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Role of zinc in microglial phenotypes

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Summary

Microglia are resident immune cells of the central nervous system (CNS) that continuously survey the local microenvironment by extending and withdrawing their cellular processes in the resting state. When activated by tissue injury or other signals, microglia retract their processes and transform into an activated amoeboid morphology. These activated cells are known to polarize into M1 pro-inflammatory or M2 anti-inflammatory phenotypes during neuropathological conditions, including stroke, which suggests that this polarization might play a role in the development and progression of brain disorders. Furthermore, zinc homeostasis in the CNS is integral to normal CNS function, such as learning and memory. Although the effects of zinc on microglial activation are not well known, recent studies have demonstrated that zinc affects microglial activation as well as neuronal function. In this review, we discuss in detail the effects of extracellular and intracellular zinc levels on microglial activation and the M1 and M2 microglial phenotypes. Extracellular zinc might act as a novel trigger for the microglial morphological changes via a zinc-induced microglial activation signaling pathway, where intracellular zinc accumulation via Zrt-Irt-like protein 1 is the initial step. Additionally, extracellular zinc might promote the inflammatory M1 phenotype, while increased intracellular free zinc levels in interleukin-4-induced M2a microglia might negatively regulate arginase-1 expression. The zinc-promoted M1 phenotype is involved in post-ischemic cognitive decline and suppression of astrocytic engulfing activity, whereas zinc-modulated arginase-1 expression might regulate the phagocytic activity of M2a microglia.

Key words: zinc, microglial activation, M1/M2 polarization

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Introduction

Microglia, a type of glial cell derived from the embryonic yolk sac, are resident innate immune cells of the central nervous system (CNS) [1-3]. Under normal physiological conditions, they are ubiquitously distributed in the mature CNS and exist in a resting state characterized by a ramified morphology with highly motile, active, and long cellular processes [2]. Two-photon microscopy studies have demonstrated that resting microglia survey their microenvironment with highly motile processes in

the healthy brain [4, 5], supporting the proposition that microglia are involved in the maintenance of brain homeostasis as well as host defense [6, 7]. Conversely, in response to neuronal injury or pathogen-derived molecules, resting microglia transform into an activated state comprising an amoeboid morphology, increased proliferation, and release of several types of mediators [8-11]. Furthermore, activated microglia under pathological conditions, such as brain ischemia and Alzheimer's disease, can exert either detrimental or protective effects, suggesting that these cells may acquire opposing phenotypes, termed M1 and M2 activation states [12-14]. However, the concept of M1/M2 microglial activation remains controversial. The M1 phenotype is a pro-inflammatory and cytotoxic phenotype, which is activated mainly by pathogens and pro-inflammatory factors (including lipopolysaccharide [LPS], interferon gamma, and tumor necrosis factor-alpha [TNF-α]) and produces pro-inflammatory cytokines, such as interleukin (IL)-1, IL-1β, and IL-6 [15]. Conversely, the M2 phenotype is an alternative activation state involved in the fine-tuning of inflammation, tissue remodeling, and repair [15]. Therefore, understanding the regulation of the polarization and function of these phenotypes may facilitate the development of effective therapeutic strategies for brain disorders.

Zinc is the second most abundant essential trace element in the brain at an estimated concentration of 150 mM, which is 10-fold of that of the serum zinc levels [16]. Zinc homeostasis in the brain is tightly regulated by primarily two families of transporters, Zrt-Irt-like proteins (ZIPs) and zinc transporters, along with zinc-bound proteins. Accumulating evidence indicates that zinc homeostasis in the brain contributes to normal brain functions, such as learning and memory [17-19]. Conversely, dysregulated zinc homeostasis has been implicated in the pathogenesis of a wide range of neurological diseases. However, the effects of zinc on microglial activation are not well known. In this review, we discuss the effects of extracellular and intracellular zinc levels on microglial activation and the M1 and M2 microglial phenotypes.

