcium channels, type N) channels (Eder, 1998; Espinosa-Parrilla *et al.*, 2015; Tokuhara *et al.*, 2010). For voltage-dependent calcium channels of the L-type, there is a mechanism of inhibition involving interaction with the protein STIM1 upon a decrease in the concentration of Ca^{2+} within the ER (C. Y. Park *et al.*, 2010). Despite the conflicting data regarding the expression of L-VDCCs in microglial cells (Kettenmann *et al.*, 2011), antagonists of these calcium channels exhibit anti-inflammatory effects in vivo across various models of neuroinflammation and neurodegeneration (Hopp, 2021).

4) Proteins of the TRPV subfamily (transient receptor potential cation channel, subfamily V) that are expressed in microglia (Chakraborty & Goswami, 2022; Maksoud *et al.*, 2021). This channel type has various activation factors like mechanical (Hu *et al.*, 2023; Redmon *et al.*, 2021) and thermal stimuli (Chakraborty & Goswami, 2022). Presumably, these channels play a significant role in regulating the migration of microglial cells, including their thermosensitive mobility (Nishimoto *et al.*, 2021). Inhibition of TRPV4 channels in microglia disrupts their cytoskeletal remodeling essential for the microglial "patrolling" function in the healthy brain (Beeken *et al.*, 2022). Activation of TRPV1 by capsaicin increases calcium concentration in microglial mitochondria, stimulates autophagy, activates uptake of beta-amyloid (C. Wang *et al.*, 2022) and alpha-synuclein (Yuan *et al.*, 2022) by polarized microglial cells in experimental models of Alzheimer's and Parkinson's diseases, respectively. Suppressing the expression of TRPV1 in microglial cells disturbs the formation of NLRP3 inflammasomes and induces an anti-inflammatory effect in experimental autoimmune encephalomyelitis (Y. Zhang *et al.*, 2021). Thus, one could suggest that TRPV channels in microglial cells are required for the wide spectrum of the functions (movement, autophagy, mitochondrial respiration, NLRP3 assembly, etc.) and should be considered as promising targets for pharmacother-

apy in conditions associated with abnormal microglial activity (neurodegeneration and neuroinflammation).

5) Intracellular calcium concentrations in microglial cells could be affected by some other molecules like sodium-calcium exchangers (NCXs) – a family of membrane proteins that facilitate the exchange of Ca^{2+} and Na^{+} across concentration gradients. There are three isoforms of NCX – NCX1, NCX2, and NCX3. According to data obtained from in vitro studies, microglia primarily express NCX1 (Newell *et al.*, 2007). Under normal conditions, NCXs promote the efflux of calcium from the cytoplasm to the extracellular space. However, under pathological conditions, these transporters can exhibit reverse activity, contributing to a significant increase in intracellular calcium concentrations leading to the promotion of microglial polarization and migration (Newell *et al.*, 2007; Noda *et al.*, 2013).

Figure 1 displays data on the functioning of calcium-dependent proteins in microglia, along with intracellular calcium-dependent signaling cascades that lead to the activation of factors associated with microglial polarization.

Calcium Signalling in the (Patho)Physiology of Microglia and MLCs

Elevated calcium concentrations in microglia are associated either with physiological activity or pathological conditions. It should be noted that events involving prominent calcium release from intracellular stores into the cytosol of quiescent microglia are exceedingly rare. Alternatively, significantly increased calcium levels are usually associated with the establishment of polarized microglia in neuroinflammation, hypoxia, and tissue damage (Umpierre *et al.*, 2020). Rapid elevation of cytosolic calcium levels initiates a series of Ca^{2+} -dependent protein kinase cascades and activation of various transcription factors. This leads to gene expression changes associated with alterations in microglial morphology, mobility, and secretory profile (Nagamoto-Combs & Combs, 2010; S.Y. Park *et al.*, 2018). However, chronic elevation of basal cytoplasmic calcium concentrations could result in the suppression of intracellular signaling (Hoffmann *et al.*, 2003).

