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How microglia kill neurons

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ABSTRACT

Microglia are resident brain macrophages that become inflammatory activated in most brain pathologies. Microglia normally protect neurons, but may accidentally kill neurons when attempting to limit infections or damage, and this may be more common with degenerative disease as there was no significant selection pressure on the aged brain in the past. A number of mechanisms by which activated microglia kill neurons have been identified, including: (i) stimulation of the phagocyte NADPH oxidase (PHOX) to produce superoxide and derivative oxidants, (ii) expression of inducible nitric oxide synthase (iNOS) producing NO and derivative oxidants, (iii) release of glutamate and glutaminase, (iv) release of TNFα, (v) release of cathepsin B, (vi) phagocytosis of stressed neurons, and (vii) decreased release of nutritive BDNF and IGF-1. PHOX stimulation contributes to microglial activation, but is not directly neurotoxic unless NO is present. NO is normally neuroprotective, but can react with superoxide to produce neurotoxic peroxynitrite, or in the presence of hypoxia inhibit mitochondrial respiration. Glutamate can be released by glia or neurons, but is neurotoxic only if the neurons are depolarised, for example as a result of mitochondrial inhibition. TNFα is normally neuroprotective, but can become toxic if caspase-8 or NF-κB activation are inhibited. If the above mechanisms do not kill neurons, they may still stress the neurons sufficiently to make them susceptible to phagocytosis by activated microglia. We review here whether microglial killing of neurons is an artefact, makes evolutionary sense or contributes in common neuropathologies and by what mechanisms.

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Abbreviations: BDNF, brain-derived neurotrophic factor; casp8, caspase 8; Cox-2, cyclooxygenase-2; CXCL1, chemokine (C-X-C motif) ligand 1; CX3CR1, CX3C chemokine receptor 1; DAP12, DNAX-activation protein 12; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HMGB1, high-mobility group protein B1; HSP60, heat shock protein 60; IGF1, insulin-like growth factor 1; INF-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; mito, mitochondria; MERTK, Mer tyrosine kinase; MFG-E8, milk fat globule-EGF factor 8 protein; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptor; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; MMP-9, matrix metallopeptidase 9; PHOX, phagocytic NADPH oxidase; PS, phosphatidylserine; RIP1, receptor-interacting protein 1; RIP3, receptor-interacting protein 3; MLKL, mixed lineage kinase domain-like protein 1; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; TNFα, tumour necrosis factor alpha; TLR, toll-like receptor; VNR, vitronectin receptor; XIAP, X-linked inhibitor of apoptosis protein.

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1. Introduction

There is evidence that brain inflammation and/or activated microglia contribute to acute pathologies such as stroke, trauma and meningitis, psychiatric diseases such as schizophrenia, depression and autism, and neurodegenerative diseases such as AIDS dementia, multiple sclerosis, Alzheimer's disease, Parkinson's disease and motor neuron disease (Bal-Price and Brown, 2001; Bal-Price et al., 2002; Klegeris et al., 2007; Lucas et al., 2006; McNaught and Brown, 1998; Zipp and Aktas, 2006). These pathologies have different causes and consequences, but they all involve brain inflammation, and there is evidence that blocking inflammation can either delay onset or reduce symptoms (Klegeris et al., 2007; Lucas et al., 2006; Zipp and Aktas, 2006; Block et al., 2007; Brown and Bal-Price, 2003; Wyss-Coray, 2006). In general, inflammation may have beneficial and/or detrimental effects in any particular disease and in any particular phase of a disease. The beneficial effects are mainly due to elimination of pathogens, clearing debris, recruiting other cells, aiding repair and providing neurotrophins; and the detrimental effects may be unintended side-effects of the beneficial processes (Klegeris et al., 2007; Lucas et al., 2006; Zipp and Aktas, 2006; Block et al., 2007; Brown and Bal-Price 2003; Wyss-Coray 2006).

Inflammation can damage the brain in a variety of ways, including: (i) inflammation in the vascular wall may drive atherosclerosis, leading to stroke and vascular dementia, (ii) inflammation in the blood brain barrier may compromise barrier function and allow thrombin, albumin and antibodies into the brain, (iii) inflammation and/or blood brain barrier breakdown may recruit/allow lymphocytes, monocytes and neutrophils into the brain (Engelhardt and Ransohoff, 2005), (iv) antibodies generated against brain antigens may induce immune attack as occurs in multiple sclerosis, (v) inflammation may induce brain oedema (swelling), (vi) some types of inflammation may suppress neurogenesis, (vii) cytokines may inflammatory activate astrocytes, which may then kill neurons, and (vii) pathogens, protein aggregates, damaged neurons and/or cytokines may inflammatory activate microglia, which may then kill neurons. It is this last type of damage, common to many brain pathologies, that we shall be concerned with here.

