J Neuroimmune Pharmacol (2013) 8:66–78
DOI 10.1007/s11481-012-9347-2

INVITED REVIEW

Roles of Activated Microglia in Hypoxia Induced Neuroinflammation in the Developing Brain and the Retina

Charanjit Kaur · Gurugirijha Rathnasamy · Eng-Ang Ling

Received: 16 September 2011 / Accepted: 9 February 2012 / Published online: 26 February 2012

Abstract Amoeboid microglial cells (AMCs) in the developing brain display surface receptors and antigens shared by the monocyte-derived tissue macrophages. Activation of AMCs in the perinatal brain has been associated with periventricular white matter damage in hypoxic-ischemic conditions. The periventricular white matter, where the AMCs preponderate, is selectively vulnerable to hypoxia as manifested by death of premyelinating oligodendrocytes and degeneration of axons leading to neonatal mortality and long-term neurodevelopmental deficits. AMCs respond vigorously to hypoxia by producing excess amounts of inflammatory cytokines e.g. the tumor necrosis factor–α (TNF-α) and interleukin-1β (IL-1β) along with glutamate, nitric oxide (NO) and reactive oxygen species which collectively cause oligodendrocyte death, axonal degeneration as well as disruption of the immature blood brain barrier. A similar phenomenon is observed in the hypoxic developing cerebellum in which activated AMCs induced Purkinje neuronal death through production of TNF-α and IL-1β via their respective receptors. Hypoxia is also implicated in retinopathy of prematurity in which activation of AMCs has been shown to cause retinal ganglion cell death through production of TNF-α and IL-1β and NO. Because AMCs play a pivotal role in hypoxic injuries in the developing brain affecting both neurons and oligodendrocytes, a fuller understanding of the underlying molecular mechanisms of microglial activation under such conditions would be desirable for designing of a novel therapeutic strategy for management of hypoxic damage.

Keywords Amoeboid microglia · Hypoxia · Developing brain · Retina · Oligodendrocyte/neuronal damage · Inflammatory cytokines · Reactive oxygen species

Abbreviation

AMCs	Amoeboid microglial cells
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
CNS	Central nervous system
CR3	Complement type 3 receptors
CSF-1	Colony stimulating factor
GluR2-4	AMPA glutamate receptors
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IL-1 β	Interleukin-1β
IL-1R	Interleukin 1 receptor
iNOS	Nitric oxide synthase
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage-colony stimulating factor
MHC I	Major histocompatibility class I antigens
MHC II	Major histocompatibility class II antigens
NMDA	N-methyl-D-aspartate
NR1, NR2A-D	NMDA receptor subunits
NO	Nitric oxide
PWM	Periventricular white matter
PWMD	Periventricular white matter damage
RGC	Retinal ganglion cell
RGCs	Retinal ganglion cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TβRI and TβRII	Transforming growth factor receptors I and II

Charanjit Kaur and Eng-Ang Ling contributed equally to this manuscript.

C. Kaur (✉) · G. Rathnasamy · E.-A. Ling (✉)
Department of Anatomy, Yong Loo Lin School of Medicine,
National University of Singapore,
4 Medical Drive, MD10,
Singapore 117597, Singapore
e-mail: antkaurc@nus.edu.sg
e-mail: antlea@nus.edu.sg

TGF-β1	Transforming growth factor β1
TNF-α	Tumor necrosis factor –α
$TNF\text{-}R_1$, $TNF\text{-}R_2$	TNF receptor 1 or 2

Introduction

The brain consists of the neurons and three major types of glial cells, namely the astrocytes, oligodendrocytes and microglia. In the latter, the first description dates back to the late 19th century when Nissl (1899) described them as "the rod cells". Following this, Cajal (1913) referred to them as the 'third element' other than the neurons and neuroglia. del Rio-Hortega (1932) had identified the glial type with the weak silver carbonate staining, considered then to be a reliable staining method for microglia. The identification and characterization of microglia was established later by electron microscopy, histochemical and immunohistochemical staining. Microglial cells are ubiquitous in the central nervous system (CNS). In the mature brain, they are characterized by a small flattened cell body giving rise to a variable number of branching processes or ramifications. The occurrence of the nascent form of microglia is well documented in the developing brain (Ling and Wong 1993). Termed the amoeboid microglial cells (AMCs), the cells have a rounded cell body which emits filopodial and pseudopodial processes.

AMCs are distributed preferentially in large numbers in the developing white matter tracts notably in the supraventricular region of the corpus callosum, also called the periventricular white matter (PWM). The PWM in the early postnatal brain consists of unmyelinated nerve fibres and glial cells including the astrocytes, oligodendrocytes, glioblasts and AMCs. It is well documented that the developing brain is highly susceptible to hypoxic damage, the PWM being selectively vulnerable to hypoxic-ischemic damage in premature infants (Johnston 1997; McQuillen and Ferriero 2004; Volpe 2003; Follett et al. 2004; Folkerth 2006). Hallmark features of PWM damage are death of immature oligodendrocytes before the onset of myelination and axonal degeneration (Dammann et al. 2001; Ness et al. 2001). Although several factors such as increased release of glutamate, oxidative and nitrosative stress and inflammation have been implicated in the pathogenesis of PWM damage, the contributory role of AMCs to the damage have remained elusive until recently.

Amoeboid microglial cells

Distribution

Although ubiquitously distributed in the brain, the AMCs are mostly located in the developing white matter tracts forming a conspicuous colony in the PWM (Fig. 1a); other areas near the PWM where the AMCs preponderate include the cavum septum pellucidum and the subventricular zone (Fig. 1b). The cells exist either singly or in clusters and may be closely associated with the blood vessels (Fig. 1c). A majority of the AMCs have a rounded cell outline bearing a few stout cytoplasmic processes. At the ultrastructural level, the abundant cytoplasm shows dense granules of various sizes, scattered mitochondria, stringy cisternae of rough endoplasmic and a well-developed Golgi complex (Fig. 2a). Some membrane bound vacuoles and a few lipid droplets are mainly confined to the periphery of the cytoplasm. The round or oval nucleus showing condensed chromatin masses is usually placed eccentrically. By scanning electron microscopy, the cell surface exhibits blebs and filopodial projections (Fig. 2b).