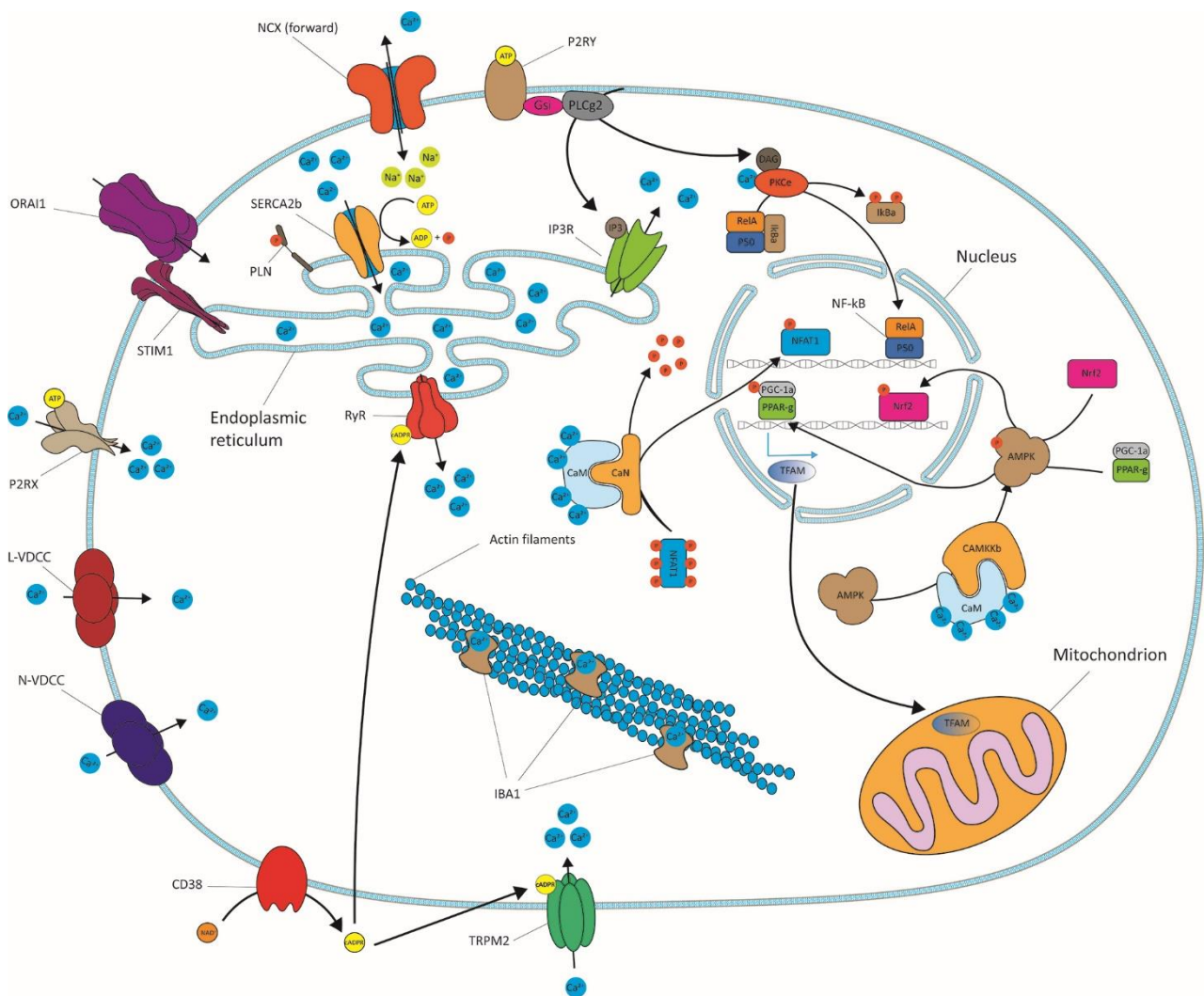


Fig. 1. Current view on key mechanisms of Ca^{2+} signaling in microglia.

