Microglial phagocytosis of live neurons

Guy C. Brown and Jonas J. Neher

Abstract | Microglia, the brain's professional phagocytes, can remove dead and dying neurons as well as synapses and the processes of live neurons. However, we and others have recently shown that microglia can also execute neuronal death by phagocytosing stressed-but-viable neurons — a process that we have termed phagoptosis. In this Progress article, we discuss evidence suggesting that phagoptosis may contribute to neuronal loss during brain development, inflammation, ischaemia and neurodegeneration.

How and why do neurons get phagocytosed in the brain? The prime suspects for carrying out this phagocytosis are microglia, which are the resident macrophages of the brain and spinal cord. Microglia are of myeloid origin and invade the CNS from the yolk sac during development and are maintained by self-renewal throughout an animal's lifespan[1,2]. They occupy non-overlapping territories and continuously survey the brain parenchyma through extensive movement of their processes, which enables efficient detection of any alterations in their microenvironment, including signals ranging from changes in neuronal activity to damage-associated ligands and pathogens[3,4]. Microglia are also the professional phagocytes of the brain and are able to engulf whole neurons within hours[5,6]. Phagocytosis of dead and dying neurons and neuronal debris is beneficial in part because it reduces inflammation[7,8]. However, microglia can also phagocytose live neurons[5,9–13], live neuronal progenitors[14–17], live neutrophils[18] and live glioma cells[19], causing death of the engulfed cell. We have termed the cell death caused by phagocytosis of live cells 'phagoptosis' (BOX 1). In this article, we outline the signalling between neurons and microglia that enables or prevents microglial phagocytosis of neurons, and discuss the evidence that phagocytosis of live neurons or parts of live neurons occurs in physiological and pathological conditions.

Phagocytic signalling

Microglia constantly palpate the surface of neurons[6,20]. When microglia detect exposed 'eat-me' signals, rapid recognition and engulfment of neurons or parts of neurons exposing such signals follows[5,6]. Eat-me signals can be recognized by opsonins, which are soluble, extracellular proteins that promote cell engulfment by binding to eat-me signals on the target cell as well as phagocytic receptors on the phagocyte. Through a sequence of signalling events, activation of phagocytic receptors then induces remodelling of the actin cytoskeleton, often through the recruitment of members of the RHO-family of small GTPases, which leads to target engulfment and, eventually, digestion through the fusion of phagosomes with lysosomes[21,22]. Importantly, for phagocytosis to proceed, the absence or removal of inhibitory neuronal cell surface signals, so-called 'don't-eat-me' signals, may also be required[23] (FIG. 1).

Eat-me signals. The phospholipid phosphatidylserine, when exposed on the cell surface, acts as a crucial eat-me signal that is required for microglial phagocytosis of both dying and viable neurons[5,11,12,23,24]. Phosphatidylserine is normally confined to the inner leaflet of the plasma membrane because it is continuously pumped from the outside to the inside by phosphatidylserine translocases. In neurons, these translocases have been identified as the type 4 P-type ATPases (P4-ATPases) ATP8A1 and ATP8A2 (REFS 24,25). Interestingly, loss of ATP8A2 enables neuronal phosphatidylserine exposure, which results in axonal loss and neurodegeneration in mice[24].

Alternatively, phosphatidylserine exposure can occur as a result of the following: inhibition of phosphatidylserine translocases by oxidative stress, an increase in calcium levels or ATP depletion[26,27]; fusion of intracellular vesicles with the plasma membrane; calcium-activated phospholipid scramblases, which have been identified as TMEM16 proteins[26,28]; or caspase-mediated cleavage of the transmembrane protein XK-related protein 8 (XKR8), which causes irreversible phosphatidylserine exposure during apoptosis[28]. However, the particular activities responsible for phosphatidylserine exposure on neurons in various conditions are currently unclear.

Importantly, reversible phosphatidylserine exposure can occur on stressed-but-viable neurons as a result of non-toxic levels of glutamate[9,29], oxidative stress[5,9] or growth-factor withdrawal[29]. Phosphatidylserine exposure is not itself toxic to neurons but marks the neuron for selective removal by microglia, resulting in phagocytosis of the phosphatidylserine-exposed neurons if activated microglia are present, but neuronal survival if microglia are absent[5,9–11] (BOX 1). Activation of group 1 metabotropic glutamate receptors by glutamate may block phosphatidylserine exposure on neurons and thereby prevent their phagocytosis by microglia[30].

