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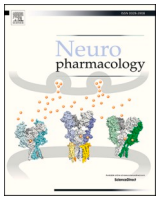

Contribution of microglia to the epileptiform activity that results from neonatal hypoxia

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ABSTRACT

Microglia are described as the immune cells of the brain, their immune properties have been extensively studied since first described, however, their neural functions have only been explored over the last decade. Microglia have an important role in maintaining homeostasis in the central nervous system by surveying their surroundings to detect pathogens or damage cells. While these are the classical functions described for microglia, more recently their neural functions have been defined; they are critical to the maturation of neurons during embryonic and postnatal development, phagocytic microglia remove excess synapses during development, a process called synaptic pruning, which is important to overall neural maturation. Furthermore, microglia can respond to neuronal activity and, together with astrocytes, can regulate neural activity, contributing to the equilibrium between excitation and inhibition through a feedback loop.

Hypoxia at birth is a serious neurological condition that disrupts normal brain function resulting in seizures and epilepsy later in life. Evidence has shown that microglia may contribute to this hyperexcitability after neonatal hypoxia. This review will summarize the existing data on the role of microglia in the pathogenesis of neonatal hypoxia and the plausible mechanisms that contribute to the development of hyperexcitability after hypoxia in neonates.

This article is part of the Special Issue on "Microglia".

1. Introduction

Microglia were first described by N. Achucarro and P. del Rio-Hortega (both alumni of S. Ramon y Cajal) in 1919. In the four original publications, Rio-Hortega described different types of microglia based on their morphology, from homeostatic to amoeboid microglia (Fig. 1), and their similarities with phagocytic immune cells (del Río--Hortega, 1919; Sierra et al., 2016; Tremblay et al., 2015).

Microglia are considered the permanent resident immune cells of the central nervous system, and as originally described, they share characteristics with macrophages. Microglia, like macrophages, originate in the fetal yolk sac, distinguishing them from the other cells in the nervous system, which originate in the neural ectoderm. Microglia mature slowly during embryonic and postnatal periods. They infiltrate the brain in several waves during E9.5-E17.5 as macrophage-like cells, and in the brain environment, they transform into mature microglia. Once they enter the brain, microglia are long-lived cells, with a slow turnover over the lifespan and remain functionally active (Reu et al., 2017). However, due to their long-lasting life, early-life insults result in pathological and chronically activated microglia which may have a long-lasting impact on neuronal circuitry and brain function (Bilbo et al., 2018).

During development, microglia can be divided into 3 subtypes based on their gene expression; early microglia (between E10-14 in mice); pre-microglia (E15-postnatal day 9 in mice); and adult microglia, which are fully established from postnatal day 28 in mice (Matcovitch-Natan et al., 2016). This process of microglial development occurs in synchrony with wider brain development, and throughout this, microglia coordinate the neuronal and immune responses in a time-dependent manner, which is critical to maintaining homeostasis of the brain. Thus, any disruptions of the maturation process, either genetically, pharmacologically, or environmentally, have been implicated in the onset of neurological conditions. This was observed by the depletion or overactivation of microglia. During the embryonic state, deletion of the colony-stimulating factor 1 receptor (Csf1r), which is critical for the survival of microglia, results in abnormal brain development (Erblich et al., 2011). Activation of microglia during the perinatal period can also lead to the development

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of neurological conditions, such as depressive disorders (Cao et al., 2021) and over-activated microglia later in life, resulting in an excessive inflammatory response to otherwise subthreshold insults (Williamson et al., 2011). Similar results were seen in human studies conducted on post-mortem brain tissue from patients with neurodevelopmental conditions such as autism, where microglia were shown to have an amoeboid shape (Morgan et al. 2010, 2012; Pardo et al., 2005; Vargas et al., 2005).

Microglia are relatively uniformly distributed in the adult brain, with the highest concentrations in areas of high plasticity, including the reward circuits and hippocampus (Lawson et al., 1990; Tan et al., 2020), suggesting that they are critical for synaptic refinement. It is important to note that microglia are very heterogeneous to accommodate the diversity of physiological and pathological functions (Hanisch, 2013; Stratoulias et al., 2019; Tan et al., 2020). This heterogeneity is seen in the spatiotemporal location (De Biase and Bonci, 2019; Olah et al. 2011, 2012; Silvin and Ginhoux, 2018; Tay et al., 2017; Thion and Garel, 2017), colonisation (Tan et al., 2020; Thion and Garel, 2017), abundance (Tay et al., 2017; Tan et al., 2020; Thion and Garel, 2017), and morphological features affecting mobility and motility (Smolders et al., 2019; Thion and Garel, 2017). This review will not describe functions and microglia states, these have been well described in the review by Paolicelli et al., (2022).