Role of zinc in microglial morphological changes

Resting microglia retract their processes and transition into an activated amoeboid morphology within several hours in response to numerous neuropathological conditions, leading to a dramatic change in appearance [20]. In the adult mammalian brain, zinc is concentrated in the synaptic vesicles within a specific subset of glutamatergic neurons in the hippocampus and cerebral cortex [21] and released into the extracellular space in an impulse- and calcium-dependent manner to modulate neurotransmission [17-19]. However, under pathological conditions, including transient brain ischemia and hypoglycemia, massive amounts of vesicular zinc are released into the extracellular space and accumulate in the postsynaptic neurons, resulting in neuronal cell death [22, 23]. Accordingly, treatment with zinc chelators has been shown to attenuate neuronal cell death in animal models of brain disorders. These findings suggest that the dysregulation of extracellular zinc release promotes brain injury [23-26]. In line with these findings, our group demonstrated that ischemia-induced morphological changes in microglia were blocked in mice receiving intraventricular calcium ethylenediaminetetraacetic acid (CaEDTA), an extracellular zinc chelator [27]. Additionally, the addition of 15-60 μM $ZnCl_2$ to microglial cultures caused the retraction of the microglial processes and transition to the round amoeboid morphology, with the changes reaching nearly maximal stage within 2 h [27, 28]. Therefore, the release of massive amounts of extracellular zinc might act as a signal that triggers morphological changes in microglia (**Fig. 1**). However, extracellular actual concentration of zinc released in the brain under pathological conditions remains uncertain. Ischemia-induced zinc release has been calculated to raise extracellular zinc concentrations up to 300 μM. Direct measures of extracellular free zinc during ischemia have identified elevations to only the nanomolar range [22]. On the other hand, treatment of microglial cultures with 120 μM $ZnCl_2$ led to cell death [28], indicating that the range of extracellular zinc concentration which induces microglial morphological changes is limited. Therefore, it is necessary to determine the concentration of extracellular zinc that promotes microglial morphological changes in brain under pathological conditions.

Increasing evidence has shown that extracellular zinc is taken up into the cytosol of neurons via Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [29], L-type voltage-gated Ca^{2+} channels [29, 30], N-methyl-D-aspartate (NMDA) receptors [31], and transporters [32]. Moreover, excessive amounts of extracellular zinc have been reported to induce glial cell death [33] and affect normal astrocytic functions [34]. However, the glial transmembrane routes of zinc are not fully understood. Recently, the ZIP family, which consists of 14 members in the humans and mice, have been investigated for their role in zinc uptake in glial cells because of their expression in various glial cells, including microglia [28, 35], astrocytes [36, 37], and oligodendrocytes [38]. Accordingly, the studies have shown that ZIP1, which is localized on the plasma membrane, is highly expressed in mouse microglia [28], while nickel (a competitive inhibitor of ZIP1) and ZIP1 knockdown decrease zinc uptake