2. Microglia and their 'activation' states

Microglia, the brain's main resident macrophages, are the predominant immune cells in the healthy brain, and main regulators of brain inflammation (Block et al., 2007; Ransohoff and Perry, 2009). The healthy, non-inflamed brain contains almost entirely 'resting' microglia, which are highly ramified, with a small, static cell body, but with dynamic and branched processes actively seeking out signs of pathogens or damage in the brain (Hanisch and Kettenmann, 2007). When microglia detect such signs, they become 'activated' (Fig. 1). Activation is normally accompanied by partial retraction of processes to the cell body, proliferation, and expression and release of pro-inflammatory cytokines, including TNFα, IL-1β, IL-6 and IFN-γ. These cytokines recruit and activate other microglia. In fact in the inflamed brain, most microglia will encounter pro- or anti-inflammatory cytokines, or chemokines or other chemotactic factors first before they encounter a pathogen or damaged neuron, and this first encounter will 'prime' or programme their response to subsequent encounters (Perry and Holmes, 2014). Highly activated microglia may completely retract processes to the cell body producing rounded ('amoeboid') microglia that are highly mobile and phagocytic.

Microglia can be 'activated' into a variety of states, of which the best characterised are the M1 or classically activated state (induced by pro-inflammatory cytokines and/or TLR activation) and the M2 or alternatively activated state (induced by IL-4) (Ransohoff and Perry, 2009; Chhor et al., 2013). This classification is derived from the Th1 (releasing IFN-γ) and Th2 (releasing IL-4) responses of T cells, which was then extended to macrophages as M1 (induced by

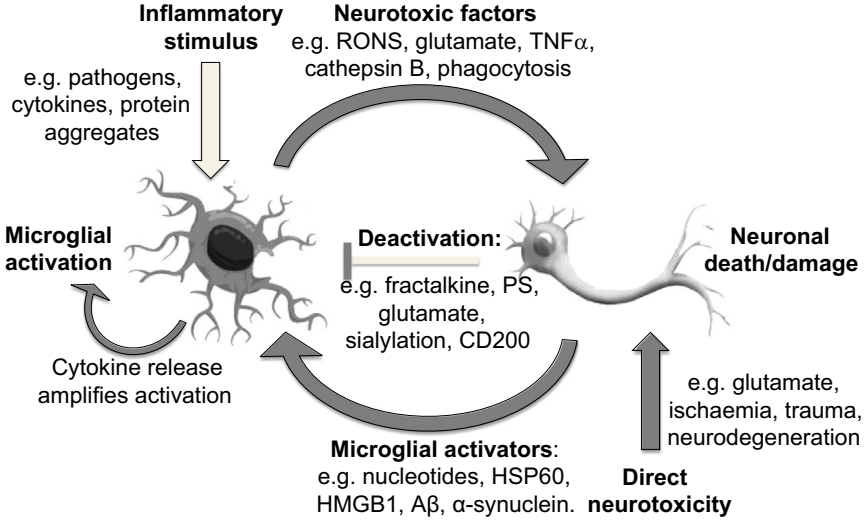

Fig. 1 – Factors regulating neuroinflammation and associated neurotoxicity. Microglia are activated by inflammatory stimuli, amplified by cytokine release, causing release of neurotoxic factors that damage or kill neurons. This or direct neurotoxicity may further activate microglia. However, the potential vicious cycle can be deactivated by a variety of factors or resolution of the original stimuli.

IFN-γ) and M2 (induced by IL-4) responses. However, this classification has become too simple for T cells and macrophages (Martinez and Gordon, 2014), and is unlikely to cover the diversity of states of microglia activation which can lead to a large array of stimuli acting via multiple receptors and transcription factors on thousands of genes (Boche et al., 2013). But it remains the case that TLR agonists and pro-inflammatory cytokines put microglia into a broadly similar state – the classically activated state – with greatly increased expression of iNOS, Cox-2, TNFα, IL-1β, IL-6, IL-12 and CXCL1, and with greatly reduced expression of CD206, IGF-1 and CX3CR1 (Chhor et al., 2013).

Classically (M1) activated microglia have been equated with neurotoxicity, and alternatively (M2) activated microglia with neuroprotection. However, things are unlikely to be this simple, for example IL-4 activation of microglia may result in neuronal death via PHOX activation (Park et al., 2008), and classically activation appear to encourage neurogenesis (Shigemoto-Mogami et al., 2014).