Microglial L-type (L-VDCC) and N-type (N-VDCC) voltage-dependent calcium channels are activated in response to membrane depolarization, allowing calcium ions to enter the cytosol from the extracellular space. Under normal conditions, the Sodium-Calcium Exchanger (NCX) facilitates the influx of Na^+ into the cytoplasm and the efflux of Ca^{2+} into the extracellular space. In microglial activation, NCX action is reversed, thereby leading to calcium ions entering the cytoplasm and sodium ions exiting into the extracellular space. P2X channels are activated by purinergic nucleotides, predominantly ATP, resulting in Ca^{2+} influx into the cytoplasm. P2Y receptors being also sensitive to purinergic nucleotides activate $\text{PLC}\gamma 2$ which cleaves membrane phospholipids into IP3 and DAG. IP3 interacts with IP3 receptors (IP3R) and induces the release of calcium ions from intracellular stores. DAG and Ca^{2+} activate $\text{PKC}\epsilon$, which in turn activates the transcription factor NF- κB and up-regulates the expression of pro-inflammatory genes. CD38 hydrolyzes NAD^+ to cADPR which is internalized to activate the membrane calcium channel TRPM2 and to stimulate ryanodine receptors (RyR) at the ER, thereby causing calcium entry from the extracellular space and calcium release from the intracellular stores, respectively.

Upon depletion of microglial intracellular stores, the regulatory protein STIM1 activates Orai1, thus causing calcium ions to enter the cytoplasm and inhibiting L-VDCC channels. SERCA2b transports Ca^{2+} from cytosol into intracellular stores in ATP-dependent manner. Elevated cytosolic calcium levels result in the reorganization of cytoskeletal actin filaments through the action of Iba1 molecules. Ca^{2+} induces the activity of the regulatory protein CaM needed for the activation of two protein kinases – CaN and AMPK. CaN dephosphorylates the NFAT1 transcription factor leading to its activation and expression of pro-inflammatory genes. Conversely, AMPK activates transcription factors PPAR- γ , PGC1-1a, and NRF2, thereby promoting the expression of anti-inflammatory genes as well as mitochondrial biogenesis.

The results of numerous studies indicate that microglial activation is linked to the mobilization of intracellular calcium and the maintenance of chronically elevated concentrations in the cytoplasm (Campagno *et al.*, 2021; Fomina *et al.*, 2021; Hoffmann *et al.*, 2003; Mizuma *et al.*, 2019). Such abnormal elevation of Ca^{2+} levels in the cytosol has been observed during microglial activation induced by LPS (Kraft *et al.*, 2004; F. Liu *et al.*, 2014; L. Liu *et al.*, 2021), IFN- γ (Franciosi *et al.*, 2002; Horikawa *et al.*, 2010), and IL-1 β (Hayes *et al.*, 2002). Although the mechanisms underlying the increase in cytoplasmic calcium concentration may vary, the principal pathways for M1 polarization of microglia seem to involve calcium-dependent activation of transcription factors such as NFATc2 (Jiang *et al.*, 2022; Nagamoto-Combs & Combs, 2010) and NF- κ B (Dresselhaus & Meffert, 2019; M. Liu *et al.*, 2018). According to the recent findings (Guo *et al.*, 2022), the elevation of intracellular calcium concentration serves as one of the activators of the CaM-CaMKK β -AMPK signaling pathway, which triggers the activation of transcription factors associated with M2 polarization of microglia. Particularly, NRF2 (S. Y. Park *et al.*, 2018; Velagapudi *et al.*, 2018) and PPAR- γ (X. Li *et al.*, 2021; Song *et al.*, 2016) are involved in this type of polarization. Activation of AMPK suggests involvement of specific metabolic response in M2-polarized microglia since AMPK activity usually promotes anti-inflammatory effect (Saito *et al.*, 2019) coupled to altered mitochondrial dynamics in microglial cells with activated P2X7 receptors (Sekar *et al.*, 2018). The complex formed by the transcription factors PPAR- γ and PGC-1 stimulates the expression of the mitochondrial factor TFAM, thus promoting mitochondrial biogenesis (Jamwal *et al.*, 2021).