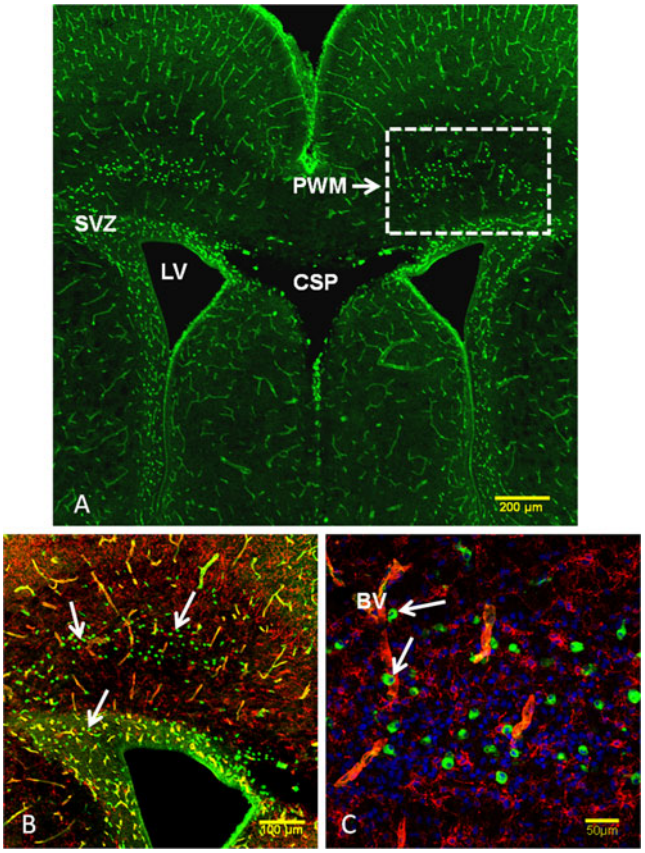

Fig. 1 **A** Confocal image of lectin stained brain section showing the distribution of amoeboid microglial cells (AMCs) in the developing brain of a 5-day old rat. Note the concentration of AMCs (*green*) in the periventricular white matter (PWM, boxed area) immediately above the lateral ventricle (LV). AMCs are also localized in the subventricular zone (SVZ) as well as the cavum septum pellucidum (CSP). Scale bar=200 µm. **B** shows enlarged view of AMCs (*arrows*) in the PWM and SVZ. Merged image of lectin (*green*) and NG2 (*red*) staining. Scale bar=100 µm. **C** AMCs (*green*) are mostly round in outline; some of them (arrows) are closely associated with the NG2 (*red*) labeled blood vessels (BV). Merged image of lectin (*green*) and NG2 (*red*) labeling. Scale bar=50 µm

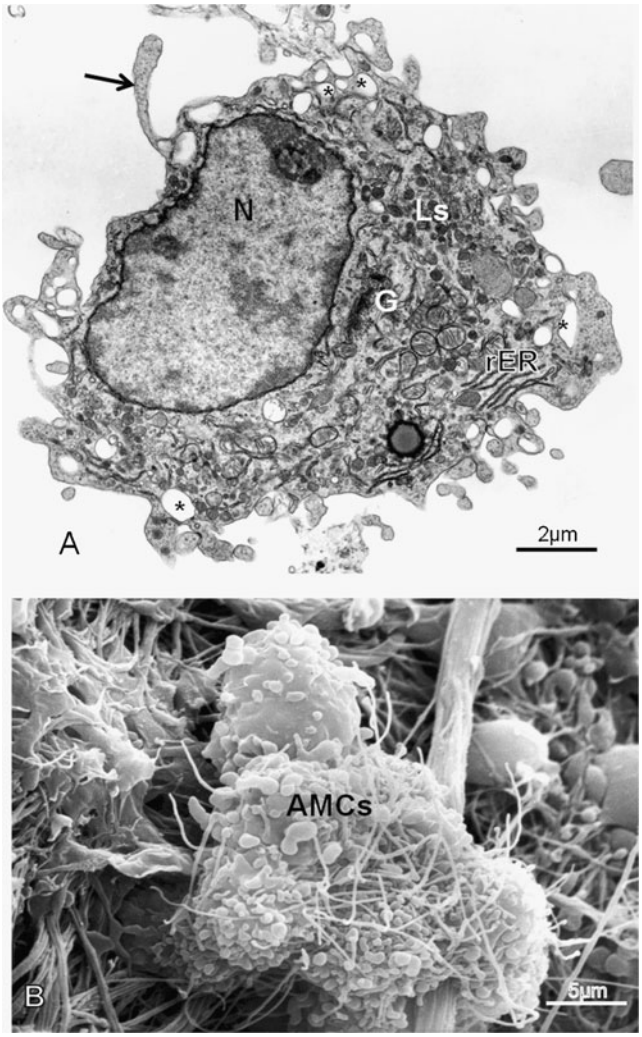

Fig. 2 **A** Transmission electron micrograph shows an AMC in the periventricular white matter. The abundant cytoplasm shows a Golgi complex (G), lysosomes (Ls), cisternae of rough endoplasmic reticulum (rER) and vacuoles (*asterisk*). The nucleus (N) shows margination of chromatin. Note the cell shows surface projections including filopodia (*arrow*). Scale bar= 2 μm. **B** Scanning electron micrograph showing a cluster of six AMCs in the cavum septum pellucidum. Note surface projections including microvilli, blebs and slender filopodial processes. Scale bar=5 μm

Tracer studies have shown that the AMCs transform into ramified microglia with advancing age assuming an oval cell body with concomitant reduction in cytoplasm (Leong and Ling 1992; Wu et al. 1994). In the course of microglial development, some of the AMCs undergo apoptosis and appear to be engulfed by their companion AMCs; others emigrate to other areas (Ling and Tan 1974; Imamoto and Leblond 1978). The mechanism guiding this 'self-elimination process' of the microglial cell population has remained obscure.