As will be discussed in this review, microglia are vital regulators of neuronal function throughout development and in the mature brain. Microglia continuously conduct surveillance of their surroundings to detect pathogens or damage, and they have also been shown to monitor and regulate synaptic activity and neuronal morphology (Kettenmann et al., 2011; Graeber, 2010; Schafer et al., 2013; Tremblay and Majewska, 2011). Importantly, when microglia are activated in response to stress, cell damage or pathogens, they cannot carry out their normal homeostatic functions to the same degree. This may result in aberrant neural circuit formation and the development of neurological conditions; this is especially critical during early development.

1.1. Microglia and early life development

During early life, microglia play a pivotal role in brain maturation. Microglia regulate neuronal numbers and the early wiring of neural circuits. Microglia localize to several neurogenic niches in the rat brain, including the subventricular zone and neocortex, where they play a pivotal role in regulating neurogenesis (Cunningham et al., 2013). In the context of neonatal hypoxia, neurogenesis is not affected after neonatal hypoxia in mice (Quinlan et al., 2019) or in the more severe ischemic model in rats (Ehlting et al., 2022), showing that microglia's main pathological pathway is not via regulation of neurogenesis.

Microglia regulate early circuit formation, via specific pathways, including complement and purinergic pathways. In normal physiology, microglia transiently associate with specific axonal tracts, including dopaminergic axons and corpus callosum axons, where microglia then regulate axon progression and fasciculation (Squarzoni et al., 2014; Pont-Lezica et al., 2014).

The role of microglia in regulating neuronal circuits is not restricted to the regulation of neural activity and maturation. Microglia also interact with oligodendrocytes and astrocytes and through this communication maintain homeostasis. Microglia are critical for the process of myelination, where they support oligodendrocytes progenitor cells (OPCs) and oligodendrocytes. In the developing white matter, microglia are found in clusters (Hagemeyer et al., 2017; Wlodarczyk et al., 2017). At these clusters, microglia secrete TNF-α, IL-1β, IL-6 and INF-γ. The secretion of this subset of cytokines promotes the development of oligodendrocytes and the synthesis of galactolipid sulfatide and the myelin proteins MBP and PLP, necessary for the formation of myelin (Althaus et al., 2008; Shigemoto-Mogami et al., 2014). Importantly, microglia may regulate neurogenesis and oligodendrogenesis in parallel. This is seen in the accumulation of microglia in the subventricular zone in the early postnatal stage, and the fact that microglial cytokine release enhances both processes (Shigemoto-Mogami et al., 2014).

In the developing brain, microglia-astrocyte communication plays an important role. The astrocyte-released cytokine IL-33 activates microglia, promoting synaptic pruning and neuronal maturation (Vainchtein et al., 2018). In the adult brain, activated microglia can induce an A1 phenotype of astrocytes, through the release of IL-1α, TNF-α and C1q (Liddelow et al., 2017). This communication is bidirectional, reactive astrocytes release pro-inflammatory cytokines, IL-1β, TNF-α and IL-6, which result in the activation of microglia (Li et al., 2015). However, it is not known if the same communication between microglia and astrocytes occurs in the developing brain whether a different set of factors are required, or how it may be regulated over time.

1.2. Microglia and neonatal hypoxia

Perinatal hypoxia-ischemia encephalopathy is a perinatal insult mainly caused by a lack of oxygen to the brain. The intracellular cascade activated after neonatal hypoxia is well characterised and divided into several phases: primary energy failure, latent period and secondary and tertiary energy failure. The primary energy failure is initiated by the original reduction in oxygen, which causes a disruption of the respiratory chain in mitochondria and decreased ATP production (Millar et al., 2017). The subsequent depletion of ATP pools is the primary acute energy failure that disrupts the cell machinery, particularly the Na^+/K^+ ATPase pump. Upon failure of the pump, the resting membrane potential becomes difficult to maintain, resulting in membrane depolarization and the release of neurotransmitters, such as glutamate, into the synaptic cleft (Gunn et al., 1997; Tan et al., 1996). Within the neuron, glutamate binds to AMPA (N-methyl-D-aspartate receptor) and NMDA (N-methyl-D-aspartate receptor) receptors, causing an elevated concentration of intracellular calcium, which acts as a secondary messenger. As a result of these events, the caspase cascade, Nitric Oxide (NO), and Reactive Oxygen Species (ROS) pathways are activated, inducing neuronal death and neuroinflammation, mainly via activation of microglia (Bainbridge et al., 2014; Bennet et al., 2007). The high release of glutamate affects microglial and astrocyte function and will be further described in the next section. After reperfusion, there is a latent period followed by a secondary energy failure, which results in further inflammation and damage to the brain (Millar et al., 2017).