As we have mentioned above, M2 microglia has lower migratory activity than M1 cells. In general, microglial motility can be classified into two types: baseline and directed motility (Franco-Bocanegra *et al.*, 2019; Madry & Attwell, 2015). Baseline motility involves the constant reshaping of protrusions that scan the intercellular space for cellular debris and path-

ogens. On the other hand, directed motility refers to the ability of activated microglia to migrate based on the mechanisms of chemotaxis and thermotaxis. Microglial migration is associated with the activation of purinergic metabotropic receptors P2RY4, P2RY12, and P2RY13 (Jairaman *et al.*, 2022; Ohsawa & Kohsaka, 2011) which leads to an increase in intracellular calcium concentrations through the IP₃-mediated mechanism. During monocyte-to-MLC transformation, the expression of P2RY12 is increased, becoming similar to bona-fide microglia (Quek *et al.*, 2022). There is a report (Pozner *et al.*, 2015) which revealed that the motility of microglial cells is linked not only to the (Muzio *et al.*, 2021; Shao *et al.*, 2022). These functions include phagocytosis of pathogens and products of cell destruction, release of cytokines and metabolites, involvement into inflammatory processes, patrolling of the brain tissue and completion of synaptic pruning (Morini. Another study showed that GM-CSF- and IL-34-induced response in monocyte-derived microglia (iMG) is coupled to the higher expression of Iba1 than in GM-CSF-stimulated conventional tissue macrophages (A. Banerjee *et al.*, 2021). Furthermore, this study demonstrated the expression of the above-mentioned microglia-specific purinergic metabotropic receptor P2RY12 in iMG. Despite the crucial role of intracellular calcium signaling in microglial migration processes, the influence of extracellular calcium on microglial motility has not been observed (Eyo *et al.*, 2014). Apart from chemotaxis, microglia also exhibit positive thermotaxis which is mediated by the vanilloid-sensitive calcium channel TRPV4. Inhibition of this channel leads to reduced microglial motility and filopodia movement in vitro (Beeken *et al.*, 2022; Nishimoto *et al.*, 2021). Dysfunction of another thermosensitive channel, TRPM8, results in impaired baseline motility of rat microglial cells in vitro (Chakraborty & Goswami, 2022).

As we have mentioned before, rise in cytosolic calcium concentrations is required for the assembly of NLRP3 inflammasome in microglial cells. It was demonstrated that inflamm-

asome assembly is mediated by both the release of Ca^{2+} ions from intracellular stores and the influx of calcium ions from the extracellular space (Murakami *et al.*, 2012), although the precise mechanism of calcium-mediated NLRP3 subunit oligomerization remains unknown. In the recent study, it was shown that TRPV1 calcium channel is involved into a two-step mechanism of inflammasome assembly. The priming signal activates the transcription factor NF- κ B, thereby leading to the expression of the NLRP3 gene, followed by the activating signal that initiates inflammasome assembly: inhibition of TRPV1 with the specific antagonist capsazepine leads to a reduction in NLRP3 inflammasome activation, cessation of IL- 1β production, decreased TNF- α secretion, and reduced caspase-1 activity in an in vitro model of LPS-induced neuroinflammation in mice (Y. Zhang *et al.*, 2021): Treatment of microglia with the agonist capsaicin leads to a decrease in NLRP3 inflammasome activation which might be in agreement with the data on the inhibition of intracellular calcium signaling under chronic elevation of basal cytoplasmic calcium concentrations (Lin *et al.*, 2022). However, application of the toxin antagonist (5'-iodo-resiniferatoxin iRTX) results in increased expression of NLRP3, IL- 1β , and caspase-1 in microglia in an oxygen-glucose deprivation model used to reproduce mechanisms of ischemic-reperfusion brain injury in mice (Lin *et al.*, 2022). In sum, deciphering the molecular mechanisms of Ca^{2+} -dependent inflammasome assembly in the microglia might provide novel approaches to the treatment of brain disorders associated with excessive or inefficient microglia activation.