The term 'resting' microglia has been criticised based on the finding that 'resting' microglia in the healthy brain have highly mobile and active processes, and that they have a variety of physiological activities such as synaptic pruning (Paolicelli et al., 2011). However, this is not a criticism of the classification system, but rather that the name used is inappropriate. They can alternatively be termed 'ramified' or 'non-activated' microglia. The term 'activated' microglia has also been criticised based on the diversity of activation states and the lack of specificity or meaning of the term. However, we probably do not know enough yet to produce a mature classification of activation states. Recently, bipolar/rod-shaped microglia (Tam and Ma 2014) and multinucleated giant cells (Hornik et al., 2014) were identified as potential other states of microglia.

3. Microglial killing of neurons: fact or artefact? Loss or gain of function? Direct or indirect?

Activated microglia can kill and/or remove pathogens, but they may also kill neurons, at least in culture. However, it has recently been suggested that microglial killing of neurons may be an artefact of in vitro studies or may be misdiagnosed by in vivo studies that merely correlate microglia with pathology, rather than determining causality (Biber et al., 2014). And it is certainly important to test whether mechanisms found in vitro apply in vivo by blocking the candidate mechanism and determining whether this prevents the pathology and we review some such studies below.

Microglia normally function to protect neurons by for example killing pathogens, removing debris, removing amyloid β or supplying nutritive factors such as BDNF and IGF1 (Biber et al., 2014). Thus neuronal death may occur if, for whatever reason, microglia are unable to provide these protective functions. For example, microglia express and release IGF1 that supports neurons, and this expression is greatly reduced by lipopolysaccharide (LPS), resulting in neuronal death if there are no other neurotrophins present (Chhor et al., 2013). This review will concentrate on those factors released by microglia that directly kill neurons, but it should be born in mind that neurotrophin release from microglia or other cells may affect the sensitivity of neurons to such neurotoxic factors.

Factors released by microglia may kill neurons *directly* or *indirectly*. By direct killing, we mean that the factor acts on the neuron itself to induce death. By indirect killing, we mean that the factor acts on some non-neuronal cell to induce neuronal death via some other factor. For example, IL-1β is released by activated microglia; however it does not act on neurons to induce death directly, but rather amplifies the inflammatory response such that other microglia and astrocytes may release factors that are directly toxic to neurons. Thus for all the factors discussed below that are released by microglia, we need to consider whether they are directly or indirectly toxic to neurons. Our aim is to identify those factors that are directly toxic.

4. Does it make evolutionary sense for microglia to kill neurons?

It has been suggested that 'microglial killing of neurons' makes no evolutionary sense, because killing neurons is unlikely to benefit the organism, and therefore it could not have evolved (Biber et al., 2014). This line of reasoning is an important constraint when thinking about microglial killing of neurons. However:

1. Microglial killing of neurons has been invoked mainly in the context of age-related degenerative pathology. It is thought that there has never been significant selection pressure against ageing and age-related disease, because most animals in the wild and humans before civilisation bred and died before significant ageing. Therefore there never was a significant selection pressure on microglia in the context of the aging brain or degenerative pathology.
2. Evolutionary rationalisations of pathology can be given in terms of trade-offs and side-effects i.e. a process that may be beneficial in one context may be detrimental in another context, so that detrimental processes persist if the net effect on fitness is positive. So microglial killing of neurons might be rationalised as an unintended side-effect of microglial process designed to kill pathogens.
3. In a small number of conditions, microglial killing of neurons might be beneficial. Such conditions might include: killing/removal of excess neurons during development, killing/removal of infected neurons to prevent spread of infection, or killing/removal of damaged neurons to prevent network disruption/seizures.

5. TNFα and other cytokines

Activation of microglia results in their expression and release of pro-inflammatory cytokines, which act to *amplify* the inflammatory response, by further recruiting, activating and proliferating microglia irrespective of whether they have encountered pathogens or damage (Fig. 1). There is strong evidence that pro-inflammatory cytokines such TNFα and IL-1β can lead to neuronal death in vitro and in vivo (Glass et al.,

2010; McCoy and Tansey, 2008). However, it is unclear that pro-inflammatory cytokines can cause direct neurotoxicity, rather than causing indirect neurotoxicity via activating microglia, astrocytes or other cells. For example, TNFα was found to cause death specifically of dopaminergic neurons in embryonic rat midbrain cultures, but it was not tested whether this neurotoxicity was mediated by glia (Clarke and Branton, 2002; McGuire et al., 2001). We found that TNFα could induce delayed neuronal loss that was prevented if microglia were removed from the cultures, and was mediated by microglial phagocytosis of neurons (Neniskyte et al., 2014). Others found that TNFα and IL-1β were synergistic in inducing neuronal death in brain cell cultures, mediated by iNOS and NMDA receptors, suggesting that neuronal death was indirect via glial activation (Chao et al., 1995).