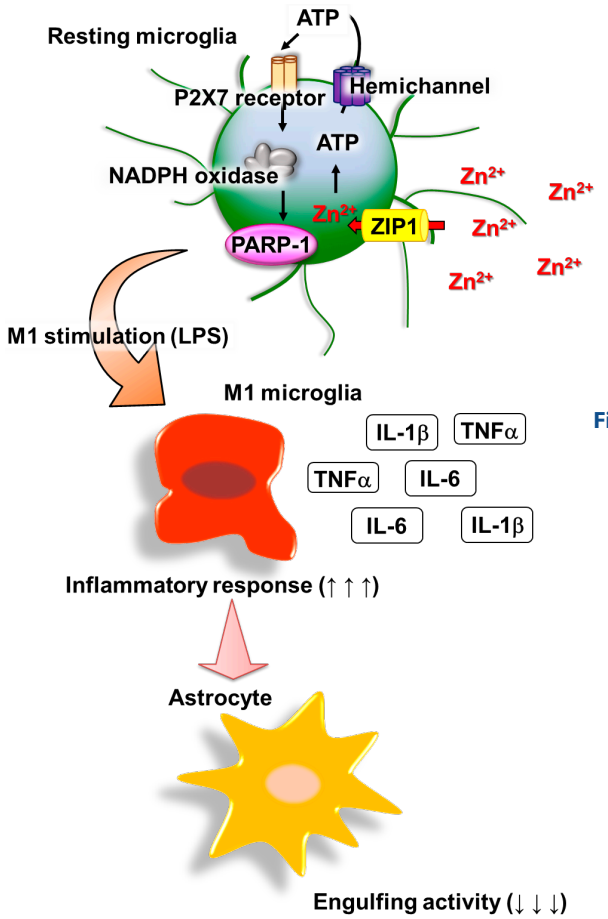

Fig. 1. **Promotion of pro-inflammatory M1 phenotype of microglia by extracellular zinc**
Extracellular zinc triggers microglial morphological changes via the zinc-induced microglial activation signaling pathway (ZIP1-mediated zinc uptake induces release of ATP, followed by P2X7 receptor activation and the sequential activation of NADPH oxidase and PARP-1). This pathway mediates the aggravation of pro-inflammatory M1 phenotype of microglia in response to M1 stimuli, including LPS. These aggravated microglia then suppress astrocytic engulfing activity and produce pro-inflammatory cytokines. ZIP1, Zrt-Irt-like protein 1; PARP-1, poly(ADP-ribose) polymerase; LPS, lipopolysaccharide; IL-, interleukin-; TNF-α, tumor necrosis factor-alpha.

by microglia [35]. Additionally, intracellular zinc chelation by *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) suppresses zinc-induced morphological changes in microglia [28]. This indicates that ZIP1 is the major transporter responsible for zinc uptake in microglia, and ZIP1-mediated zinc transport may be the first step in the microglial morphological changes.

After brain disorders, such as brain ischemia and trauma, ATP is released from the damaged and dying cells into the extracellular space, where it serves as a signal for microglial activation and chemotaxis. Furthermore, microglia release ATP in response to stimuli such as LPS, lysophosphatidic acid, amyloid β, and hypotonic stress [39-42]. Microglial ATP release is primarily mediated by hemichannels, vesicular nucleotide transporter-dependent exocytosis, or cystic fibrosis transmembrane conductance regulators [43-45]. Our earlier study showed that zinc stimulation induced an increase in extracellular ATP in primary microglia culture, and this ATP increase was inhibited by a non-specific hemichannel inhibitor, carbenoxolone (CBX) [28]. Furthermore, the pretreatment of microglia with CBX suppresses zinc-induced morphological changes [28]. Additionally, we revealed that a P2X7 receptor-selective antagonist, oxATP, abolished zinc-induced microglial morphological changes. Conversely, exposure to ATP or a relatively highly selective P2X7 receptor agonist, BzATP, transformed microglia into its activated amoeboid form [28]. Thus, hemichannels might be a potential pathway for ATP release in zinc-stimulated microglia, and this hemichannel-mediated ATP release is involved in the subsequent cascade of microglial morphological changes via autocrine and/or paracrine activation of P2X7 receptors.

The activation of P2X7 receptors in many types of brain cells stimulates NADPH oxidase activity, a major source of reactive oxygen species (ROS) [41, 46, 47]. Microglial ROS production by NADPH oxidase promotes microglial activation [48]. The exposure of microglia to zinc induces an increase in ROS levels, whereas the inhibition or genetic disruption of NADPH oxidase blocks zinc-induced increase in ROS levels and morphological changes [27]. Additionally, the zinc-induced increase in microglial ROS levels is suppressed by the inhibition of intracellular zinc accumulation, hemichannel-mediated ATP release, and P2X7 receptor activation in microglia after zinc stimulation [28]. Therefore, a necessary step for zinc-induced morphological changes in microglia might involve the activation of NADPH oxidase, which lies downstream of intracellular zinc accumulation and

P2X7 receptor activation via hemichannel-mediated ATP release.

ROS acts as a secondary messenger in many cell types. Oxidative DNA damage modulates the activation of microglia via poly(ADP-ribose) polymerase (PARP)-1 [49-51], which is an ADP-ribosylating enzyme involved in various DNA and RNA metabolic processes, including DNA repair. When activated by oxidative DNA damage, PARP-1 contributes to the regulation of the expression of inflammation-associated genes, such as cyclooxygenase-2, and inducible nitric oxide synthase (iNOS) [52, 53]. Conversely, PARP-1 inhibitors suppress the morphological changes in microglia that are induced by treatment with TNF-α, amyloid β, S100B, and NMDA [54-57]. In our earlier study, PARP-1 activation led to the transition to the amoeboid form in microglia treated with zinc [27]. However, the zinc-induced PARP-1 activation and morphological changes were suppressed by the inhibition of NADPH oxidase and PARP-1 [27], indicating that zinc-induced morphological changes in microglia are mediated by the sequential activation of NADPH oxidase and PARP-1. Furthermore, we demonstrated that zinc-induced PARP-1 activation was abolished when microglia were pretreated with a hemichannel inhibitor and P2X7 receptor antagonist [28].