Monocytic and macrophagic nature of AMCs

The description of source of microglial precursors had been a debated issue and focus of many investigators in the past few decades. In animals such as rodents and in humans two different pools of myeloid cells are thought to be the precursors of microglia—progenitors invading the early embryonic brain from the yolk sac (Alliot et al. 1999; Cuadros et al. 1993), and monocytes invading the brain during late embryonic or early postnatal period (Ling and Tan 1974; Imamoto and Leblond 1978; Ling et al. 1982; Kaur et al. 1984). Using lectin labeling we have shown that the first colonization of macrophages in the fetal mouse brain was independent of its vascularization and that the cells appear to originate from some lectin-labeled precursor cells in the yolk sac (Kaur et al. 2001). Several studies carried out in our laboratory using histochememical and immunohistochemical techniques have demonstrated the entry of monocytes into the developing brain to become AMCs (Ling et al. 1980, 1982; Kaur et al. 1984; Ling et al. 1990). The finding that microglial cells express receptors for colony stimulating factors (CSFs), a group of cytokines known to regulate cells of monocytic lineage, and that CSF-1 induces proliferation and morphological changes in microglia provides support to the monocytic nature of microglia (Sawada et al. 1990; Suzumura et al. 1990; Shafit-Zagardo et al. 1993). As in monocytes, CSF-1 has been reported to increase the lysosomal activity in microglia, indicating the similarity of microglia to the mature cells of monocytic lineage (Suzumura et al. 1990). Additionally, we have shown that AMCs are labeled by ED1, a cellular marker for monocytes/macrophages (Kaur and Ling 1995, 1999). It needs to be pointed out that the early proposal of the monocytic and macrophagic nature of AMCs (Ling and Wong 1993) is fundamental to the understanding of the roles of these cells both in physiological and pathological conditions as described below.

Functions of AMCs

Phagocytosis and antigen presentation

The ability of AMCs to phagocytose degenerating axons and cells is evident during normal brain development (Kaur et al. 1985) as well as in experimental/pathological conditions where they have been found to be engaged in removal of apoptotic or necrotic cells (Kaur et al. 2006a). The macrophagic nature of AMCs is further established by the fact that they possess a repertoire of hydrolytic enzymes such as acid phosphatase, aryl sulphatase, non-specific esterase and 5′- nucleotidase (Ling 1977; Ling et al. 1982; Kaur et al. 1984) that are also present in macrophages at other sites in the body. Internalization of intraperitoneally or intravenously administered exogenous agents such as rhodamine isothiocyanate and horseradish peroxidase or *E coli* reaching the immature brain through the vascular route by AMCs (Kaur et al. 1986, 2004; Xu et al. 1993) further strengthens the notion that they are active phagocytes. Expression of

complement type 3 receptors (CR3) on the AMCs, known to be involved in endocytosis (Ling et al. 1990) further corroborates the macrophagic lineage of these cells as CR3 are also expressed on other tissue macrophages (Newman et al. 1980; Beller et al. 1982; Abrahamson and Fearon 1983) and are known to be involved in endocytosis (Ling et al. 1990)

Expression of major histocompatibility (MHC) class I antigens (Ling et al. 1991) under normal conditions and MHC class II under pathological and experimental conditions (Xu and Ling 1994a) ascertains the possibility that the cells could present antigens to lymphocytes in the event of an infection or lymphocytes entering the brain through a breach in the vascular walls. In vitro studies have shown that in response to inflammation, activated microglia up regulate the expression of CD45 along with co-stimulatory molecules such as CD40, CD80, CD86 which are essential for antigen presentation and T cell activation (Aloisi et al. 1998). Activation of intracerebrally recruited T cells depends on the ability of MHC class II positive microglia to endocytose and present antigens to the T cells (Aloisi 2001). For example, in human fetal microglial cultures, following toll like receptor 3 induced activation, microglia expressing MHC class II were reported to activate CD4+ T cell response (Jack et al. 2007). Several in vivo and in vitro studies have shown the antigen presenting function of microglia through the acquisition of macrophagic properties (see review by Aloisi 2001).

Other functions

Besides phagocytosis, AMCs also partake in certain development events such as promotion of oligodendrocyte proliferation through secretion of insulin-like growth factor-1 (IGF-1) that is required for the proper development of the neural tissues. IGF-1 is known to foster proliferation of oligodendrocytes as well as their myelin synthesis in the developing brain (Dubois-Dalcq and Murray 2000; Guan et al. 2001). Expression of IGF-I and IGF-2 on the AMCs has been reported (Kaur et al. 2006b) and it was suggested that this may also be involved in enhancing the phagocytic activity as has been reported in the peritoneal macrophages (Inoue et al. 1995). IGF-2 may play a role in myelination (Logan et al. 1994; Walter et al. 1999) or in the phagocytic activity of AMCs (Kaur et al. 2006b).

We have recently reported that transforming growth factor (TGF)-β1, a prototype of multifunctional growth factors generally considered as an anti-inflammatory cytokine, and its receptors TβRI and TβRII are expressed constitutively by the AMCs in the PWM of neonatal rats. The expression of TGF-β1 and its receptors in the AMCs was markedly upregulated after a hypoxic exposure suggesting that it may help to autoregulate microglial activation in adverse conditions via its receptors (Li et al. 2008).

The varied functions of microglia came to light from the recent studies which demonstrated the involvement of microglia in vasculogenesis, synaptic pruning, neurogenesis and astrogenesis. Microglia which invade the retina and brain prior to vascularisation are found in association with the tip cells of growing vascular plexus (Fantin et al. 2010; Rymo et al. 2011) and have an intimate association with vascular endothelium and vascular sprouts (Provis et al. 1997). The depletion of microglia in two mutant mouse models: macrophage colony-stimulating factor (M-CSF)/CSF-1 deficient mice and PU.1 null mice, resulted in sparser vascular complexity implicating the essential role of microglia in vasculogenesis (Kubota et al. 2009; Fantin et al. 2010). In addition it has been suggested that the association of AMCs with the microvessels in the postnatal PWM may have a role in maintaining the function of the blood- brain barrier by ingesting any serum- derived foreign substances (Xu and Ling 1994b; Kaur et al. 1986).

The role of microglia in synaptic elimination during postnatal synaptic remodelling has gained importance in recent years. Immunohistochemical studies in the past have revealed the presence of microglia bearing many processes in the brain (Perry et al. 1985; Fiske and Brunjes 2000) which are now known to interact with the synapses. The frequent and transient contacts established by microglial processes with the synapses suggest that microglia could have a role in monitoring and in remodelling the synapses (Wake et al. 2009; Tremblay et al. 2010). As synapses are endowed with complement component C3, the recognition of these components by microglial complement receptors was implicated in synaptic elimination (Schafer et al. 2010). In mice lacking Cx3cr1, a chemokine fractalkine receptor expressed by microglia, there was a delay in synaptic pruning due to the impairment in migration of microglia towards the neurons expressing chemokine fractalkine, Cx3cl1(Paolicelli et al. 2011).