Proliferation of microglia is important in the embryonic development, while in the adult brain, the proliferative potential of microglia is significantly lower. However, it is increased in various neurological disorders, incl. neuroinflammation and neurodegeneration (Tan *et al.*, 2022). Various calcium-dependent signaling cascades have different effects on microglial proliferation. It was demonstrated (Maksoud *et al.*, 2021) that activation of NFATc2 associated with the NO-dependent pathway and an increase in cytoplasmic calcium concentrations initiates

the expression of the p21 gene which inhibits the proliferation of mouse microglia in vitro. In another study (Ding *et al.*, 2015), the connection was found between the inhibition of mouse microglial proliferation through p21 gene expression and its stimulation by cytokines IFN- γ and TNF- α in vitro, presumably, through the elevated cytosolic calcium levels (Miyake *et al.*, 2014; Zhu *et al.*, 2018). Moreover, it was clearly shown (Franciosi *et al.*, 2002) that the deletion of NFATc2 (NFAT1), whose expression is linked to the activity of Ca^{2+} -dependent kinases, negatively affects microglial proliferation in the mouse brain in vivo. On the other hand, expression of the purinergic calcium channel P2XR7 promotes rat microglial proliferation in vitro (Monif *et al.*, 2009, 2010). It is known that the overall activation and proliferation of microglia in neuroinflammation are associated with a chronic increase in cytosolic calcium concentrations (Sharma & Ping, 2014). However, the particular mechanisms that couple calcium signaling to the proliferation of microglia in pathological conditions remain to be evaluated. Since local population of MLCs is mainly maintained by the proliferation (Cuadros *et al.*, 2022), calcium-dependent mechanisms of their recruitment and proliferation might be also important.

There are several approaches to modulating microglial activation through the action at calcium signaling machinery. For instance, one approach involves suppressing the activity of the Ca^{2+} -dependent protein kinase CaMKII by inactivating the regulatory protein calmodulin. When calmodulin activity is disrupted, signaling pathways that activate the expression of proinflammatory genes are interrupted in microglial cells (Hu *et al.*, 2012; Jeon *et al.*, 2018; Szabo *et al.*, 2016). However, this approach has several drawbacks, with the main one being the tissue non-specificity of CaM inhibitors activity, leading to global changes in other organs, including nephrotoxic effects (Finn, 1999; Kanofsky *et al.*, 2011; Karolin *et al.*, 2022). Another way to manipulate microglial polarization by altering calcium metabolism is blocking the L-type voltage-gated calcium channels. Selective calcium channel inhibitors have been shown to suppress the neurotoxic effect of activated microglia (Hash-

ioka *et al.*, 2012; Saddala *et al.*, 2020). However, according to the data obtained in the experimental model of Parkinson's disease in mice, the blockade of the Cav1.2 channel only leads to an enhancement of M1 polarization of microglia in vivo (X. Wang *et al.*, 2019). A third method is based on attempts to control the activity of CRAC channels. It was demonstrated (Mizuma *et al.*, 2019) that inhibiting Orai1 activity with the molecule CM-EX-137 prevents the increase in cytosolic Ca²⁺ concentrations in target microglial cells. This reduction leads to a decrease in the number of activated microglia and an improved neuronal survival in the in vivo model of neuroinflammation in mice. In another study (Heo *et al.*, 2015), inhibition of STIM/ORAI1 expression with small interfering RNA (siRNA) and suppression of the calcium channel activity with SKF96365 reduced the production of pro-inflammatory cytokines and completely halted the UDP-induced phagocytosis in primary in vitro mouse microglia cultures.

Mechanisms of functional coupling between mitochondria and ER in the context of calcium signaling in microglia are largely unexplored. Recent studies have shown that regulatory mechanisms of M1 polarization might be connected with the activity of ER-mitochondria associated membranes (MAMs) in microglia and could potentially be controlled by ligands of sigma-1R receptors (Ooi *et al.*, 2021). Such observations are particularly intriguing considering the role of MAMs in mitochondrial dynamics, mitophagy and inflammasome assembly in activated microglia. Particularly, it might correspond to the activation of mitophagy during mitochondrial fission, alongside the metabolic shift from mitochondrial to glycolytic energy generation pathways upon glial activation (Proulx *et al.*, 2021).