Therefore, these findings suggest that extracellular zinc acts a novel trigger for microglial morphological changes, which are initiated by intracellular zinc accumulation via ZIP1. Furthermore, these morphological changes are mediated by ATP release through hemichannels and autocrine/paracrine activation of P2X7 receptors, and followed by the sequential activation of NADPH oxidase and PARP-1 (zinc-induced microglial activation signaling pathway) (**Fig. 1**). To our knowledge, there are no reports showing the aforementioned intracellular signaling pathway induced by other stimuli. It is expected to determine whether the zinc-induced microglial activation signaling pathway is unique.

Zinc and M1 activation of microglia

Although the concept of M1/M2 activation of microglia remains controversial, increased expression of pro-inflammatory mediators and M1 cell surface markers have been demonstrated by numerous studies on animal models and patients with brain disorders, including stroke, Alzheimer's disease, amyloid lateral sclerosis, and Parkinson's disease [58-65]. These findings imply that an excess accumulation of pro-inflammatory mediators, such as IL-1β, IL-6, and TNF-α, caused by the chronic activation of M1 microglia might lead to neuronal damage. Additionally, studies have shown that conditioned media collected from ischemic neurons prime microglial polarization toward the M1 phenotype [58], while the microenvironment associated with spinal cord injuries leads to the upregulation of the M1 phenotype and downregulation of the M2 phenotype [66]. Thus, an endogenous soluble factor that regulates microglial M1/M2 polarization might exist in the brain under pathological conditions.

Since resting microglia rapidly transform into an amoeboid morphology and exhibit an M1 phenotype in response to the corresponding M1 stimuli [67], we focused on extracellularly released zinc following transient brain ischemia as a modulator of microglial polarization. In our earlier study of LPS-induced M1 polarization of microglia, the pretreatment of microglia with $ZnCl_2$ resulted in a dose-dependent increase in iNOS expression and IL-1β, IL-6, and TNF-α secretion [68]. However, the effects of zinc pretreatment on microglia were suppressed by treatment with TPEN, a cell-permeable zinc chelator; Trolox, a radical scavenger; and A438079, a P2X7 receptor antagonist [68]. This implies the involvement of a zinc-induced microglial activation signaling pathway that leads to the transformation of ramified microglia into the amoeboid form. Moreover, we found that intra-cerebroventricular pre-injection with CaEDTA attenuated ischemia-induced pro-inflammatory cytokine expression and M1 polarization of microglia in the hippocampus as well as protected against post-ischemic cognitive decline [68]. Therefore, extracellular zinc may be an endogenous factor involved in promoting the inflammatory M1 phenotype of microglia arising in response to M1 stimuli (**Fig. 1**). Moreover, considering that zinc plays an important role in brain functions, interventions targeting zinc-induced microglial activation signaling pathway may be effective strategy for preventing brain dysfunction following ischemia. Therefore, it is necessary to clarify the precise mechanism underlying zinc-promoted M1 phenotype in future studies. Furthermore, since vesicular zinc has been shown to be in the glutamatergic neurons in the several brain regions other than the hippocampus, it is expected to address regional differences of zinc-promoted M1 phenotype.

Effect of zinc-promoted microglial M1 activation on astrocytic function

Astrocytes play an important role in normal physiological brain function by providing neurons with structural, metabolic, and trophic support [69, 70]. Microglia, on the other hand are responsible for the clearance of dead cells and debris that accumulate in the affected region after brain damage. Recently, studies have demonstrated that astrocytes could also engulf