Hypoxia-induced neuroinflammation activates microglia. An increase in the number of microglia has been reported in several models of

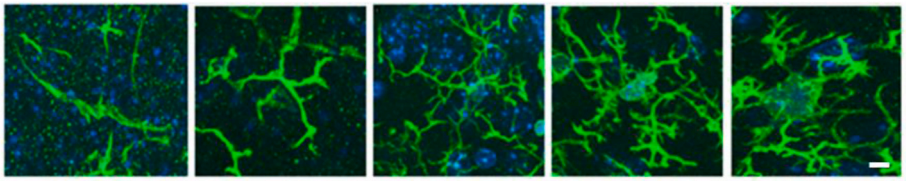

Fig. 1. Fluorescent images of Iba1 (microglial marker, green) and DAPI (nuclear marker, blue) of typical microglia observed in the hippocampi of 72-h post-hypoxia mice. Microglia morphology varies from small cell bodies and few to several processes to larger cell bodies and thick. Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hypoxia-ischemia encephalopathy (Bona et al., 1999; Cowell et al., 2003; Hellstrom Erkenstam et al., 2016; Ivacko et al., 1996; Mallard et al., 2019; McRae et al., 1995; Quinlan et al., 2019; Xu et al., 2001), with similar results observed in post-mortem tissue of human pre-term infants affected by hypoxia at birth (Verney et al., 2012).

Homeostatic microglia express a diverse set of receptors, including Damage Associated Molecular Patterns (DAMPs) and Pathogen Associated Molecular Patterns receptors (PAMPs) including Toll-like receptors, cytokine receptors, and purinergic receptors (Fig. 2). These receptors are necessary to maintain homeostasis but also will activate microglia in the presence of increased levels of DAMPs and PAMPs, such as cytokines or ATP.

Toll-like receptors are part of the innate immune receptors by responding to PAMPs and are critical for non-infectious stimuli, such as hypoxia-ischemia. Activation of Toll-like receptors has been shown to contribute to the pathophysiology of neonatal hypoxia (Quinlan et al., 2019; Stridh et al., 2013). Inhibition of Toll-like receptors 2 and 3 improves pathology and seizures acutely and reduces susceptibility to seizures later in life (Quinlan et al., 2019; Stridh et al., 2013).

This increase in microglia and activation is also associated with morphological changes (Fig. 1) and upregulated expression of the pro-inflammatory cytokines IL-1β and TNF-α (Hellstrom Erkenstam et al., 2016; Quinlan et al., 2019). This cytokine over-expression has a detrimental effect on the brain after neonatal hypoxia, as demonstrated by pharmacological inhibition of the IL-1β pathway; inhibitors of IL-1 receptors improve outcomes in animal models following hypoxia-induced injury (Girard et al., 2012; Leitner et al., 2014). Furthermore, after hypoxia in neonates, there is an increase in caspase 1 and cleaved caspase 1, an enzyme produced mainly by microglia, which is necessary for the maturation of IL-1β (Rodriguez-Alvarez et al., 2017). Deletion of caspase 1 has also been shown to attenuate brain damage after hypoxia-ischemia (Hedtjarn et al., 2002).

As mentioned earlier, microglia from the perinatal period respond differently to mature microglia from the adult brain. This is apparent in studies of the microglial receptor CD36 post-ischemia; in the adult brain CD36 depletion is neuroprotective after stroke, whereas in the neonatal brain CD36 depletion worsens the injury. This may be due to the resulting inhibition of microglial phagocytic activity, a critical function of microglia during the development and formation of neural circuits (Li et al., 2015; Woo et al., 2012). As we will see later, activation of purinergic receptors may be critical for the development of seizures after neonatal hypoxia (Rodrigues et al., 2019; Rodriguez-Alvarez et al., 2017). This data shows that microglia have a key role in the pathogenesis of neonatal brain injury. Further studies are necessary to evaluate which specific functions have a positive or detrimental effect on the brain.