Though microglia phagocytose supernumerary neurons and synapses as a process of development they also have a role in defining the neural precursor environment. The presence of microglia in the neural precursor regions has been implicated in the regulation of precursor proliferation and astrogenesis (Antony et al. 2011). Several in vitro studies have demonstrated the role of microglia in causing differentiation of neural precursor cells to neuronal phenotype (Aarum et al. 2003), basal progenitor cells to cholinergic neruons (Jonakait et al. 1996, 2000) and in directing the migration of neural precursors (Aarum et al. 2003). Also, astrogenesis was found to be reduced in PU.1$^{-/-}$ mice, owing to a reduction in microglia numbers (Antony et al. 2011) and this was attributed to the reduced availability of microglial secretory factors such as leukemia inhibitory factor (Zhu et al. 2008). Besides the above, it was suggested that the AMCs in the PWM could have a role in axon growth and guidance during development (Streit 2001).

Activation of AMCs in pathological states

In pathological conditions or injuries of the mature brain, the microglia transform into "activated microglia" or "reactive microglia" when they retract their processes and their cell bodies become larger. However, in the developing PWM, activation of AMCs following an injury does not necessarily involve a change in its shape but is manifested by alteration in the expression of surface receptors and antigens. For example, the expression of CR3 and MHC I antigens was drastically enhanced along with induction of MHC II antigens when the cells were challenged with lipopolysaccaride (Xu and Ling 1994a) or *E coli* (Kaur et al. 2004) suggesting that the phagocytic activity and antigen presentation capability of the AMCs was increased. Furthermore, they were involved actively in removing apoptotic/necrotic cells and degenerating axons in the PWM following a hypoxic injury (Kaur et al. 2006a). The expression level of proinflammatory molecules notably tumor necrosis factor (TNF)-α and interleukin (IL)-1β by the AMCs following hypoxic-ischemic injuries is markedly enhanced when compared to that under normal conditions (Fig. 3) suggesting the involvement of AMCs in an inflammatory reaction (Deng et al. 2008). Activated AMCs are known to proliferate and migrate to the site of injury so that their numbers are increased significantly (Deng et al. 2009). Other than the cytokines, activated AMCs are known to produce nitric oxide (NO) through inducible nitric oxide synthase (iNOS) which may be detrimental to the oligodendrocytes (Merrill et al. 1993; Kaur et al. 2006a). Activated AMCs can also generate reactive oxygen species (ROS) when stimulated by hypoxia (Kaur and Ling 2009). As AMCs are prevalent in the PWM, it is conceivable that excess production of a plethora of neurotoxic factors would inundate the tissue and affect the ambient cellular constituents such as oligodendrocytes, axons etc.

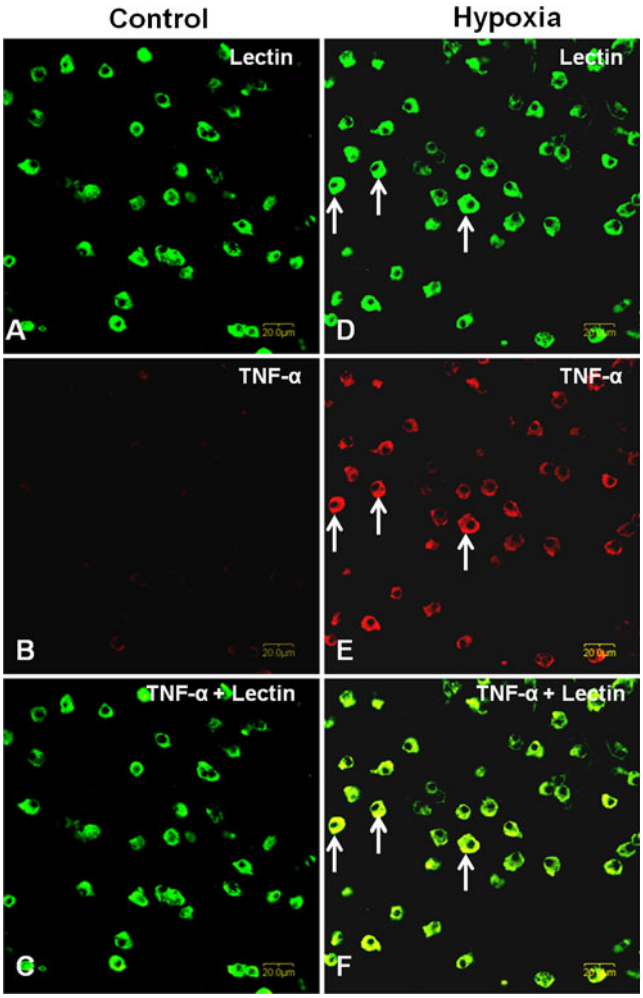

Fig. 3 An enlarged view of lectin (*green*) labeled AMCs in the periventricular white matter in neonatal rats in the control (**A**,**B**,**C**) and at 3 d after hypoxic exposure (**D**,**E**,**F**). TNF-α immunoexpression is absent in AMCs in the control (**B**), but is evidently induced after hypoxic exposure (**E**). Arrows indicate co-localization of lectin and TNF-α in AMCs as evident in the merge image (**F**). Scale bar (A–F)=20 μm

Hypoxia induced periventricular white matter damage in the developing brain

Neuropathology

Hypoxia-ischemia is a leading cause of morbidity and mortality in the perinatal period (Calvert and Zhang 2005). The developing brain is highly vulnerable to oxygen deprivation or hypoxia which affects its normal development and maturation (Perlman 2006). Placental insufficiency, decreased utero-placental blood flow and premature onset of labor or prolonged labor are risk factors which may compromise fetal oxygenation. Neonatal hypoxia results from pulmonary and/or cardiac dysfunction or neonatal stroke (Jiang et al. 2003). The PWM is selectively vulnerable to damage in premature infants (McQuillen and Ferriero 2004; Folkerth 2006).

Swollen or degenerating axons, activated microglia, astrocytosis and oligodendroglial death have been reported as the main pathological features of hypoxic-ischemic damage in the PWM in various animal models (Takashima et al. 1995; Skoff et al. 2001; Ness et al. 2001; Kaur et al. 2006a). AMCs engaged in phagocytosis of the degenerating axons and necrotic/apoptotic cells is a hallmark feature of periventricular white matter damage (PWMD) (Kaur and Ling 2009). Delayed myelination as evidenced by significantly decreased myelin basic protein (MBP) (Kaur et al. 2006a) and cystic cavities are known to occur in late stages of the PWMD. These changes may be accompanied by the presence of edema in the PWM, hemorrhages, dilatation of the lateral ventricles and structural alterations in the ependymal lining and the choroid plexus epithelial cells.