Phagocytosis of neurons with exposed phosphatidylserine can be mediated via several microglial receptors and opsonins, some of which are strongly upregulated by inflammation. For example, during inflammation, microglia and astrocytes release increased amounts of milk fat globule EGF factor 8 (MFG-E8; also known as lactadherin or SED1)[9–11], which tightly binds to exposed phosphatidylserine through its C1 and C2 domains and to microglial vitronectin receptors (VNRs) — αvβ3 or αvβ5 integrins — through an RGD motif[5,10,31]. The resulting activation of VNRs induces phagocytosis by activating a CRKII–DOCK180–RAC1 signalling pathway that causes remodelling of the microglial actin cytoskeleton. Microglia also release

annexin A1, which promotes phagocytosis of neurons by binding to neuronal phosphatidylserine and activating the microglial formyl peptide receptor 2 (REF. 32).

MER receptor tyrosine kinase (MERTK) is another receptor on microglia that can mediate phagocytosis of neurons by binding the opsonins growth arrest-specific protein 6 (GAS6) or protein S, which in turn bind to exposed phosphatidylserine on neurons[33,34]. Microglial MERTK can also be activated by the opsonins tubby and galectin 3 if they are bound to (unknown) eat-me signals on neurons[35,36]. Interestingly, VNR- and MERTK-signalling pathways seem to cross-stimulate each other, resulting in a synergistic activation of phagocytosis, at least in cell lines[33].

In inflammatory conditions, specific phosphatidylserine-binding opsonins and their receptors are expressed, enabling potent detection and phagocytosis of phosphatidylserine-exposing cells. For example, MERTK and GAS6 are also upregulated in microglia by brain inflammation[9,37]. We found that the upregulation of MFG-E8 and MERTK after focal brain ischaemia is delayed by 2–3 days[9], which might correspond to the resolution phase of inflammation. In non-inflammatory conditions, brain-specific angiogenesis inhibitor 1 (BAI1) may act as a direct receptor for exposed phosphatidylserine and possibly mediate microglial phagocytosis of live axons[38]. Thus, different phagocytic receptors and opsonins may be used in different conditions, but it is as yet unclear whether they target specific neuronal structures for removal. Note that it is important to distinguish between receptors that mediate phagocytosis directly (such as VNRs) and receptors that increase phagocytosis indirectly (such as the Toll-like receptors (TLRs)) by, for example, increasing the expression of phagocytic receptors and opsonins, although some receptors may mediate phagocytosis both directly and indirectly.

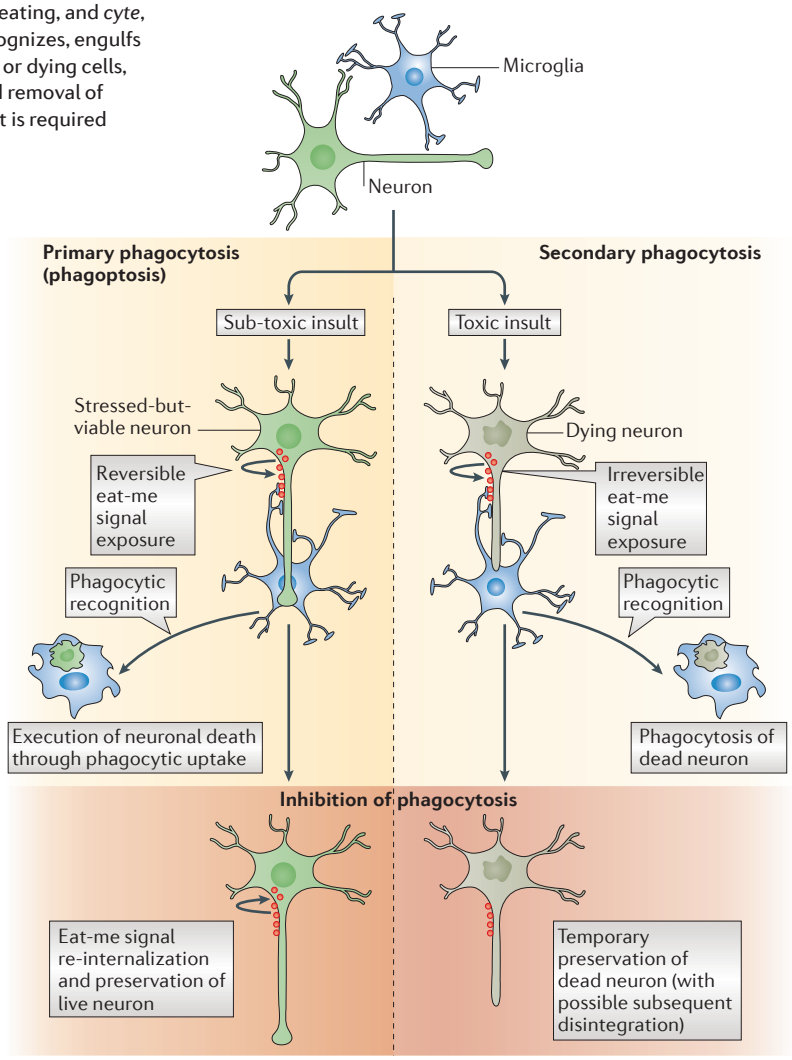
There are eat-me signals other than phosphatidylserine. Calreticulin, which is normally localized in the endoplasmic reticulum, can be exposed on the surface of non-neuronal cells as a result of endoplasmic reticulum stress or apoptosis, where it can act as an additional eat-me signal or opsonin[13,39]. On neurons, calreticulin exposure promotes their phagocytosis by binding to microglial low-density lipoprotein receptor-related protein (LRP), probably in association with other signals[13]. In addition, the complement components C1q and C3, which are produced by microglia and astrocytes, may induce phagocytosis by binding to altered neuronal surfaces. C1q mediates phagocytic recognition by binding to de-sialylated neuronal cell