Supporting the role of inflammation in neonatal hypoxia, the gold standard treatment for neonatal encephalopathy is therapeutic hypothermia (TH). While it is unclear how TH works, current data points to a decrease in metabolism, reduced radical generation, ameliorated inflammation, and inhibition of both excitotoxicity and apoptosis (Sun et al., 2019). Importantly, TH-treated neonates with moderate to severe neonatal encephalopathy still suffer sequelae, showing the importance of evaluating novel adjuvant treatments. Preclinical models have shown that the use of melatonin and allopurinol has a beneficial effect (Sabir et al., 2023). Currently, these adjuvant medications with anti-inflammatory properties have been tested in clinical trials, including erythropoietin (Juul et al., 2018; Malla and Bhat, 2018), allopurinol (Maiwald et al., 2019), melatonin (Jerez-Calero et al., 2020) and cannabidiol (EudraCT Number: 2016–000,936-17). These therapies show promising results both alone and in conjunction with therapeutic hypothermia in clinical trials. While, these studies are still ongoing, as they include long-term evaluations of the socio- and psychological development of the participants. So far, they have shown some improvement at 18–24 months of age in the Bayley Scales of Infant Development III (Jerez-Calero et al., 2020). This suggests that targeting the inflammatory system may have beneficial therapeutic effects on neonatal hypoxia.

2. Microglia regulate neuronal activity

Communication between microglia and neurons is essential to maintaining homeostasis of the brain and for the development of neural circuits. In this review, we will discuss some of the factors that contribute to neural activity regulation, maintaining a special focus on the role of neurotransmitters in microglia-neuron communication, and how microglia control neural circuits within the context of neonatal hypoxia. We will not discuss how microglia regulate neuronal migration as, while this process is certainly critical for the maturation and establishment of neuronal circuits, it has been previously described in Perez-Rodriguez et al. (2021).

Cytokines are expressed at low levels in the healthy brain, but they

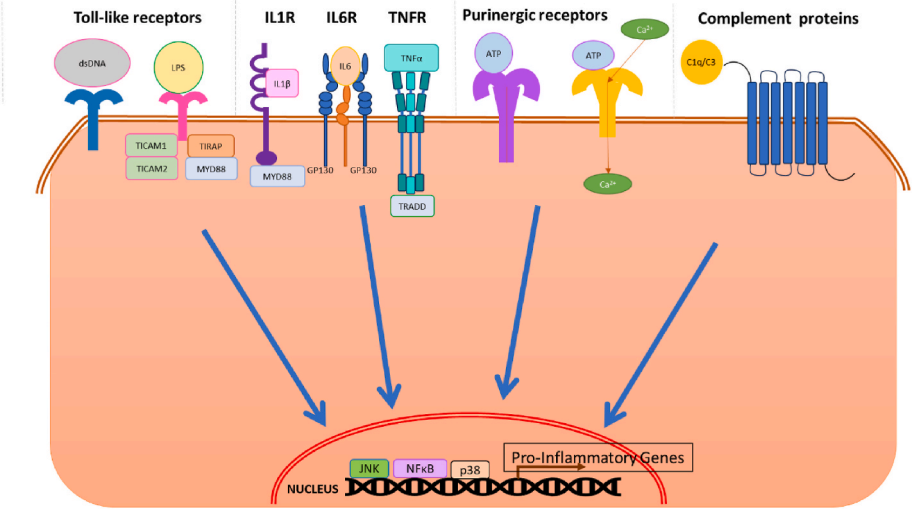

Fig. 2. Representative schematic of the main Damage Associated Molecular Patterns (DAMPs) and Pathogen Associated Molecular Patterns (PAMPs) in homeostatic microglia described in the current review. Microglia express a diverse set of receptors that allow microglia to respond to neuronal damage, including purinergic receptors and complement-associated receptors, and pathogens, such as Toll-like receptors and cytokine receptors. After activation, the intracellular cascade will end on the increased levels and release of cytokines.

can be rapidly induced in response to a variety of injuries. An increase in cytokines is a common response to hypoxic and excitotoxic insults, mainly due to the activation of microglia (Allan and Rothwell, 2003). Activation of microglia and astrocytes is the first response to seizure-like activity, and this activation can have a detrimental or beneficial effect on the brain (Gibbs-Shelton et al., 2023; Vezzani and Viviani, 2015). The release of IL-1β, TNF-α and IL-6 can modulate neuronal activity and result in the neural circuits to become more excitatory (Vezzani and Viviani, 2015). In adult mouse models, the elimination of microglia delayed the initiation of neural depolarization after neural activation (Szalay et al., 2016), suggesting that microglia may be involved in incrementing excitatory signals. On the other hand, in ex vivo experiments, depletion of microglia exacerbates NMDA-induced cell death in the hippocampus (Vinet et al., 2012). Further, microglial activation using the pro-inflammatory compound LPS protects against the induction of seizures in the pilocarpine model (Mirrione et al., 2010), and this effect was alleviated by abolition of microglia (Mirrione et al., 2010). This neuroprotective effect of microglia may be due to the release of IL-10, an anti-inflammatory cytokine (Bhandare et al., 2015). Whilst this data shows a possible dual effect of microglia in the adult brain, this cannot be fully extrapolated to the context of neonates. Administration of the microglia activator PamC3K2 before hypoxia has a detrimental effect on seizures (Quinlan et al., 2019).