In contrast to other pro-inflammatory cytokines, TNFα is also a death ligand for TNFα receptors that can induce either (i) apoptosis via activation of caspase-8, or (ii) necroptosis if RIP1, RIP3 and MLKL are activated and caspase-8 inactivated, or (iii) inflammation via NF-κB, which also inhibits apoptosis (Christofferson et al., 2014) (Fig. 2). Thus the fate of neurons exposed to TNFα and expressing TNFα receptors may depend on the relative activity of these three pathways (Kraft et al., 2009). Injection of TNFα into the brain was found to induce hippocampal neuronal loss via RIP3-mediated necroptosis of neurons (Liu et al., 2014). However, again it is unclear whether this was direct neurotoxicity as in culture TNFα-induced neurotoxicity required blockade of caspase-8 as is usual for necroptosis, but it is unclear how this would occur in vivo (Liu et al., 2014). Taylor et al. (2005) found that TNFα only induced neuronal death in the presence of microglia or conditioned media from activated microglia, apparently because the activated microglia released Fas ligand, which then induced apoptosis in the neurons. Thus Fas ligand is potentially another way that microglia can kill neurons, presumably via the death receptor Fas.

TNFα has been found to induce the expression and release of glutaminase from neurons, resulting in elevation of extracellular glutamate, causing excitotoxicity prevented by blocking the glutamate NMDA receptor (Ye et al., 2013). And neuronal loss in a model of HIV-1 encephalitis appeared to be mediated by TNFα and glutaminase (Ye et al., 2013). However, again it is not clear that the TNFα neurotoxicity was direct. The same group has also previously shown that HIV-infected microglia and macrophages express and release glutaminase, generating glutamate from glutamine extracellularly, resulting in excitotoxicity (Erdmann et al., 2009; Huang et al., 2011).

Other pro-inflammatory cytokines have been implicated in neuronal death, but again through amplifying inflammation, rather than causing direct neurotoxicity. For example, IL-1β does not cause neurotoxicity when added to neurons alone, but does when added to glia and the conditioned media from these glia added to neurons (McNamee et al., 2010).

6. Cathepsin B and other proteases

The cysteine protease B – cathepsin B – released by activated microglia has been shown to be neurotoxic in the context of neurodegenerative diseases (Gan et al., 2004; Kim et al., 2007; Kingham and Pocock 2001) (Fig. 2). Activating BV-2 microglia with Aβ upregulated expression of cathepsin B, and downregulating this expression with a siRNA or adding a specific inhibitor of cathepsin B diminished loss in primary cortical neurons (Gan et al., 2004). Similar results were obtained with primary microglia activated with peptide chromogranin A to release cathepsin B (Kingham and Pocock 2001). Conditioned medium from these microglia or pure cathepsin B caused neuronal apoptosis, prevented by caspase inhibitor or an antibody to cathepsin B (Kingham and Pocock 2001). Cathepsin D was also found to be released by LPS/IFN-γ activated microglia, and knocking its expression down prevented neurotoxicity in a co-culture of microglia and neuroblastoma cells (Kim et al., 2007), but it unclear whether this neurotoxicity was direct.

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent neutral proteases that degrade or modify extracellular matrix components. MMPs can be expressed and released by microglia and other cells, and their expression is upregulated in multiple brain pathologies (Annese et al., 2015; del Zoppo et al., 2012; Leonardo et al., 2009; Vilalta et al., 2008a, 2008b). In a hippocampal slice culture model, oxygen and glucose deprivation induced MMP-9 expression in micro-

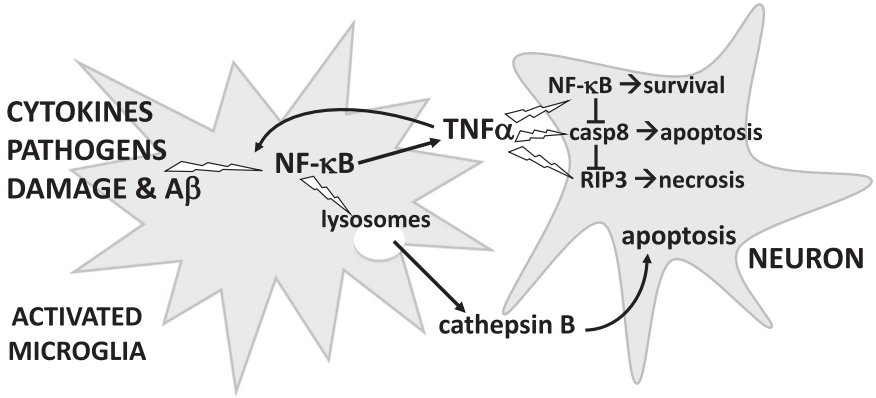

Fig. 2 – Roles of TNFα and cathepsin B in inflammatory neurodegeneration. Inflammatory stimuli cause microglia to express and release TNFα, which may bind to TNFα receptors on neurons to promote survival, apoptosis or necrosis. Activated microglia may release cathepsin B that may induce neuronal apoptosis.