Box 1 | **Phagocytosis and phagoptosis**

The term phagocytosis (derived from *phagos*, which means eating, and *cyte*, which means cell) describes the process by which a cell recognizes, engulfs and digests a target that is ≥1 μm in size[21,61], including dead or dying cells, during physiological and pathological conditions. The rapid removal of dying cells is an essential process of tissue homeostasis, as it is required to prevent disintegration of dying cells, which leads to the release of toxic components and self-antigens and thereby causes tissue injury and autoimmunity[7]. Thus, it has previously been assumed that the phagocytosis of neurons is always preceded by their commitment to cell death; however, recent data indicate that phagocytosis can execute the death of viable neurons during development, inflammation and neuropathology. We have called this form of cell death phagoptosis (derived from *phagos* and *ptosis*, which means falling but here is used with the connotation of dying). Phagoptosis means cell death caused by the cell being phagocytosed, with the defining characteristic that inhibition of phagocytosis or phagocytic signalling prevents cell death[61].

Phagoptosis has previously been called primary phagocytosis, as during phagoptosis the primary cause of cell death is phagocytosis — this distinguishes it from so-called 'secondary phagocytosis', in which the primary cause of cell death is apoptosis or necrosis and the dead or dying cell is phagocytosed (see the figure). Experimentally distinguishing between primary phagocytosis (that is, phagoptosis) and secondary phagocytosis (that is, the phagocytosis of a cell dying by apoptosis or necrosis) is relatively simple, as in the first case inhibiting phagocytosis will leave live cells, whereas in the second case it will leave dead cells (at least temporarily before their disintegration). Note that phagocytosis of neuronal structures, such as synapses or neurites, is not phagoptosis unless this phagocytosis causes the death of the neuron.

Phagoptosis is one of the most common causes of cell death in the body under physiological conditions, as it mediates the removal of senescent or activated red and white blood cells[61].



surface glycoproteins. In turn, C1q can either be recognized by LRP (in association with calreticulin) or promote the conversion of C3 to C3b. C3b then opsonizes neurons and is recognized through complement receptor 3 (CR3; consisting of the subunits CD11b and CD18) on microglia[40,41].

Phagocytosis of neurons may also require the local release of uridine diphosphate (UDP) from damaged neurons, which activates microglial purinergic P2Y6 receptors and thereby induces the formation of the phagocytic cup and, subsequently, engulfment of the target neuron[42].

Don't-eat-me signalling. Inhibitory signals can also modulate microglial phagocytosis of neurons. For example, CD47 expression on cells and myelin inhibits phagocytosis by microglia by binding to signal regulatory protein-α (SIRPα; also known as SHPS1), the CD47 receptor[43], but it is unclear whether CD47 is an important don't-eat-me signal on neurons. By contrast, polysialylated proteins on the surface of neurons have been shown to inhibit phagocytosis of those neurons through activation of members of the sialic acid-binding immunoglobulin-like lectins (SIGLECs), including SIGLEC-11 (in humans) and SIGLEC-E (in mice), on the surface of microglia[44,45]. In addition, plasminogen activator inhibitor type 1 (PAI1), which is known to act as a don't-eat-me signal on neutrophils, may be released by activated microglia and astrocytes to induce microglial migration but also to inhibit VNR-mediated microglial phagocytosis[46].

The protein C-X3-C motif ligand 1 (CX3CL1; also known as fractalkine) is normally present on the cell surface of neurons, where it may act to suppress microglial inflammatory responses by activating the microglial chemokine receptor CX3CR1. However, neuronal stress caused by nerve injury or excitotoxicity results in the cleavage of membrane-bound CX3CL1 and the release of its soluble form, which attracts microglia and may stimulate microglial phagocytosis of phosphatidylserine-exposed neurons by increasing the release of the bridging protein MFG-E8 (REFS 47,48).