Pathogenesis

The factors underlying hypoxia-ischemia induced white matter damage have been the focus of several investigations in recent years. Both in vivo and in vitro methods have been adopted to elucidate the pathogenic mechanisms leading to oligodendrocyte death and axon damage. Increased release of glutamate, free radical generation and inflammation in the PWM in response to hypoxia are culpably involved in the pathogenesis of white matter damage. These are discussed below with special reference to the involvement of AMCs in causing damage through production of inflammatory cytokines and free radicals. Along with this, our recent findings have further extended the role of glutamate induced cell death and increased iron accumulation in causing PWMD through the AMCs.

Role of AMCs in glutamate induced cell death

In hypoxic-ischemic injuries, increased accumulation of extracellular glutamate in the white matter may occur possibly due to release from damaged axons and glia (Meng et al. 1997; Fern and Møller 2000). Glutamate increase in neural tissues results in overactivation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors which are considered as major players in mediating injury to oligodendrocytes in hypoxic-ischemic conditions (Yoshioka et al. 1995; McDonald et al. 1998; Deng et al. 2003; Salter and Fern 2005; Matute et al. 2007). We have reported recently that glutamate concentration in the PWM of neonatal rats was increased following a hypoxic injury (Sivakumar et al. 2010). AMCs were shown to release increased levels of glutamate following the hypoxic injury. Concomitantly, the expression of AMPA glutamate receptors (GluR2-4) and NMDA receptor subunits (NR1, NR2A-D) was increased on the AMCs (Kaur et al. 2006a; Sivakumar et al. 2010; Murugan et al. 2011). Overactivation of glial AMPA receptors has been described to play an important role in disruption of axons (Tekkok and Goldberg 2001) and oligodendrocyte damage (Fern and Møller 2000; Fontaine et al. 2002).

Expression of NMDAR receptors on AMCs was proposed to cause damage to oligodendrocytes through activation of NF-κB pathway. Glutamate induced NMDA receptor activation in AMCs was found to induce iNOS expression via the NF-κB signalling pathway in neonatal rats following a hypoxic exposure (Murugan et al. 2011). A decrease in hypoxia induced NMDAR-mediated iNOS expression by microglia was observed when the cells were incubated with BAY, a selective inhibitor of NF-κB 30 min prior to hypoxia treatment (Murugan et al. 2011). Expression of iNOS in the AMCs is known to result in tissue damage through production of NO. In vitro studies have shown that NO produced by AMCs is highly damaging to the oligodendrocytes by causing their lysis (Merrill et al. 1993). Enhanced death of oligodendrocytes has been reported when primary oligodendrocytes were treated with conditioned medium from microglia cultures exposed to hypoxia; the cell death was reduced with NMDA receptor antagonist MK801, BAY and iNOS inhibitor1400w. In the light of these results, it was suggested that activation of NMDARs on the AMCs by glutamate following hypoxia might be involved in iNOS synthesis via activation of NF-κB leading to death of oligodendrocytes (Murugan et al. 2011). These observations are supported by previous studies which have shown that hypoxia induced the expression of iNOS on the AMCs in the PWM of hypoxic neonatal rats (You and Kaur 2000; Kaur et al. 2006a).

Besides the excitotoxic effects, increased release of glutamate may also be involved in enhancing the release of proinflammatory cytokines TNF-α and IL-1β by the AMCs. Primary cultures of AMCs subjected to glutamate showed significantly higher release of these cytokines when compared to the controls (Sivakumar et al. 2010) suggesting that increased release of glutamate under hypoxic states may activate the AMCs to produce higher amounts of cytokines. In addition to this, the AMCs may also contribute to accumulation of excess glutamate in the extracellular spaces in the hypoxic PWM as our in vitro experiments showed that the primary microglia subjected to hypoxia release higher amounts of glutamate (Sivakumar et al. 2010).

Very interestingly, glutamate was also found to suppress the release of IGF-I and IGF-II by the AMCs (Sivakumar et al. 2010). IGF-I plays a significant role in recovery from insults such as hypoxia-ischemia (Guan et al. 2003). Glutamate induced reduction in release of these growth factors by the AMCs may cause injury to the oligodendrocytes and axons due to excitotoxicity and enhanced production of inflammatory cytokines. This is especially so in view of our findings that primary microglial cells transfected with IGF-I siRNA showed a signifant increase in glutamate, TNF-α, and IL-1β production (Sivakumar et al. 2010).

Role of AMCs in inflammation

The involvement of inflammation in causing PWMD in hypoxic-ischemic conditions is well recognized (Kadhim et al. 2002). Hypoxia is an important stimulus for production of inflammatory chemokines and cytokines (Carloni et al. 2006; Guo and Bhat 2006). We have shown that activated AMCs express enhanced levels of TNF-α and IL-1β in response to hypoxia (Fig. 3) (Deng et al. 2008). In response to hypoxia, AMCs also produce other molecules such as syndecan-2, one of the major heparan sulfate glycosaminoglycan-containing cell surface proteins, and exhibit enhanced expression of ion

channels such as Kv1.1 and Kv1.2 (Li et al. 2008; Kaur et al. 2009a; Wu et al. 2009) that have been reported to promote the release of inflammatory cytokines and chemokines (Li et al. 2008; Kaur et al. 2009a, b; Wu et al. 2009).

TNF-α acts through its receptor 1 (TNF-R_1) or 2 (TNF-R_2). Activation of TNF-R_1 elicits caspase signal pathways that lead to cell apoptosis (Nakazawa et al. 2006). TNF-R_2 is known to activate Akt signaling pathway to promote cell growth and proliferation (Fontaine et al. 2002). Expression of TNF-R_1 on the oligodendrocytes in the PWM was found to be increased after a hypoxic exposure in neonatal rats suggesting that TNF-α may induce apoptosis of oligodendrocytes via binding to TNF-R_1 (Deng et al. 2008). The unmyelinated axons also showed an upregulated expression of TNF-R_1 coupled with the disruption of MBP immunopositive processes of oligodendrocytes in the PWM of hypoxic neonatal rats suggesting that overproduction of TNF-α may damage axons and delay their myelination via binding to their respective receptors (Deng et al. 2010).