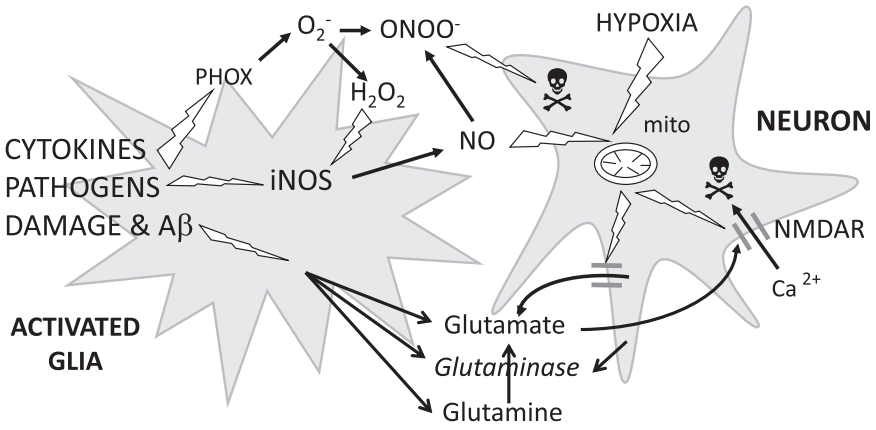

Fig. 3 – Roles of RONS and glutamate in inflammatory neurodegeneration. Inflammatory stimuli may activate PHOX to produce superoxide that dismutate to hydrogen peroxide, which further activates microglia resulting in expression of iNOS producing NO that can react with superoxide to give peroxynitrite ($ONOO^-$) that can stress or kill neurons depending on level. NO, together with hypoxia, can inhibit mitochondria (mito) at cytochrome oxidase, resulting in neuronal depolarisation, causing glutamate release and activation of NMDA receptors, causing influx of calcium that cannot be pumped out. Glial activation may also cause glutamate release, in part due to NO, and glutaminase release from glia or neurons may generate more glutamate from glutamine, potentiating excitotoxicity.

glia together with neuronal injury, which was prevented with either a MMP inhibitor or a blocker of microglial activation (Leonardo et al., 2009). However, there is no evidence that MMPs are directly toxic to neurons.

7. Glutamate and glutaminase

Glutamate can be directly toxic to neurons expressing glutamate receptors if the extracellular level is excessive and sustained, or if high extracellular glutamate is paired with sustained neuronal depolarisation, enabling sustained activation of the NMDA receptor, causing 'excitotoxic' death of the neuron. Microglia, astrocytes and neurons can all release glutamate in particular conditions, and thus have been implicated in 'excitotoxic' death of the neuron (Fig. 3). For example, in a genetic model of Rett syndrome, microglia apparently damaged neurons via hemichannel release of glutamate, causing excitotoxicity (Maezawa and Jin, 2010). Japanese encephalitis virus caused neuronal death by infecting and activating microglia, resulting in glutamate release from microglia (amplified by the TNFα release) causing excitotoxic death of neurons (Chen et al., 2012). HIV-infected microglia and macrophages expressed and released glutaminase, an enzyme that converts glutamine to glutamate, resulting in excitotoxicity (Erdmann et al., 2009; Huang et al., 2011).

8. Superoxide and hydrogen peroxide from the phagocyte NADPH oxidase

Resting and activated microglia express the phagocyte NADPH oxidase (PHOX), and classical activation increases expression of PHOX (particularly its NOX2 transmembrane, catalytic subunit). However, PHOX is not assembled and active unless acutely stimulated by for example TNF-α, IL-1β, chemokines, arachidonate, β-amyloid, LPS, ATP or phagocytosis. When activated, it produces high levels of superoxide extracellularly or into phagosomes, which may either dismutate to hydrogen peroxide (catalysed by extracellular superoxide dismutase) or react with NO to produce peroxynitrite (Bal-Price et al., 2002). These and derivative oxidants, called reactive oxygen species (ROS), contribute to the killing of pathogens by microglia, but may also damage neurons (Fig. 3).

Importantly, however, activation of microglial PHOX alone causes no acute neurotoxicity (Mander and Brown, 2005), but stimulates the microglia to proliferate (Mander et al., 2006), produce TNFα and IL-1β (Jekabsone et al., 2006; Pawate et al., 2004), and express iNOS (Pawate et al., 2004; Cheret et al., 2008). For example, we showed that fibrillar β-amyloid induces microglia to proliferate and produce cytokines via activation of PHOX (Jekabsone et al., 2006). Thus, inhibition of PHOX is sufficient to prevent classical activation of microglia (Block et al., 2007), and may promote alternative activation (Choi et al., 2012), and thus is a target for anti-inflammatory strategies.