After neonatal hypoxia, the brain may go into a vicious loop where microglia respond to increased neurotransmitter levels by releasing cytokines, and neurons respond to high levels of cytokines by releasing neurotransmitters, and so on (Fig. 3). Activation of microglia after neonatal hypoxia will result in the release of cytokines, including IL-1β and TNF-α (Quinlan et al., 2019). Elevated pro-inflammatory cytokine release can affect ionic currents in neurons (Beattie et al., 2002; Bezzi et al., 2001) and activate gene transcription in neurons and glia (Rothwell and Hopkins, 1995; Hopkins and Rothwell, 1995). IL-1β enhances NMDA receptor (N-methyl-D-aspartate receptor) activity by phosphorylation of the NR2AB subunit (NMDA Receptor subunit 2AB) (Viviani et al., 2003; Vezzani and Baram, 2007), and TNF-α regulates glutamate release in astrocytes and increases the expression of neuronal AMPA receptor (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor) (Viviani et al., 2003).

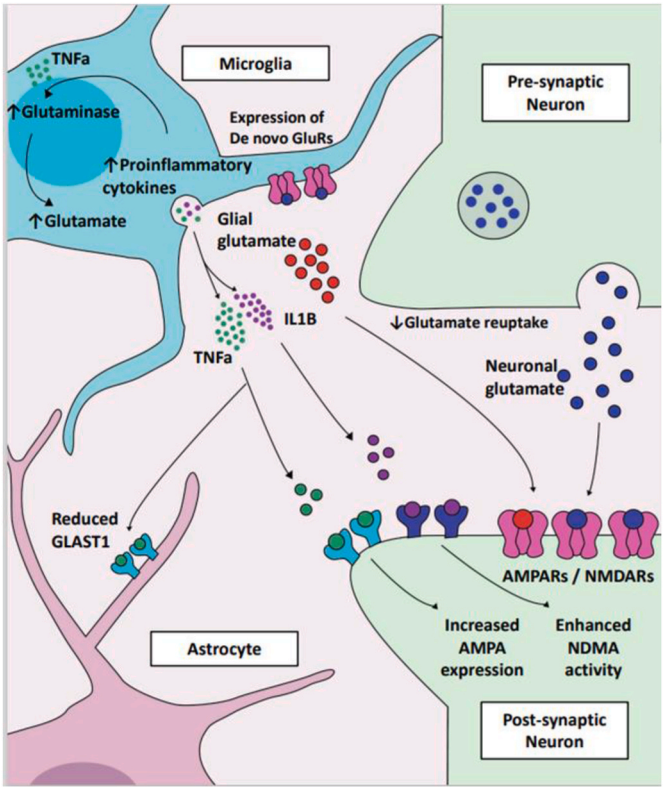

Fig. 3. Representative diagram of glutamate pathway after microglia activation. After an insult, microglia will release cytokines, this increase in cytokines will result in an increase expression of AMPA receptor and modification of NMDA electrical properties postsynaptically, and reduce levels of the glutamate transporter GLAST1 in astrocytes. Also, microglia will express de novo AMPA, Kainic Acid and metabotropic glutamate receptors (inducing further microglia activation). All these factors will result in increase of glutamate at the synapses, increase excitability and increase microglia activation.

In addition to their role as the resident immune cells of the brain (Paolicelli et al., 2022; Prinz et al., 2019), microglia have emerged as a key regulator of neuronal networks after it was shown that they respond to changes in neuronal activity (Illes et al., 2021; Prinz et al., 2019). Activation of microglia is complex, integrating both immune responses and neuronal function. To sense danger signals and/or homeostatic imbalance, microglia express a variety of transporters and receptors, including neurotransmitters and neuromodulators (Illes et al., 2021). Microglia-neuronal communication is particularly critical for the formation of neuronal circuits during development. When pathological activation of microglia even partially disrupts this communication, this can result in neurological disorders such as epilepsy (Di Nunzio et al., 2021). Indeed, mounting evidence has shown that microglia affect neuronal networks by inhibiting excitatory neurons and enhancing synaptic inhibition in healthy conditions (see next section). In adults after a brain insult, microglia express de novo ionotropic and metabotropic glutamate receptors (including AMPA, Kainic Acid (KA), metabotropic GluR2-5 receptors), and glutamate transporters (GLAST and GLT1) (Mead et al., 2012). The expression of AMPA and KA receptors on microglia stimulates the production of cytokines, contributing to inflammation and further neuronal damage (Gras et al., 2012). mGluR2 in microglia promotes neurotoxicity by stimulating further release of cytokines, glutamate, and Nitric Oxide (Taylor et al., 2005). This increase in inflammation, activated by glutamate, further contributes to damage and hyperexcitability through the activation of cytokine receptors in neurons. Importantly, the release of cytokines also adds to the glutamate system in microglia and astrocytes. High concentrations of intracellular TNF-α in microglia stimulate the activity of the enzyme glutaminase, resulting in a higher conversion of glutamine to glutamate (Takeuchi et al. 2006, 2008). A high release of TNF-α results in reduced expression of the astrocytic glutamate transporter GLAST1 (Takeuchi et al. 2006, 2008) and a reduction of glutamate re-uptake in the synapses. Additionally, the release of BDNF (Brain-derived neurotrophic factor) by microglia modulates dendritic spine density and increases the expression of AMPA and BDNF receptors (Parkhurst et al., 2013; Pascual et al., 2012). Consequentially, the combination of these factors contributes to further hyperexcitability and inflammation.