IL-1β acts through binding to type I and type II IL-1R. The expression of IL-$1R_1$ on the oligodendrocytes in PWM in hypoxic rats was found to be increased significantly (Deng et al. 2008). Although IL-1β was reported as being non toxic to oligodendrocyte lineage cells as oligodendrocyte apoptosis was not induced through this receptor, it can block oligodendrocyte proliferation at the late progenitor/pro-oligodendrocyte stage (Vela et al. 2002) suggesting that white matter development and recovery in hypoxic conditions via inhibition of oligodendrocyte progenitor proliferation by IL-1 β may be delayed. In addition to these observations, IL-1β and TNF-α may also be involved in the activation of iNOS gene (Lopez-Figueroa et al. 2000; Kadhim et al. 2006) and hence generation of NO. In support of these observations, AMCs were found to express iNOS in response to hypoxia (Kaur et al. 2006a).

AMCs may also be involved in exacerbation of the inflammatory response through secretion of monocyte chemoattractant protein-1 (MCP-1) and macrophage-colony stimulating factor (M-CSF). MCP-1 is a chemokine which modulates migration of activated microglial cells and leukocytes to the inflammatory sites in the CNS (Lu et al. 1998; Rankine et al. 2006; Yan et al. 2007) through binding with its G protein-coupled receptor CCR2. It was reported that the primary source of MCP-1 in the neonatal brain was the AMCs (Deng et al. 2009). MCP-1, its receptor CCR2 and the numbers of AMCs increased in the PWM in response to hypoxia. This was attributed primarily to the migration of AMCs from the neighboring areas of the brain or from invasion of the monocytes into the hypoxic brain (Deng et al. 2009).

M-CSF, also known as colony stimulating factor (CSF)-1, is a cytokine released mainly by macrophages, T cells, B cells, microglia (Lee et al. 1993) and astrocytes (Hao et al. 1990; Lee et al.1993) and is an important mediator of inflammation (Hao et al. 2002; Deng et al. 2010). M-CSF exerts its actions by binding to its receptor CSF-1R and is known to have a role in microglial inflammatory response (Murphy et al. 2000). Following brain injury or in diseases such as Alzheimer's disease, up-regulation of M-CSF in activated microglia is accompanied by CSF-1R expression (Murphy et al. 2000; Takeuchi et al. 2001). The overexpression of M-CSF receptors on the microglia is suggested to exacerbate the inflammatory process by propagating the proinflammatory signals to the nearby resting microglia and astrocytes through increased production of proinflamamtory cytokines (Hao et al. 2002). Our recent study showed enhanced expression of M-CSF by AMCs in the PWM in response to hypoxia and, concurrently the astrocytes expressed amplified CSF-1R, TNF-α and IL-1β (Deng et al. 2010). It was suggested that the interaction between AMCs and astrocytes via the M-CSF and its receptor led to release of proinflammatory cytokines such as TNF-α and IL-1β from the astrocytes augmenting the inflammatory response in the PWM of the hypoxic neonatal rats.

Though CSF-1R was suggested to be a definitive marker of cells of mononuclear phagocyte lineage which includes microglia (Sasmono et al. 2003) previous studies have demonstrated the proximal c-*fms* promoter activity in the astrocytes (Tkachuk and Gisler 1997) and expression of the mRNAs of receptors for M-CSF and GM-CSF in astrocytes (Sawada et al. 1993). The discrepancy might be due to the description of the c-*fms* promoter region in different studies. Additionally the presence of GM-CSF receptors on the cell membrane of the cultured simian astrocytes was demonstated by Guillemin et al. (1996) using immunofluorescence technique.

Role of AMCs in oxidative stress

Under normal conditions, the generation of reactive oxygen species (ROS) in the tissues of the body is balanced as the endogenous antioxidants either neutralize or eliminate them. Oxidative stress occurs when the balance between the formation of ROS and the ability of cells to defend against them is disrupted. ROS is a product of the multi subunit phagocyte NADPH oxidase comprising of P22 ^{phox}, P47 ^{phox}, P67 ^{phox} and gp91 ^{phox} (Bedard and Krause 2007). The abundant amount of ROS produced by the activated microglial cells is through the induction of phagocyte NADPH oxidase (Lavigne et al. 2001; Huo et al. 2011). ROS produced by microglia causes detrimental effects to the neurons and oligodendrocytes and has been implicated in causing damage to myelin sheath (van der Goes et al. 1998). Release of ROS by AMCs in the PWM is increased significantly following hypoxic injury (Kaur et al. 2009a; Rathnasamy et al. 2011). Immature oligodendrocytes in the PWM are highly susceptible to damage from oxidative stress (Haynes et al. 2005) and their

selective degeneration is the result of lipid peroxidation caused by ROS (Griot et al. 1990). Activated microglia are also known to release reactive nitrogen species (RNS) (Murphy et al. 1993; Hanisch 2002; Nakanishi 2003) which plays a crucial role in the pathogenesis of developing white matter lesions (Haynes et al. 2005). Expression of iNOS and RNS is known to be triggered by hypoxia in AMCs in the PWM of neonatal animals (You and Kaur 2000; Murugan et al. 2011; Rathnasamy et al. 2011). It has been reported that excessive production of NO from iNOS is toxic to the oligodendrocytes (Merrill et al. 1993). The synergistic activation of NADPH oxidase and iNOS could lead to peroxynitrite formation, a potent oxidising agent (Brown 2007).

We have already shown that hypoxia elicited an increase in iron levels in the PWM with specific localization of iron in AMCs along with increased expression of iron regulatory proteins and transferrin receptors (Kaur and Ling 1999; Rathnasamy et al. 2011). Intracellular iron increase in AMCs was accompanied by increase in ROS, TNF-α and IL-1β generation and increased oligodendrocyte apoptosis; these biomarkers were reduced on treatment with the iron chelator deferoxamine. Based on these results, it was suggested that increased intracellular iron in AMCs is a major contributing factor to the enhanced release of ROS and cytokines that cause apoptosis of oligodendrocytes by reducing their antioxidant defenses as manifested by the reduced glutathione and increased lipid peroxidation in hypoxic injury (Rathnasamy et al. 2011).