In vivo in mice, knockout of PHOX subunits prevents LPS-induced neuronal damage (Cheret et al., 2008), 6-hydroxydopamine-induced neurodegeneration (Hernandes et al., 2013), neuronal death after transient ischaemia (Yoshioka et al., 2011), mortality in a mutant huntingtin model of Huntington's disease (Valencia et al., 2013). And inhibition of PHOX prevents rod cell death in model of retinal degeneration (Zeng et al., 2014). Thus it is clear that PHOX can mediate neurotoxicity in vivo, but generally unclear whether this is neuronal or microglial PHOX, and if microglial PHOX, whether it acts via regulating microglial activation or more directly via producing neurotoxic ROS or peroxynitrite.

9. Nitric oxide from iNOS

In a variety of cell types, high levels of NO induce energy depletion-induced necrosis, due to (i) rapid inhibition of

mitochondrial respiration, (ii) slow inhibition of glycolysis, (iii) induction of mitochondrial permeability transition, and/or (iv) activation of poly-ADP ribose polymerase (Brown, 2010). Alternatively, if energy levels are maintained, NO can induce apoptosis, via oxidant activation of p53, p38 MAPK pathway or endoplasmic reticulum stress (Brown, 2010). GAPDH, Drp1, mitochondrial complex I, MMP-9, Parkin, XIAP and protein disulphide isomerase can also be S-nitro(sy)lated, but the contribution of these reactions to neurodegeneration remains unclear (Brown, 2010).

However, low levels of NO can block cell death via cGMP-mediated: vasodilation, Akt activation or block of mitochondrial permeability transition. While, high NO may protect by killing pathogens, activating NF-κB or S-nitro(sy)lation of caspases and the NMDA receptor. Thus NO is normally neuroprotective in the brain and it requires specific circumstances to make it neurotoxic (Brown, 2010).

Inducible NO synthase (iNOS) is not normally expressed in the brain, but inflammatory mediators such as LPS and cytokines cause its expression in microglia and astrocytes (Murphy, 2000). NO from glial iNOS has been suggested to mediate neurotoxicity, for example in MPP^+-induced death of dopaminergic, mid-brain neurons as a model of Parkinson's disease (Brzozowski et al., 2014). Once expressed, iNOS produces high levels of NO continuously (up to 1 μM NO from microglia or astrocytes (Bal-Price and Brown, 2001; Bal-Price et al., 2002)). We showed that these high levels of NO can induce neuronal death by causing inhibition of mitochondrial cytochrome oxidase in neurons (Bal-Price and Brown, 2001; Brown and Cooper, 1994). NO inhibition of mitochondrial respiration caused neuronal depolarisation and glutamate release, which together are sufficient to activate the NMDA receptor, resulting in excitotoxic death of the neurons (Bal-Price and Brown, 2001; McNaught and Brown, 1998; Golde et al., 2002; Jekabsone et al., 2007; Stewart et al., 2002). NO from iNOS also causes glutamate release from astrocytes, so that inflammatory-activated astrocytes maintained a relatively high extracellular glutamate level (Bal-Price et al., 2002). Non-toxic levels of extracellular glutamate can become toxic to neurons if in addition the mitochondrial respiratory chain is inhibited (Novelli et al., 1988).

However, the above mechanism requires relatively high levels of NO or iNOS expression, as occurs in the presence of IFNγ, which dramatically potentiates iNOS expression (Kinsner et al., 2006). In contrast, iNOS can be expressed at low levels in vitro (Mander and Brown, 2005) or in vivo (Han et al., 2002) apparently with little or no neuronal death. Indeed NO from iNOS may be protective by blocking brain cell death (Cho et al., 2005; Takuma et al., 2001). However, hypoxia strongly synergises with NO or iNOS expression to induce neuronal death via respiratory inhibition (Mander et al., 2005). This is because NO is a competitive inhibitor of cytochrome oxidase, the NO competing with oxygen for binding to cytochrome oxidase (Brown and Cooper, 1994; Mander et al., 2005), so that NO greatly increases the apparent K_M of neuronal respiration for oxygen. This sensitisation to hypoxia is potentially important in brain pathologies involving both inflammation and hypoxia (Fig. 3).