3. Microglia sense neuronal hyperexcitability

Microglia constantly monitor the microenvironment and throughout this process, microglia interact with neurons directly (Cserep et al., 2021). It is quite clear that the balance between excitation and inhibition regulates hyperexcitability, and we now know that microglia can inhibit neuronal activity in a process mediated by purines. This is demonstrated in studies of microglia-deficient mice, which present with more numerous and more severe seizures than same-age control mice in an acute model of Kainic Acid-induced seizures (Badimon et al., 2020). During neuronal activity, neurons release the co-transmitter ATP (Burnstock et al., 2011), which is sensed by microglia and converted to adenosine via hydrolysis by CD39 and CD73. Adenosine released from microglia then binds to the ADO-A1R (Adenosine A1 Receptor), having an inhibitory effect on neurons (Castellano et al., 2016; Lanser et al., 2017). Therefore, the two convertase enzymes CD39 (mainly expressed by microglia) and CD73 are the main regulators of microglial inhibition of neuronal activity (Castellano et al., 2016; Lanser et al., 2017).

Additionally, neuronal activity can regulate gene expression in microglia. CD39 can hydrolyse neuronal ATP to AMP, producing cAMP as a by-product of this reaction. Increased cAMP production activates the transcription factor CREB (cAMP response element-binding protein), which mediates transcriptional regulation of CD39 (Hu et al., 2023; Cao et al., 2023).

Inhibition of neuronal activity by microglia is also regulated by the microglial purinergic receptor P2Y12. P2Y12 is mainly expressed in homeostatic microglia (Rodrigues et al., 2019; Beamer et al., 2021), and during hyperexcitability ATP activates P2Y12, contributing to the inhibition of glutamatergic neurons (Badimon et al., 2020). Depletion of P2Y12 causes more severe seizures in a mouse model of adult epilepsy, showing that activated microglia may not have the same inhibitory capabilities as homeostatic microglia, and microglial negative feedback may also support interneurons in pathology (Badimon et al., 2020). Interestingly, reduced expression of P2Y12 has been seen in neurological disorders such as Alzheimer's or Huntington's disease, inferring that a reduction in microglia-driven inhibition of neuronal activity could explain the pathological excitability observed in these conditions.

Purinergic signalling is key to facilitating microglia-neuron communication. Over the past number of years, the ionotropic purinergic receptor P2X7 has received special attention for its role in the pathophysiology of neonatal hypoxia (Rodriguez-Alvarez et al., 2017; Smith et al., 2023). P2X7 expression is regulated during brain development, with more abundant expression found in the adult cortex and hippocampus when compared to expression in these regions during the early postnatal days (Rodriguez-Alvarez et al., 2017). Elevated expression of P2X7 is seen after neonatal hypoxia in both preclinical models and human studies of infants suffering from hypoxia at birth (Rodriguez-Alvarez et al., 2017). Other members of the P2X family, such as P2X2 and P2X1, are not affected by neonatal hypoxia, showing that the upregulation is specific to the P2X7 receptor (Rodriguez-Alvarez et al., 2017). Interestingly, genetic or pharmacological inhibition of P2X7 reduces seizure severity during hypoxia (Rodriguez-Alvarez et al., 2017; Smith et al., 2023), and the susceptibility of mice to develop provoked seizures later in life (Smith et al., 2023). How does P2X7 regulate hyperexcitability in mice? Activation of P2X7 triggers an intracellular cascade, resulting in the release of the proinflammatory cytokine IL-1β, which, as discussed earlier, increases glutamatergic neurotransmission (Rossi et al., 2012; Vezzani and Baram, 2007; Deuchars et al., 2001; Leon et al., 2008; Sperlagh et al., 2006). More recent evidence suggests that glutamatergic signalling is downregulated after neonatal hypoxia in P2X7-depleted mice (Smith et al., 2023), demonstrating that activation of P2X7 may increase glutamatergic signalling, and, consequentially, hyperexcitability. Importantly, P2X7 inhibition may have a dual action by downregulating both inflammation and excitatory signalling, which contributes to reduced hyperexcitability after neonatal hypoxia (Smith et al., 2023).