dead cells and small axonal or myelin debris to restore impaired neuronal neural circuits and attenuate the inflammatory impact of damaged neuronal cells [71-73]. Therefore, astrocytes can also function as phagocytes together with microglia under pathological conditions. However, in a rodent model of transient brain ischemia, phagocytic astrocytes exhibited an ischemic spatiotemporal pattern distinct from that of microglial cells [71]. Furthermore, attenuation of the engulfing activity of cultured astrocytes occurred under sub-lethal oxidative stress [74]. This indicates that astrocytic engulfing activity is impaired or limited by the disruption of the local neural microenvironment. Recently, Hamada et al. revealed that astrocytic engulfing activity was suppressed by conditioned medium derived from zinc-pretreated M1 microglia [75]. Other studies have shown that the chronic or aggravated inflammatory M1 phenotype of microglia exacerbates brain injury [58, 76] and that extracellular zinc released after brain ischemia promotes this M1 microglial phenotype in the hippocampus [68]. Therefore, zinc might be involved in the suppression of astrocytic engulfing activity by promoting the M1 phenotype and disrupting the microenvironment (**Fig. 1**). Conversely, recent accumulating evidence indicates that astrocytes can affect microglial function (for example, microglial neuroinflammation is inhibited by exosomes derived from astrocytes) [77]. However, further studies are needed to determine whether astrocytes modulate zinc-induced promotion of M1 activation.

Zinc and M2 activation of microglia

In contrast to the M1 activation of microglia, the M2 activation state is subdivided into the M2a, M2b, and M2c subtypes. Although these subtypes have some biochemical overlaps, they have different activation mechanisms and function [78]. M2a activation is induced by IL-4, which can induce CD206 expression and is associated with the upregulation of anti-inflammatory mediators, such as arginase-1 [79]. Arginase-1 prevents NO production by competing with iNOS for the substrate L-arginine. These responses of M2a microglia contribute to the attenuation of brain damage caused by excessive inflammation and to the promotion of tissue remodeling and repair [80, 81]. In contrast, excessive arginase-1 activity has been reported to cause endothelial dysfunction in traumatic brain injury [82] and retinopathy [83] models. In support of these findings, studies in mouse models of Alzheimer's disease have revealed accumulation of arginase-1 in the subiculum and CA1 regions of the hippocampus, the major areas of amyloid β deposition and neurodegeneration. Furthermore, the pharmacological inhibition of arginase activity protected the model mice from Alzheimer's disease-like pathologies [84]. Thus, arginase-1 might exert either beneficial or detrimental effects depending on the degree of its expression.

In the brain cells, including neurons and glial cells, most zinc binds to proteins such as enzymes, signaling molecules, and transcription factors, which in turn are involved in maintaining the efficient performance of the brain cells. Consequently, intracellular free zinc is considered to be excessively low under normal physiological conditions in the CNS [85]. Additionally, transient changes in intracellular free zinc concentration have been reported to play an important role in signal transduction and cell function [86-88]. Moreover, free zinc concentration in neurons and astrocytes is shown to increase in response to several pathophysiological stimuli, such as hypoosmolality [89, 90], glucocorticoids [91], and oxidative stress [36, 92], and this increase in intracellular free zinc is mediated by zinc influx from the extracellular space via zinc importers or by release from cytosolic zinc-binding proteins (intracellular zinc release) [93]. In our earlier study, when microglia were treated with IL-4, intracellular free zinc concentration increased after the induction of arginase-1 expression, whereas the induction of arginase-1 expression was prevented by an intracellular zinc chelator (and not an extracellular zinc chelator) [94]. Additionally, chelation of intracellular zinc resulted in a dramatic increase in both mRNA levels and enzymatic activity of arginase-1 in IL-4-induced M2a microglia. Therefore, IL-4 might induce intracellular zinc fluctuation in M2a microglia through intracellular zinc release, and the increased intracellular zinc level may in turn act as a negative regulator that prevents excessive expression of arginase-1 in M2a microglia [94].

In addition to regulating arginase-1 expression, M2a microglia can phagocytose cell debris to promote reconstruction of the extracellular matrix, tissue repair, and neuronal survival [78]. However, the overexpression of recombinant murine IL-4 in the hippocampus of a mouse model of Alzheimer's disease led to an increase in the "M2-like" microglial phenotype, along with downregulation of microglial phagocytosis [95]. Chelation of intracellular zinc suppressed IL-4-induced phagocytic activity in microglia, which was then reversed by L-arginine supplementation [94]. L-arginine, a substrate for arginase-1, is an important factor that promotes phagocytosis in peripheral immune cells [96-98]. Therefore, these findings suggest that microglial intracellular zinc release after IL-4 stimulation may play an important role as a negative regulator of arginase-1 expression, and this negative regulation of arginase-1 expression may be essential for maintaining the normal phagocytic activity of M2a microglia (**Fig. 2**).