As described above, neither activation of NADPH oxidase (PHOX) nor production of (moderate levels of) nitric oxide through iNOS expression is sufficient to induce neurotoxicity. However, we found that when both were activated together, this resulted in apparent apoptosis of most neurons mediated by peroxynitrite. We showed that inflammatory neurodegeneration induced by TNFα, IL-1β, prion peptide, LPS, IFNγ, arachidonate, ATP and/or PMA was mediated by this dual-key (iNOS and PHOX) mechanism of inflammatory neurodegeneration in particular conditions (Mander and Brown, 2005). Simultaneous activation of PHOX and iNOS in microglia resulted in the disappearance of NO, appearance of peroxynitrite (Bal-Price et al., 2002; Mander and Brown, 2005), and apoptosis of co-cultured neurons that was prevented by inhibitors of iNOS or PHOX, or by scavengers of superoxide or peroxynitrite (Mander and Brown, 2005). Microglial iNOS and ROS were also found to be required for LPS-induced neurotoxicity by others (Shie et al., 2005). As physiological levels of NO maintain blood flow and are neuroprotective by multiple mechanisms and PHOX activation removes extracellular NO, it is possible that PHOX activation is neurotoxic in part by removing neuroprotective NO.

10. Microglial phagocytosis of neurons

Inflammatory activated microglia can phagocytose stressed-but-viable, resulting in neuronal death by phagoptosis, i.e. cell death caused by phagocytosis of the cell (Brown and Neher, 2014). We delineated a pathway by which TLR-activated microglia release oxidants that cause neurons to transiently expose phosphatidylserine (Fig. 4). Phosphatidylserine is a phospholipid normally confined to the inner leaflet of the plasma membrane, but elevated calcium, oxidants or caspase activity can cause its export to the outer leaflet, thus 'exposing' phosphatidylserine to the outside of the cell. The activated microglia also released the opsonin MFG-E8, which bound exposed phosphatidylserine and activated phagocytosis via the vitronectin receptor (VNR) expressed on microglia (Fricker et al., 2012; Neher et al., 2013, 2011; Neniskyte et al., 2011). Consequently, microglia activated by TLR agonists, LPS, lipoteichoic acid or amyloid-β (Aβ), caused a slow progressive loss of neurons by phagocytosis in neuron–microglia co-cultures. Blocking VNR, MFG-E8 or exposed phosphatidylserine prevented all neuronal loss, leaving viable neurons in vitro without inhibiting inflammation (Fricker et al., 2012; Neher et al., 2013, 2011; Neniskyte et al., 2011). Thus, in cultures from Mfge8 knockout mice, LPS- or Aβ-induced neuronal loss was absent, but this could be reconstituted by adding MFG-E8, without any effect on inflammation (Fricker et al., 2012; Neniskyte and Brown, 2013). Analogously, LPS injection into the striatum of rats and mice in vivo caused strong microglial inflammation and neuronal loss, but this neuronal loss was much reduced in Mfge8 knockout mice or after co-injection of a VNR inhibitor (Fricker et al., 2012).

From these results, it appears that inflammatory stress can induce neurons to expose phosphatidylserine, resulting in the phagocytosis of stressed-but-viable neurons by activated microglia. Consistent with this view, we found that low levels of peroxynitrite, hydrogen peroxide or glutamate induced reversible phosphatidylserine exposure on viable neurons. In the absence of microglia, these neurons were

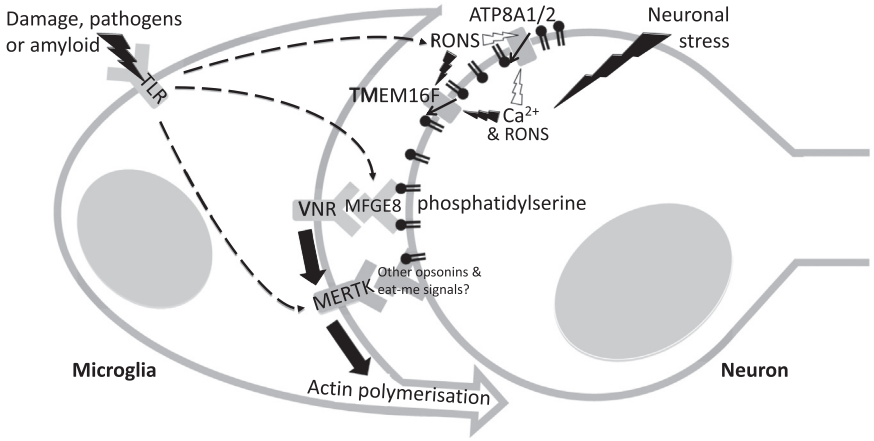

Fig. 4 – Microglial phagocytosis of stressed-but-viable neurons. Activated microglia release reactive oxygen and nitrogen species (RONS) that activate the phosphatidylserine scramblase TMEM16F and inactivate the phosphatidylserine translocases ATP8A1 and ATP8A2, causing neuronal exposure of phosphatidylserine, which is bound by the opsonin MFG-E8 that then activates phagocytosis of the neurons via the microglial vitronectin receptor (VNR) in concert with Mer tyrosine kinase (MERTK), which may bind other opsonins such as Gas-6. Direct neuronal stress, e.g. from hypoxia and/or glutamate, can also promote phosphatidylserine exposure.

able to recover and internalise this eat-me signal, but in the presence of microglia, these neurons with exposed phosphatidylserine were lost due to phagocytosis.