4. Microglia and circuit remodelling

Neurodevelopmental hyperexcitability can result from a lack of equilibrium between excitation and inhibition or pathological neural circuits. Microglial cells are critical for neuronal pruning and the removal of excess synapses during brain development. While several mechanisms have been implicated, the complement pathway plays a critical role during this process in development, including neurogenesis and neuronal migration (Rahpeymai et al., 2006; Coulthard et al., 2018; Gorelik et al., 2018) and synaptic refinement (Schafer et al., 2012; Schafer et al., 2013; Stephan et al., 2012). Glial cells (astrocytes and microglia) express high level of complement proteins in the developing CNS. In particular, microglia express complement receptors which facilitate phagocytosis and secrete cytokines, thus activating the complement cascade in neighbouring cells (Stephan et al., 2012).

The complement system is composed of a large family of circulating and membrane-bound proteins, all of which act together in a sequential, cascade-like manner to execute and regulate its function. The activation of complement results in structural modifications, proteolytic cleavage and the assembly of enzyme complexes (Stephan et al., 2012). The complement system can be divided into three major pathways based on differences in initiation: the classical, the alternative and the lectin pathway (Kang et al., 2009). Importantly, following their distinct initiation sequence, all of them converge on the C3 protein, a key protein on the complement pathway that drives complement function, including neuronal pruning during development. The classical pathway is activated by C1q, a receptor that binds to a diverse range of antigens, such as C reactive protein, DNA, RNA and apoptotic cells (Tan et al., 2011; Kang et al., 2009) and as previously seen, critical for microglia-astrocyte communication (Liddelow et al., 2017). The lectin pathway is traditionally activated by polysaccharides and glycoproteins (Degn et al., 2007; Fujita, 2002). In contrast to the classical and lectin pathways, the alternative pathway is spontaneously and continuously activated, and its function is to amplify the signal of the classical and lectin pathways (Stephan et al., 2012).

As seen before, C3 is critical for the function of the complement pathway, and upon activation, C3 is cleaved in C3a and C3b. C3a is a potent neuroinflammatory fragment that recruits microglia to the site of the injury (Nordahl et al., 2004; Zhou, 2012). This active fragment is delivered in a non-specific manner, and excessive activation may have a detrimental effect, so the progression and activation must be highly controlled. Microglia have a battery of complement regulatory proteins which are necessary to protect other cells. An equilibrium must be maintained between activation and inhibition of the complement cascade to prevent either over-pruning or under-pruning of neurons (Wang et al., 2020; Paolicelli et al., 2011). Inappropriate or uncontrolled complement activation can promote pathology, as seen in studies of C1q and C3 knock-out mice, which present with sustained deficits in synaptic refinement and elimination (Stevens et al., 2007). Furthermore, microglia mediate synaptic pruning during normal development in retinal ganglion cells via a mechanism dependent on the Complement Receptor 3 (CR3)/C3 pathway, whereby, following complement activation, microglia phagocytose neuronal presynaptic terminals in an activity-dependent manner. Genetic or pharmacological inhibition of CR3 and/or C3 results in disrupted microglia function, aberrant synaptic remodelling, and defective synaptic circuitry (Schafer et al., 2012).

In the context of neonatal hypoxia, local activation of complement was observed acutely after neonatal hypoxia-ischemia in rats, represented by an increase of the microglial C3b fragment and deposition of C3 and C9 proteins on the injured neurons. Inhibition of C3 with cobra venom factors shows a reduction in the size of the injured area and fewer C3 and C9 depositions (Cowell et al., 2003). Similarly, C1q depletion (classical pathway) in a mouse model of hypoxia-ischemia is neuroprotective and reduces C3 deposition in the infarct site (Ten et al., 2005). These results are supported by findings that the protective effects of therapeutic hypothermia have been linked to complement activation. Therapeutic hypothermia is the only approved therapy for neonatal hypoxia and involves inducing a mild state of hypothermia in the acute phase post-injury. In a rat model of hypoxia-ischemia followed by therapeutic hypothermia, C3a expression was upregulated and C5 expression was downregulated, implying that the protective effects of therapeutic hypothermia may result from the regulation of the complement pathway (Shah et al., 2017). In a follow-up study using the same unilateral model of hypoxia-ischemia, treatment with a combination of C1q1 inhibitors and therapeutic hypothermia attenuated cerebral damage and improved hippocampal function, demonstrated in the Barnes Maze and Novel-Object location test (Kumar et al., 2021). The improvements in cerebral damage and cognitive functions were similar between the two groups treated with C1q1 inhibitor either with or without therapeutic hypothermia, demonstrating that the therapeutic benefits of therapeutic hypothermia are mainly driven by the complement pathway.