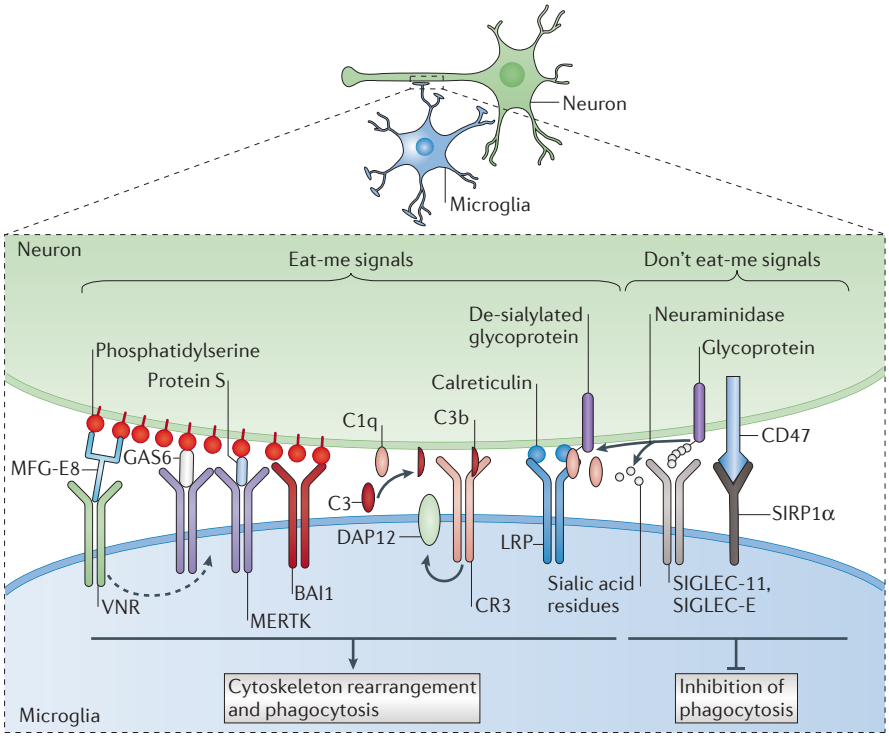


Figure 1 | **Signalling pathways implicated in the phagocytosis of neurons and neuronal structures.** Microglial phagocytosis of neurons is regulated by the neuronal presentation and microglial recognition of 'eat-me' (left) and 'don't eat-me' (right) signals. However, note that the utilization of different signals, opsonins and receptors is dependent on the specific (patho)physiological context (also see text and FIG. 2). Neuronal eat-me signals are recognized by microglial phagocytic receptors either directly or following their binding by opsonins, which are in turn recognized by microglial receptors. Phosphatidylserine that is exposed on neurons can be bound by the opsonins milk fat globule EGF factor 8 (MFG-E8), growth arrest-specific protein 6 (GAS6) or protein S, which can induce phagocytosis by binding to and activating a vitronectin receptor (VNR) (in the case of MFG-E8) or MER receptor tyrosine kinase (MERTK) (in the case of GAS6 or protein S). Note that stimulation of MERTK can also occur downstream of VNR activation (dashed arrow). Alternatively, neuron-exposed phosphatidylserine may directly bind to brain-specific angiogenesis inhibitor 1 (BAI1) on microglia, and neuron-exposed calreticulin or neuron-bound C1q can induce phagocytosis by activating the microglial low-density lipoprotein receptor-related protein (LRP). C1q can also bind to glycoproteins from which sialic acid residues have been removed by the enzyme neuraminidase. C1q deposition on de-sialylated glycoproteins in turn leads to the conversion of C3 to the opsonin C3b, which activates neuronal phagocytosis via the microglial complement receptor 3 (CR3) and its signalling partner DNAX-activation protein 12 (DAP12). By contrast, neuronal don't eat-me signals inhibit phagocytosis and can in some instances also suppress inflammation. Neuronal CD47 and sialylated glycoproteins inhibit phagocytosis of neurons by binding to the microglial receptors signal regulatory protein 1α (SIRP1α) and sialic acid-binding immunoglobulin-like lectins (SIGLECs), respectively.

Phagocytosis of neuronal precursors

Evidence now exists that microglia phagocytose viable neuronal precursors and neuronal structures during development (FIG. 2). In the nematode *Caenorhabditis elegans*, loss-of-function mutations in phagocytosis-related genes cause survival of neuronal precursors that are normally lost during development; and this protective effect is enhanced if the caspases activated in these cells during programmed cell death are partially inactivated by gene mutations[14,15]. Thus, in some conditions, caspase activation may be insufficient to cause apoptotic death but sufficient to cause eat-me signal exposure that induces phagocytosis of the cell[14,15]. Phagocytosis also causes the death and removal of multiple cell types in *C. elegans* if they are subjected to sub-toxic insults[17] or show phosphatidylserine exposure owing to the expression of mutant phosphatidylserine translocases[49]. Interestingly, sub-toxic caspase activation seems to have vital roles in mammalian neurons during development and synaptic plasticity, including neurite pruning[50].