Hypoxia induced damage in the developing cerebellum

Motor functions such as co-ordination, posture and equilibrium are controlled by the cerebellum. Cerebellar damage results in ataxia or an incoordination of movements (Gilman 1992) due to loss of Purkinje neurons which have been described to be exceptionally vulnerable to hypoxic and ischemic insult (Barenberg et al. 2001). In addition, cerebellar injury in preterm infants or in the neonatal period has been suggested to result in cognitive-behavioral dysfunction (Limperopoulos and du Plessis 2006). Several risk factors similar to those mentioned above for the PWM and the developing retina such as abnormal placental insufficiency and early postnatal cardiorespiratory instability resulting in fetal hypoxemia affect the development and growth of the cerebellum (Mallard et al. 1998). Hypoxia causes apoptosis of the Purkinje neurons and a significant decrease in the thickness of molecular and granular layers in the developing cerebellum (Biran et al. 2011; Sivakumar et al., unpublished data). Although the mechanisms responsible for the death of Purkinje neurons in hypoxic injuries in the perinatal period are not known, our recent results suggest that hypoxia induced increased production of TNF-α and IL-1β by the AMCs, which are present in large numbers in the cerebellar white matter as well as in the vicinity of the Purkinje neurons, may be an important underlying factor (Sivakumar et al., unpublished data). In parallel to the increased cytokine production, the expression of their receptors, TNF-R_1 and IL-R_1 on the Purkinje neurons was augmented by hypoxia (Sivakumar et al., unpublished data) implying that the AMCs play a pivotal role in the pathophysiological mechanism of hypoxic damage to the Purkinje neurons in the neonatal cerebellum through increased production of inflammatory cytokines.

Hypoxia induced damage in the developing retina

The retina, being an extension of the brain developmentally, is made up of basically similar cell types as the brain i.e.

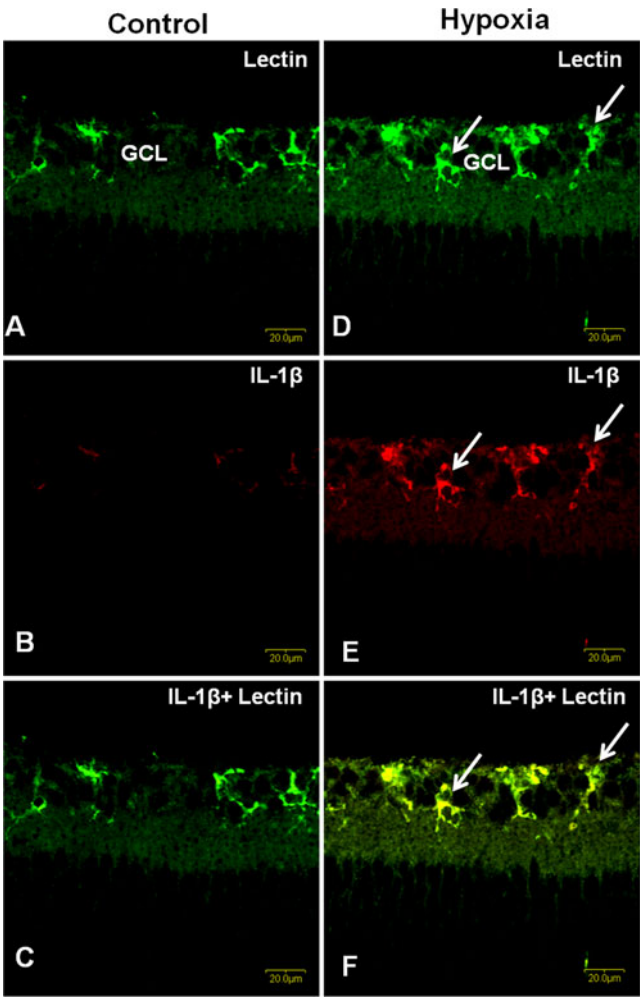

Fig. 4 An enlarged view of lectin (*green*) labeled AMCs in the ganglion cell layer (GCL) in the retina of neonatal rat in the control (**A**,**B**,**C**) and at 3 d after hypoxic exposure (**D**,**E**,**F**). Note IL-1β expression is absent in AMCs in the control (**B**), but is induced in these cells in hypoxia (**E**, *red*). Arrows indicate co-localization of lectin and IL-1β in the AMCs which is evident in the merged image in F. Scale bar (A–F)=20 μm

neurons and glial cells. The neurons are further classified into several types: the retinal ganglion cells (RGCs), amacrine cells, bipolar cells, horizontal cells and the photoreceptors. Three types of glial cells are found in the retina: the Müller cells, astrocytes and microglia. The pigment epithelium which forms the outermost layer of the retina is closely adherent to the photoreceptor layer. The multilayered neural retina consists of: the retinal pigment epithelium, photoreceptor layer, outer nuclear layer, outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fibre layer and the inner limiting membrane. Here we will focus on the changes in AMCs and the RGCs in the hypoxic developing retina.

As in the brain, microglial cells in the developing retina also appear as round and amoeboidic displaying thick stout pseudopodial processes (Ling 1982). These cells, regarded as the counterpart of AMCs in the PWM, are distributed mainly in the nerve fiber and ganglion cell layers (Ling 1982) and are known to differentiate into ramified microglia in the late postnatal period (Santos et al. 2008). They have been described as similar to cells of the mononuclear phagocyte system (Chen et al. 2002). Although initially confined to the nerve fibre and the ganglion cell layer, they migrate to outer layers barring the outer nuclear layer in the second postnatal week (Ling 1982; Santos et al. 2008). Microglial cell numbers have been shown to increase in ischemia-induced retinopathy in the mouse retina (Davies et al. 2006).

Like the PWM, the immature retina is extremely susceptible to hypoxia-ischemia resulting in the development of retinopathy (Jacobson and Dutton 2000; Kaur et al. 2009b). As in the PWM, fetal and maternal factors mentioned above such as premature birth, placental insufficiency, pulmonary or cardiac dysfunction can result in hypoxia. Hypoxic damage in the developing retina has been characterized by retinal ganglion cell (RGC) death, swelling of Müller cells, changes in the retinal pigment epithelium and increased vascular leakage (Kaur et al. 2009b). Inflammation has been suggested as a major factor involved in the pathogenesis of hypoxia induced retinopathy, the AMCs playing a pivotal role in the process (Sivakumar et al. 2011).