Table 1 summarizes the data on some currently available modulators of calcium regulatory machinery that have been proved to affect microglial activity in (patho)physiological conditions.

Future Directions

The elucidation of cellular and molecular mechanisms of calcium signaling in microglia and MLCs is crucial for several reasons. Firstly, it pertains to the exploration of the potential to modulate intracellular calcium levels as a therapeutic approach for neurodegenerative disorders associated with neuroinflammation. Despite substantial progress made in this direction in recent years, certain aspects remain unexplored, such as the expression patterns of calcium channels in microglia and MLCs during neuroinflammation, alterations in this pattern over time upon MLC transition from the quiescent bona fide microglia branching phenotype, and subcellular mechanisms of calcium signaling in microglial cells and MLCs. Also, the question of whether intracellular calcium signaling in MLCs is identical to that in microglia remains open. Given the substantial presence of MLCs within nervous tissue and the recruitment and activation of microglia and MLCs in neuroinflammation, an intriguing inquiry arises regarding the proliferation of these two subpopulations and a role of Ca²⁺-dependent mechanisms in this process. Further exploration is extremely needed on calcium signaling contribution to the M1/M2 polarization and migration of activated microglia. In this context, special attention should be paid to the expression of NAD⁺-glycohydrolase/CD38 and CD157 in microglia and MLCs since their activity might link metabolic plasticity of microglial cells and their polarization.

Table 1

Modulators of microglial calcium signaling

Target	Active agent	Interaction type	Dosage and application	Model description	Results	Source
ORAI1/STIM1	CM-EX-137	antagonist	CM-EX-137 - 1-20 μ M; administered once into the culture medium, one hour prior to the addition of LPS, Poly(I:C), IFN- γ , or PMA to the medium.	In vitro model of LPS, POLY(I:C), IFN- γ , or PMA-induced neuroinflammation in BV-2 microglial cell line.	Reduction of iNOS, NFAT, and NF- κ B expression; dose-dependent decrease in NO concentration upon LPS, Poly (I:C), IFN γ , PMA stimulation.	Mizuma <i>et al.</i> (2019)
		antagonist	Continuing the translation: CM-EX-137 - 5 mg/kg daily; administered intraperitoneally for either 3 or 14 days.	In vivo model of traumatic brain injury in male C57/B6 mice aged 3-4 months.	Reduction in the size of the damaged brain area, decrease in the number of activated microglia/macrophages, decrease in the number of STIM1+/ORAI1+ microglia; reduction in cognitive function damage.	
		antagonist	CM-EX-137 - 5 mg/kg; administered intraperitoneally as a single dose, concurrently with LPS.	In vivo model of LPS-induced neuroinflammation in the brain of male C57/B6 mice aged 3-4 months. LPS dosage - 5 mg/kg.	Decrease in the number of activated microglia/macrophages and reduction in iNOS expression upon LPS administration.	
	SKF96365	antagonist	-----	In vitro model of LPS-induced activation in primary microglial cell culture from neonatal ICR mice.	Reduction in TNF- α and IL-6 production, inhibition of calcium efflux from intracellular stores.	Heo <i>et al.</i> (2015)
L-VDCC	Nimodipine, Verapamil	antagonist	30 μ M nimodipine or 30 μ M verapamil into the medium for 15 minutes prior to LPS and IFN- γ stimulation, and 48 hours before the MTT assay.	In vitro model of LPS and IFN- γ -induced activation of microglia obtained from surgical explants of the human brain.	L-VDCC blockers had no effect on microglial survival but significantly increased survival of SH-SY5Y neuronal cells incubated for 72 hours in LPS and IFN- γ -stimulated microglial-conditioned medium.	Hashioka <i>et al.</i> (2012)