An active contribution of microglia to the developmental death of neuronal precursors has also been shown in the mouse brain. In organotypic slices of the developing mouse cerebellum, the selective elimination of microglia led to an increase in the number of mature Purkinje cells[51]. Similarly, in the developing hippocampus *in vivo*, knockout of the microglial genes encoding the CR3 subunit CD11b or DNAX-activation protein 12 (DAP12; also

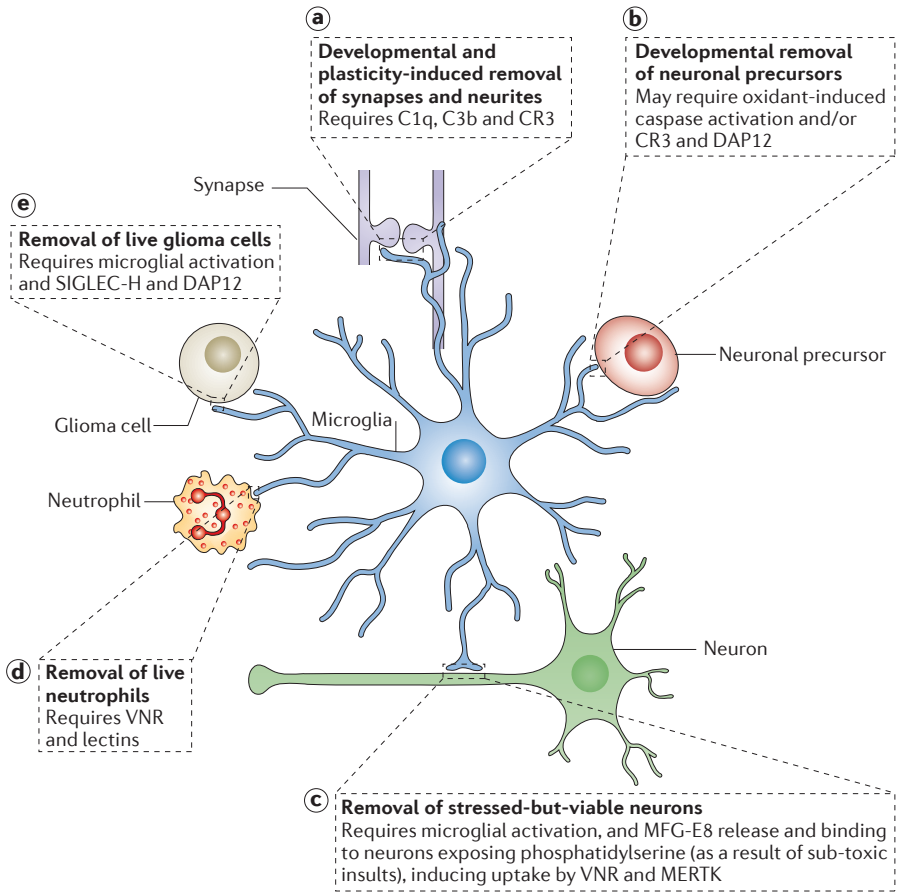


Figure 2 | **Microglial phagocytosis of live cells and neuronal structures.** The figure illustrates situations in which phagocytic recognition leads to the removal of neuronal structures (synapses and neurites) or live cells (glioma cells, neutrophils, neuronal precursors and stressed-but-viable neurons) in the CNS. The shown pathways have been implicated in mediating phagocytic recognition of each target, but other signals may contribute to these processes. **a** | During development as well as in the adult animal, weak synapses are removed through a process that is dependent on the complement components C1q and C3 and the microglial complement receptor 3 (CR3)[20,40,53,54]. **b** | During development, microglia phagocytose live neuronal progenitors, and this involves local release of reactive oxygen and nitrogen species (RONS) by microglia. RONS may induce caspase 3 activation in the targeted neuronal progenitor, which may be phagocytosed via the CR3-subunit CD11b and the adaptor protein DNAX-activation protein 12 (DAP12)[16,51,52]. **c** | During brain pathology, sub-toxic neuronal insults (such as inflammation, oxidative stress, excessive levels of glutamate or energy depletion) can induce the reversible exposure of the neuronal eat-me signal phosphatidylserine[5,9–11]. Phosphatidylserine is recognized by the opsonin milk fat globule EGF factor 8 (MFG-E8), which induces phagocytosis through activation of the microglial vitronectin receptor (VNR)[5,9–11]. In addition, MER receptor tyrosine kinase (MERTK)[9] also contributes to phagocytic signalling under these circumstances, either through its activation downstream of VNR or through the recognition of unidentified opsonins or eat-me signals (FIG. 3). **d**,**e** | In addition to the removal of neurons, neuronal precursors and neuronal structures, microglia can phagocytose live neutrophils through activation of the microglial VNR and lectins[18], or live glioma cells[19] through microglial sialic acid-binding immunoglobulin-like lectin-H (SIGLEC-H) and DAP12.

known as TRYOBP), which are required for complement-mediated phagocytosis, reduced the number of neurons with activated caspase 3 (REF. 52), 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[45], it may be that phagocytosis promotes the death of the cell being engulfed through oxidant-induced apoptosis.