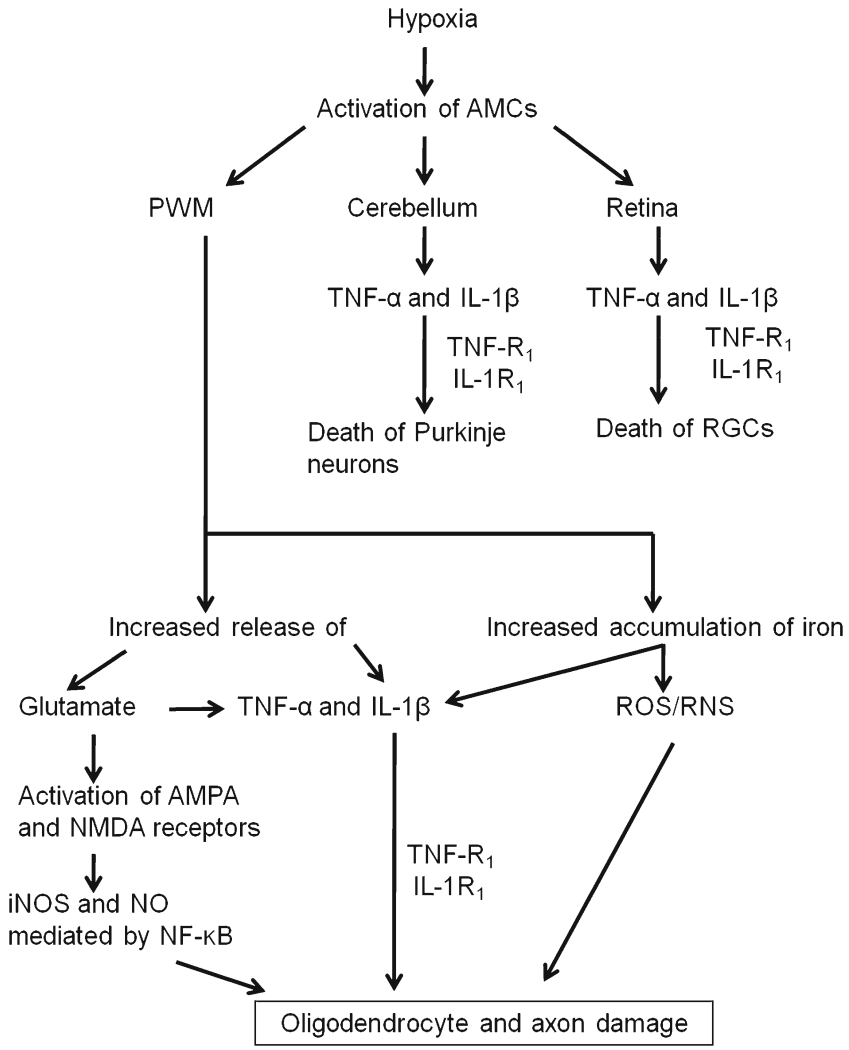

Fig. 5 A schematic diagram showing hypoxia induced robust activation of AMCs in the developing PWM, cerebellum and the retina. The ensuing excess release of TNF-α and IL-1β by the activated AMCs acting through their respective cytokine receptors results in the death of oligodendrocytes, Purkinje neurons and the RGCs. This is coupled with a surge in microglial production of glutamate and NO and accumulation of iron. Collectively this leads to ROS/RNS production which further exacerbates the neuroinflammation initiated by perinatal hypoxia

Increased apoptosis of RGCs in the neonatal retina following a hypoxic exposure has been reported (Kaur et al. 2009b; Sivakumar et al. 2011). Involvement of AMCs in RGC death was evidenced by the enhanced expression and excess release of TNF-α and IL-1β by these cells in hypoxic conditions (Fig. 4) (Sivakumar et al. 2011). It was suggested that hypoxia may initiate inflammation by direct activation of AMCs and, hence, may play a pivotal role in the pathophysiological mechanism of hypoxic damage to the RGCs in the neonatal retina through increased production of proinflammatory cytokines. Increased production of TNF-α and IL-1β by the activated AMCs was accompanied by an up regulated expression of TNF-R_1 and IL-R_1 on the RGCs suggesting that binding of the cytokines to their respective receptors would be one of the major factors involved in RGC death.

It was further shown that the level of MCP-1, known to regulate the migration of microglia, macrophages and monocytes to the hypoxic and the inflammatory sites in the CNS (Deng et al. 2009), was increased by hypoxia and the AMCs were its main cellular source in the retina (Sivakumar et al. 2010). It was suggested that the increased expression of MCP-1 in the hypoxic neonatal retina had a similar function i.e. to attract macrophages and induce migration of microglia to the vicinity of RGCs thereby augmenting the inflammatory response. We have reported an increased expression of CCR2 that is known to control mononuclear phagocyte infiltration into the brain and regulate accumulation of microglia at sites of inflammation (El Khoury and Luster 2008), on the AMCs in the developing retina following hypoxic exposure (Sivakumar et al. 2011). It was suggested that the enhanced CCR2 expression may likely be associated with the active migration of AMCs from various sites to accumulate near the RGCs.

Conclusion

Increased production of inflammatory cytokines such as TNF-α and IL-1β by the AMCs in the developing PWM, cerebellum and retina appears to be involved in the apoptosis of the premyelinating oligodendrocytes, the RGCs and Purkinje neurons through upregulated expression of TNF-R_1 and IL-R_1 (Fig. 5). Further infiltration of macrophages/migration of AMCs may occur in the vicinity of the oligodendrocytes, Purkinje neurons and RGCs through augmented release of MCP-1 by the AMCs thus amplifying the inflammatory response.

In the PWM, besides inflammation, increased release of glutamate appears to damage the oligodendrocytes and the axons through mechanisms involving activation of AMPA and NMDA receptors on AMCs and release of inflammatory cytokines and NO. Iron induced increased ROS generation by the AMCs may also be involved in cell death in the hypoxic PWM through potentiating the release of inflammatory cytokines. The various mechanisms appear to be interlinked and culminate in heightened inflammation directly or indirectly through activation of AMCs. An understanding of these mechanisms would offer the prospect of development of a novel therapeutic strategy for manipulation of activated AMCs in hypoxic lesions of the developing brain and the retina.

Acknowledgements This study was supported by a research grant (R181-000-120-213) from National Medical Research Council of Singapore. We thank Dr. Viswanathan Sivakumar for providing the technical assistance. There is no conflict of interest among the authors.

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