Will activation of the complement pathway affect neural circuits

after neonatal hypoxia? While no direct evidence has shown whether aberrant complement activation affects neuronal morphology, neuron maturation is certainly affected in preclinical models of hypoxia in neonates (Quinlan et al., 2019; Sheikh et al. 2019, 2022). In a mouse model of neonatal hypoxia, subplate neurons from the auditory cortex isolated 3 weeks post-hypoxia appear to be more complex, with an increased number of nodes and ramifications, all of which are changes associated with abnormal functional connectivity (Sheikh et al. 2019, 2022). Similar results were observed in another mouse model of hypoxia, which found that when pyramidal neurons in the CA1 region of the hippocampus are studied 5 weeks post-hypoxia, they have more dendrites and appear more ramified than CA1 pyramidal neurons in same-age control mice (Quinlan et al., 2019). Furthermore, these more ramified pyramidal neurons have a lower seizure threshold, showing that the circuit was more susceptible to developing hyperexcitability (Quinlan et al., 2019). Future studies are needed to clarify whether activation of the complement pathway after neonatal hypoxia is responsible for the pathological neuronal differences.

5. Current challenges and future directions

Microglia are dynamic cells that change throughout life, these changes are critical for microglia to respond appropriately to the needs of the brain during development. In adult brains, the microglia response to insults and stress has been extensively studied, however, less is known about microglial responses to injurious stimuli in the developing brain. This is likely due to the challenges posed by neurodevelopmental conditions, which are characterized by subtle changes over time, small changes in microglia response that are maintained over time, potentially resulting in erroneous neuronal maturation and neurodevelopmental conditions.

Differences between the mature and developing brain are seen in models of ischemia. Microglia inhibition may have a protective role in the adult brain after ischemia (Jin et al., 2014; Shang et al., 2020; Shi et al., 2021), however, mounting evidence suggests that microglia inhibition has a detrimental effect on the developing brain, functions as phagocytosis are critical for neural circuits maturation. Murine models of adult ischemia shows an increase in neurogenesis (Jin et al., 2014), however, neurogenesis is not affected in neonates after hypoxia or hypoxia ischemia (Quinlan et al., 2019; Ehlting et al., 2022). This evidence points to basic differences between adults and neonates, and future studies may compare side-by-side difference responses to insult depending on age.

In pathology, microglia activation can have both positive and detrimental effects. Additional research is needed to pinpoint the exact functions of microglia that are key for normal brain development. Microglia malfunction is not capable of carrying out their homeostatic functions, which may be a key factor in the development of neurological disorders or, maybe, gaining of pathological functions following neuroinflammation is the trigger of pathological features.

It is well known the role of microglia in the synapses in the adult brain, but little is known of its contribution to the developing brain, further research will be necessary to evaluate if microglia during development express neurotransmitter receptors, and if the receptors contribute to the communication between neurons and microglia or their contribution to pathology after an early life insult.

Within the hippocampus, the main area affected by neonatal hypoxia is the CA1 region (Quinlan et al., 2019). Future experiments may evaluate if this response is due to the differences in regional microglia as seen in aging, where microglia from CA1 and CA3 differ from each other and contribute differently to neurodegeneration (Lana et al., 2023).

Finally, while the mechanisms described in this review have been evaluated individually, future studies may also explore which processes are key to the development of neurological conditions after hypoxia, or early life insults during the perinatal period.

6. Conclusion

Brain maturation is a complex, finely tuned, time-dependent process, requiring continuous communication between astrocytes, neurons, microglia, and oligodendrocytes. Neural circuit formation is a complex process regulated by synaptic activity that is dependent on the contribution of glial cells, particularly microglia. Furthermore, microglia are critical for the refinement of neurons, with both excessive and limited removal of synapses resulting in neurological conditions. In this review, we have summarized some of the mechanisms underlying the process of brain maturation during the pre- and post-natal periods, and how these processes may exacerbate hyperexcitability after neonatal hypoxia.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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