Continued table 1

Target	Active agent	Interaction type	Dosage and application	Model description	Results	Source
	Amlodipine, Zinc20267861, Zinc18204217	antagonist	Amlodipine 3.5 μ M or Zinc20267861 2.0 μ M or Zinc18204217 2.0 μ M or Zinc33254827 2.5 μ M into the culture medium, administered once, 96 hours prior to the mobility assessment.	In vitro model of BV-2 microglial cell migration.	L-VDCC inhibitors significantly reduced microglial migration ability, and F-actin protein staining revealed the absence of intracellular filaments under calcium channel blockade conditions.	Saddala <i>et al.</i> (2020)
	Zinc20267861, Zinc18204217	antagonist	Amlodipine 3.5 μ M or Zinc20267861 2.0 μ M or Zinc18204217 2.0 μ M or Zinc33254827 2.5 μ M along with LPS into the culture medium, administered once, 24 hours prior to conducting the experiments.	In vitro model of LPS or IL-4-induced activation of BV-2 microglia. LPS dosage - 1 μ g/ml; IL-4 - 20 ng/ml.	Zinc20267861 and Zinc18204217 inhibitor molecules contributed to the reduction in COX-2 and phospho-ikB α expression upon LPS stimulation, and Arg1 upon IL-4 stimulation. A decrease in active oxygen species in the culture stimulated by amlodipine, Zinc20267861, Zinc18204217 was recorded.	
	Zinc20267861	antagonist	Continuing the translation: Zinc20267861 10 μ g subconjunctivally, daily, for 7 days starting from the day of model establishment.	Continuing the translation: In vivo model of laser-induced corneal neovascularization (CNV) in C57BL/6J and CX3CR1 ^{gfp/wt} mice aged 6-8 weeks.	Administration of Zinc20267861 led to a reduction in the size of retinal damage, decreased expression of retinal microglia activation markers COX-2 and phospho-ikB α , and reduced microglia infiltration in damaged areas.	
	Nifedipine, diltiazem	antagonist	10 μ M nifedipine or 10 μ M diltiazem one hour prior to the addition of LPS and IFN- γ , or IL-4, followed by incubation for 1 day before conducting the experiments.	In vitro model of LPS+IFN- γ or IL-4-induced microglial activation in MG6 mouse microglial cell line. LPS dosage - 2.78 ng/ml; IFN- γ - 0.56 ng/ml; IL-4 - 100 ng/ml.	Increase in iNOS expression in LPS+IFN- γ -activated microglia and decrease in Arg1 expression in IL-4-activated microglia.	Wang <i>et al.</i> (2019)

Continued table 1

Target	Active agent	Interaction type	Dosage and application	Model description	Results	Source
TRPV4	GSK2193874, HC067047, RN9893	antagonist	Continuing the translation: GSK2193874, HC067047, or RN9893 at 1 μ M or 10 μ M each into the medium.	Continuing the translation: Model of primary culture of ramified microglia from the cortex of CX3CR1 ^{eGFP/+WT} and CX3CR1 ^{eGFP/+Trpv4 KO} mice.	reatment with GSK2193874, HC067047 led to a reduction in basal mobility and adoption of a more spherical shape by microglia.	Beeken <i>et al.</i>
TRPM2, TRPM4, TRPV4	9-phenantrol (9-фенантрол)	antagonist	30 μ M 9-phenanthrol in the culture medium.	In vitro model of temperature-dependent directed motility in primary microglial cell culture from C57BL/6NCr mice.	Loss of microglial thermotaxis ability in the presence of the inhibitor molecule.	Nishimoto <i>et al.</i> (2021)
TRPV1	Capsazepine	antagonist	10 μ M capsazepine administered once into the culture medium, followed by subsequent exposure to LPS (3 hours) and then nigericin (1 hour) or ATP (30 minutes).	In vitro model of LPS-induced microglial activation with inflammasome activation in primary microglial cell culture from C57BL/6 mice. LPS dosage - 100 ng/ml; nigericin - 10 μ M; ATP - 5 mM.	Reduction in IL-1 β , TNF- α , caspase-1 expression.	<u>Zhang <i>et al.</i> (2021)</u>
	Capsaicin	agonist	1 μ M capsaicine administered once into the culture medium.	In vitro model of oxygen and glucose deprivation/re-oxygenation (OGD/R) on primary microglia from neonatal Sprague-Dawley rats.	Increase in autophagosome count; decrease in NLRP3, IL-1 β , caspase-1 expression.	Lin <i>et al.</i> (2022)
	5'-iodoresiniferatoxin	antagonist	5'-iodoresiniferatoxin 1 μ M administered once into the culture medium.		Decrease in autophagosome count; increase in NLRP3, IL-1 β , caspase-1 expression.	
P2RX7	AZ106	antagonist	AZ106 10 μ M administered once into the culture medium, 30 minutes before measurements.	In vitro model of P2RX7 purinoreceptor inhibition on BV-2 microglial cell line.	Disturbance of ATP-induced calcium signaling in microglia.	Gilbert <i>et al.</i>