In monkeys and rats, microglia have been found to phagocytose live neural precursor cells in the cortex[16]. 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 the number of neural precursors[16]. Thus, microglia regulate the size of the neuronal precursor cell pool in the developing cerebral cortex, and changes in the microglial activation state potentially affect brain development through the phagoptotic uptake of neural precursors.

Phagocytosis of synapses and neurites

During development, microglia do not only phagocytose whole neurons and neuronal progenitors but also selectively remove synapses of live neurons (synaptic pruning). This does not normally cause cell death and therefore is not a form of phagoptosis; however, it is clearly a related activity of microglia, with similar mechanisms.

In the developing hippocampus, synaptic neuronal proteins are taken up by microglia, and a transient increase in dendritic spines and immature synapses was observed in CX3CR1-knockout mice compared with wild-type mice, which was attributed to a reduction in microglial density[20,53]. Furthermore, this failure to prune synapses appropriately during development was found to cause autism-like behaviour in the adult mice[54]. Analogously, immediately after birth, synaptic inputs from the eyes are remodelled in the thalamus according to synaptic activity by complement-mediated microglial phagocytosis. Mice deficient in one of three complement components — namely, C1q, C3 or CR3 — have decreased microglial phagocytosis of synapses with less synaptic activity, leading to a reduction in the segregation of eye-specific synaptic fields[40]. Thus, unwanted synapses seem to be tagged for microglial removal by deposition of complement proteins during development.

Of note, live imaging of the visual cortex also showed that microglia prune inactive synapses in the adult brain[20]. In addition, complement-mediated synaptic pruning may

contribute to pathology because, after sciatic nerve injury, mice deficient in C3 showed reduced synapse elimination of spinal motor neurons and improved functional recovery[55].

Recently, it was also found that removal of sialic acid residues from the neuronal glycocalyx (by the enzyme neuraminidase) is essential for C1q binding to neurites and their subsequent microglial phagocytosis in culture[41]. By contrast, polysialylated proteins on the surface of neurons are bound by microglia through SIGLEC-11 (in humans) and SIGLEC-E (in mice), resulting in inhibition of phagocytosis, inflammation and neuronal loss[44,45]. Thus, polysialylation of neuronal cell surface proteins may act as a don't-eat-me signal for neurons, whereas de-sialylation may promote phagocytosis of neuronal structures. However, note that SIGLEC-H (in mice) can promote microglial phagocytosis[18].

In a culture model of AIDS dementia, the phagocytosis of axons from live hippocampal neurons occurred after addition of the HIV-1 Tat protein to microglia. This pathological phagocytosis was apparently mediated by increased expression of the phosphatidylserine receptor BAI1 on microglia and phosphatidylserine exposure on the axons, and was blocked by inhibiting leucine-rich repeat kinase 2 (LRRK2), which has also been implicated in Parkinson's disease (PD) (see below)[38]. *In vivo*, tissue damage can recruit microglia through P2Y12 receptors and result in microglial phagocytosis of myelinated axons of both injured and uninjured neurons[56]. Thus, it seems that microglia are capable of phagocytosing at least the synapses and axons of live neurons under specific conditions.

Phagoptosis in pathology

Inflammation. We and others have found that *in vitro* stimulation of microglia with TLR ligands impairs their ability to discriminate between dead and viable neurons for phagocytosis, resulting in phagoptosis during inflammation[5,9–13,32]. We delineated a pathway by which TLR-activated microglia release oxidants that cause neurons to expose phosphatidylserine transiently[5,10,11] (FIG. 3). Importantly, activation of microglia through TLRs led to upregulation and release of MFG-E8, which bound exposed phosphatidylserine and activated phagocytosis via VNRs expressed on microglia[5,10,11]. Consequently, microglia activated by one of three TLR agonists — lipopolysaccharide (LPS), lipoteichoic acid or amyloid-β (Aβ) — caused a slow, progressive loss of neurons by phagocytosis in neuron–microglia co-cultures. Strikingly, blocking VNR, MFG-E8 or exposed phosphatidylserine prevented all neuronal loss, leaving viable neurons *in vitro* without inhibiting inflammation[5,10,11]. Accordingly, 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[10,11]. 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[10].