After brain ischaemia, neurons in peri-infarct areas have been shown to reversibly expose phosphatidylserine, and we have found that MERTK and MFG-E8 are upregulated 3 days after transient focal ischaemia (Neher et al., 2013). Mice lacking MFG-E8 or MERTK, compared with wild-type animals, showed a marked reduction in brain atrophy 7–28 days after brain ischaemia, leading to a pronounced reduction in motor deficits. Thus, the brain damage induced by ischaemia was greatly reduced in the absence of these phagocytic proteins. Although the total number of microglia and the levels of inflammatory mediators were the same in wild-type and mutant animals, Mertk- or Mfge8-deficient animals had fewer microglia that contained neuronal material, confirming that lack of these phagocytic proteins inhibits the engulfment of neurons after ischaemia (Neher et al., 2013). The protection of neurons in Mertk- and Mfge8-deficient animals for up to 4 weeks after transient focal brain ischaemia and the improved functional outcome indicates that the neurons lost in the wild-type animals must have been alive when they were phagocytosed.

Others have shown that microglial phagocytosis mediates neuronal loss/death in models of frontotemporal dementia (Kao et al., 2011), AIDS dementia (Marker et al., 2012), Parkinson's disease (Barcia et al., 2012), Motor Neuron Disease (Liu et al., 2012), retinal degeneration (Zhao et al., 2015) and developmental loss of neuronal precursors (Cunningham et al., 2013). Microglia have also been shown to remove live synapses (Paolicelli et al., 2011), dendrites, axons, myelin, neutrophils and glioma cells (Brown and Neher, 2014).

A contribution of microglia to the developmental death of neurons or neuronal precursors has also been shown in the mouse brain. In developing cerebellum in vivo Purkinje cells were removed by microglia, and in organotypic slices of the developing mouse cerebellum, the selective elimination of microglia led to an increase in the number of mature Purkinje cells (Marin-Teva et al., 2004). Similarly, in the developing hippocampus in vivo, knockout of the microglial genes encoding the CR3 subunit CD11b or DNAX-activation protein 12 (DAP12; also known as TRYOBP), required for complement-mediated phagocytosis, reduced the number of neurons with activated caspase-3 (Wakselman et al., 2008), suggesting that phagocytosis contributes to the induction of neuronal death. In these studies, scavenging of microglia-produced superoxide increased the number of mature Purkinje cells in cerebellar slices and reduced the number of caspase-3-positive neurons in the developing hippocampus. Furthermore, lack of DAP12 or CD11b reduced microglial production of reactive oxygen species in vivo. As phagocytosis is known to activate the microglial NADPH oxidase (PHOX), which produces superoxide (Claude et al., 2013), it may be that phagocytosis promotes the death of the cell being engulfed via oxidant-induced apoptosis.

In monkeys and rats, microglia have been found to phagocytose live neural precursor cells in the cortex (Cunningham et al., 2013). Microglia engulfed precursor cells that were proliferating but showed no signs of apoptosis in vivo. Accordingly, time-lapse microscopy in organotypic cortical slices demonstrated that microglia were eating neural precursors, and eliminating the microglia increased the number of viable neural precursor cells. Interestingly, this process was dependent on microglial activation, as anti-inflammatory treatment with tetracyclines also increased neural precursor numbers both in slices and in vivo, whereas activating microglia in utero through maternal immune activation markedly decreased neural precursor number (Cunningham et al., 2013). Thus, microglia regulate the size of the neuronal precursor cell pool in the developing cerebral cortex, and thus changes in the microglial activation state potentially affect brain development through the phagoptotic uptake of neural precursors.

11. Conclusions

Microglial activation can cause neurotoxicity by a variety of mechanisms. The severity of the insult may determine the type of cell death, with less severe insults resulting in phagoptosis, because the stress or damage is sufficient to cause exposure of eat-me signals without triggering apoptosis or necrosis (Block et al., 2007; Hanisch and Kettenmann, 2007; Brown and Neher, 2012). More severe and/or prolonged inflammation results in strong iNOS induction in both microglia and astrocytes, which may protect neurons, but if combined with hypoxia may inhibit mitochondria resulting in excitoxicity, or if combined with PHOX activation produces peroxynitrite that can kill neurons by a variety of means. Excitotoxic glutamate may contribute to neuronal death induced by activated microglia in many different circumstances. TNFα can amplify neuroinflammation, but may also be directly neurotoxic to specific neurons.

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