Continued table 1

Target	Active agent	Interaction type	Dosage and application	Model description	Results	Source
Ca ²⁺ /CaM-dependent signaling cascades	Clozapine	antagonist	Clozapine 10 mg/kg intraperitoneally for 3 days once daily. One hour after the last injection, administration of LPS 5 mg/kg	In vivo model of LPS-induced neuroinflammation.	Decrease in MHC-II expression in the cortex and hippocampus of the brain in response to LPS stimulation.	Jeon <i>et al.</i> (2018)
			Clozapine 5-20 µM administered once into the culture medium. LPS 0.5 µg/ml one hour after clozapine application. Assessments conducted 12 hours after LPS exposure.	Continuing the translation: In vitro model of LPS-induced activation of BV-2 microglial cell line and primary rat microglia.	Dose-dependent decrease in nitric oxide, IL-1β, IL-6 production; reduction in iNOS, COX-2 expression. Reduction in LPS-induced NF-kB subunit binding.	
			Clozapine 0.01-10 µM administered once into the culture medium, with LPS added one day later. Neuron counting performed 7 days after LPS exposure.	In vitro model of LPS-induced neuroinflammation in mixed cultures of rat neurons and microglia. LPS dosage - 2.5 ng/ml.	Increase in neuron survival, normalization of dopamine uptake by dopaminergic neurons, decrease in iBa1 and p65/RelA-positive microglia.	Hu <i>et al.</i> (2012)
			0.1 µM clozapine administered once into the culture medium one day after adding MPP ⁺ . Dopamine uptake assay performed 7 days after MPP ⁺ addition.	Continuing the translation: In vitro model of MPP ⁺ -induced neurotoxicity on three mixed cultures of rat microglia, neurons, and other glial cells with varying component ratios. MPP ⁺ dosage - 0.25 µM.	Microglia-mediated normalization of dopamine uptake by dopaminergic neurons.	
			0.1-1 µM clozapine administered once into the culture medium along with LPS. Assessment	In vitro model of LPS-induced microglial activation using the HAPI rat microglial cell line.	Reduction in MHC-II expression in LPS-stimulated microglia.	

End of table 1

Target	Active agent	Interaction type	Dosage and application	Model description	Results	Source
			conducted 7 days after the administration of compounds.			
			0.1-1 μ M clozapine administered once into the culture medium along with LPS. Assessment conducted 7 days after the administration of compounds.	In vitro model of LPS-induced microglial activation in a mixed culture of rat neurons and glia.	Reduction in the number of iba1-positive microglia.	
			0.1-1 μ M clozapine administered once one day before adding LPS. Assessment conducted one day after LPS administration.	Continuing the translation: In vitro model of LPS-induced neuroinflammation in an enriched mixed culture of rat neurons and glia, with microglial enrichment. LPS dosage - 2.5 ng/ml.	Inhibition of LPS-induced reactive oxygen species production.	
			0.1-1 μ M clozapine administered once one day before adding LPS. Assessment conducted one day after LPS administration.	In vitro model of LPS-induced neuroinflammation in a mixed culture of rat neurons and glia. LPS dosage - 2.5 ng/ml.	Decrease in LPS-induced TNF- α and NO secretion.	

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