From these results, it seems that inflammatory stress can induce neurons to expose phosphatidylserine, resulting in the phagocytosis of stressed-but-viable neurons. Consistent with this view, we found that low levels of peroxynitrite or hydrogen peroxide induced reversible phosphatidylserine exposure on viable neurons. In the absence of microglia, these neurons were able to recover and internalize this eat-me signal, but in the presence of microglia, these neurons with exposed phosphatidylserine were lost owing to phagocytosis[5,9].

Recently, we found that microglial activation caused a proportion of microglia to turn into multinucleated giant cells (that is, large cells with multiple nuclei), which had a greater capacity to phagocytose large beads and cells[57]. However, the relative contribution of such cells to phagoptosis is as yet unclear.

Microglial activation can cause neurotoxicity by various mechanisms other than phagoptosis, including nitric oxide (NO) generation by inducible NO synthase (iNOS), which inhibits neuronal mitochondria, or oxidant formation by PHOX, causing direct neurotoxicity[58–60]. Phagoptosis may dominate in conditions in which TLRs are activated but pro-inflammatory cytokine levels are relatively low, as the latter can temporally inhibit phagocytosis and strongly induce iNOS. Accordingly, phagoptosis is normally delayed by several days after TLR activation, when pro-inflammatory responses may be subsiding[5,9,12]. In addition, 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[5,9,61].

Stroke and epilepsy. Brain ischaemia causes direct neuronal death in regions with very low oxygen levels through neuronal energy depletion and depolarization, followed by excessive release of glutamate, which causes excitotoxicity. In these areas, phagocytosis of dead or dying neurons may be beneficial, as it clears harmful cellular components and decreases inflammation. However, in regions of mild ischaemia, neurons may be stressed but viable. Importantly, neurons in peri-infarct areas have been shown to expose phosphatidylserine in a reversible manner[62], and we have found that MERTK and MFG-E8 are upregulated after transient focal ischaemia, with levels peaking after 3 days[9]. Strikingly, 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[9].

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 indicate that the neurons lost in the wild-type animals must have been alive when they were phagocytosed. Accordingly, *in vitro* experiments revealed that non-toxic levels of glutamate caused neurons to expose phosphatidylserine transiently, which promoted MERTK- and MFG-E8-dependent phagocytosis and death of these stressed neurons[9]. Thus, blocking phagocytosis seems to be beneficial after mild ischaemia, as this prevents the phagocytosis of stressed-but-viable neurons.

In a model of epilepsy, it was found that kainate-induced seizures caused UDP release from hippocampal neurons, which stimulated microglial phagocytosis through purinergic P2Y6 receptors, and this was prevented by knockdown or inhibition of P2Y6 (REF. 42). However, the authors did not speculate or test whether such phagocytosis might contribute to the neuronal death caused by the kainate-induced seizures.

Neurodegenerative diseases. Neuronal and synaptic loss occurs in neurodegenerative diseases by unclear means. Interestingly, recent genome-wide association studies have implicated mutations in various phagocytosis-related genes as risk factors for neurodegenerative diseases, including triggering receptor expressed on myeloid cells 2 (*TREM2*), *CD33*, apolipoprotein E (*APOE*),

clusterin (*CLU*), complement receptor 1 (*CR1*), ATP-binding cassette transporter A7 (*ABCA7*) and progranulin (*PGRN*)[63–65]. Although the effects of mutations in these genes remain largely unclear, it is conceivable that phagoptotic loss of neurons or the phagocytic removal of synapses may contribute to the pathology of neurodegenerative conditions, and some tantalizing evidence exists for some of these diseases to tentatively support this view.

Frontotemporal degeneration (FTD) can be caused by loss-of-function mutations in *PGRN*[63]. PGRN inhibits phagocytosis of apoptotic or phosphatidylserine-exposing cells in culture and *in vivo*, and knockdown of PGRN leads to the loss of stressed-but-viable neurons, suggesting that neuronal loss in FTD may be due to phagoptosis that is normally suppressed by PGRN[61]. Polymorphisms in *PGRN* are also associated with PD and amyotrophic lateral sclerosis, suggesting that excessive neuronal phagocytosis contributes to a range of neurodegenerative diseases[63].