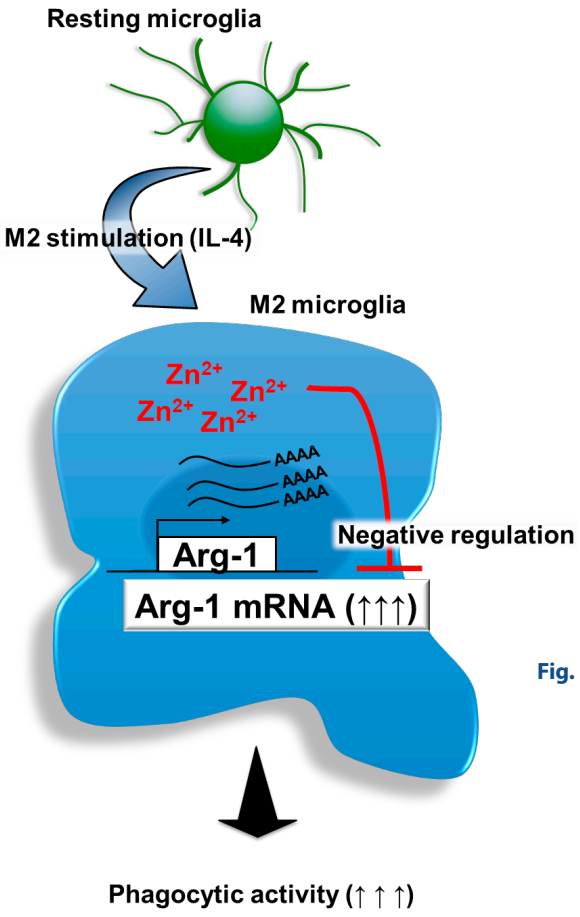

Fig. 2. **Regulation of M2a microglial function by accumulation of intracellular free zinc**
Intracellular zinc accumulated in IL-4-induced M2a microglia acts as a negative regulator to prevent the excessive expression of arginase-1. This regulation contributes to the maintenance of the phagocytic activity of M2a microglia. IL-4, interleukin-4.

However, it is unknown how much concentration of zinc is released in microglia after IL-4 stimulation. In future, it is necessary to determine the concentration of intracellular free zinc negatively regulates excess arginase-1 expression in M2a microglia, and what mechanism underlying the negative regulation of intracellular zinc-induced arginase-1 expression. Considering that M2 microglia are subdivided into subtypes, including the IL-4-induced M2a, immune complex-induced M2b, and IL-10/transforming growth factor-beta-induced M2c, studies examining the effect of zinc on the induction of the other subtypes of M2 microglia are required.

Conclusion

This review highlighted studies investigating the role of zinc in microglial phenotypes. A brief description of the main findings follows. Extracellular zinc triggers morphological changes in microglia via the intracellular zinc signaling cascade and promotes the inflammatory M1 phenotype. M1 microglia are involved in post-ischemic cognitive decline and suppression of the engulfing activity of astrocytes. In case of the M2 phenotype, M2a microglia activation is induced by IL-4. Furthermore, the increase in intracellular free zinc in M2a microglia after IL-4 stimulation acts as a negative regulator of arginase-1 expression, which then contributes to the regulation of the phagocytic activity of M2a microglia. Therefore, zinc plays different roles in microglia depending on their activation state. Numerous studies have revealed that the balance between the M1 and M2 phenotypes is disrupted during chronic inflammation conditions, such as those associated with ischemia, traumatic brain injury, Parkinson's disease, and Alzheimer's disease [58, 99-101]. Moreover, alterations in zinc homeostasis in the brain are involved in the pathophysiological progression of these disorders [24, 102-104]. All these discussed findings suggest that zinc has an important role in the microglial phenotypes and that the disruption of zinc homeostasis in the brain is one of the major causes of imbalance in the M1/M2 phenotype. Therefore, more advanced studies investigating the role of zinc in the microglial phenotypes under pathophysiological conditions are required for clarifying these findings and developing an effective strategy for the prevention and alleviation of brain disorders.

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