Alzheimer's disease (AD) is characterized by insoluble Aβ aggregates, activated microglia and extensive loss of neurons and synapses by mechanisms that are unclear[66]. *In vitro*, high (micromolar) concentrations of recombinant Aβ can induce direct toxicity in neurons, but low (nanomolar) concentrations, which may be more relevant to AD, induce neuronal loss through inflammatory activation of glia[5,11,12]. We found that nanomolar concentrations of Aβ caused microglia to phagocytose viable neurons and synapses in culture, and if we blocked this phagocytosis (by blocking phosphatidylserine exposure or VNRs), neuronal loss and death were prevented[5,11,12]. Nanomolar concentrations of Aβ also caused MFG-E8 release, which bound to phosphatidylserine-exposing neurons, and Aβ-induced neuronal loss was prevented in cultures from *Mfge8*-knockout mice but was reconstituted by the addition of recombinant MFG-E8 (REF. 11) (FIG. 3). Similarly, others have shown that Aβ induces BV-2 microglia to phagocytose live neuron-like PC12 cells[32].

Interestingly, there is an increase in phosphatidylserine-exposing neurons in AD and mild cognitive impairment[67]. This suggests that microglial phagocytosis of live synapses and neurons could contribute to AD. In support of this hypothesis, two-photon imaging showed that microglia mediate neuronal loss in a mouse model of AD, and knockout of microglial CX3CR1, which is required for microglial recruitment,

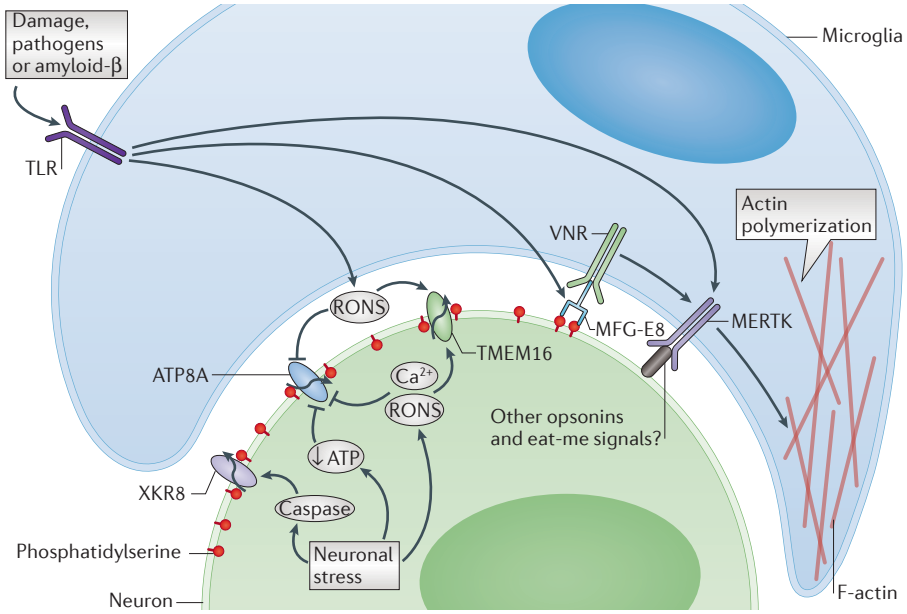


Figure 3 | **Mechanisms mediating microglial phagocytosis of stressed-but-viable neurons during inflammation.** Activation of microglial Toll-like receptor 2 (TLR2) and TLR4 by damage- or pathogen-associated molecules or by amyloid-β results in the release of reactive oxygen and nitrogen species (RONS) derived from inducible nitric oxide synthase (iNOS) and NADPH oxidase (PHOX). RONS can cause nearby neurons to expose phosphatidylserine in a reversible manner on their surface[5] through the stimulation of a phosphatidylserine scramblase (probably a TMEM16 protein[26]) and/or the inhibition of a phosphatidylserine translocase (probably type 4 P-type ATPases (P4-ATPases) ATP8A1 or ATP8A2 (REFS 24,25)). Neuronal stress induced by activated microglia or by other means may also cause exposure of phosphatidylserine on stressed-but-viable neurons via calcium- or RONS-mediated activation of a scramblase or inhibition of a translocase, via ATP depletion-induced inhibition of translocase or, in some circumstances, via caspase-mediated activation of a distinct scramblase (probably XK-related protein 8 (XKR8)[28]). Exposed phosphatidylserine is bound by milk fat globule EGF factor 8 (MFG-E8), which is released by activated microglia and astrocytes and which promotes phagocytosis of the phosphatidylserine-tagged neuron through the vitronectin receptor (VNR)[10]. The VNR may drive phagocytosis by triggering actin polymerization in synergy with MER receptor tyrosine kinase (MERTK). MERTK is upregulated by microglia activation[9] and may also bind to neurons via opsonins that bind exposed phosphatidylserine or other eat-me signals[34–36].

prevented this neuron loss[6]. However, more work is required to establish whether phagocytosis is a primary cause of neuronal death in AD.

TREM2 is a phagocytic receptor on microglia that recognizes an unknown eat-me signal on neurons and induces phagocytosis via DAP12, which is also activated downstream of several other phagocytic receptors[68]. Variants of *TREM2* increase the risk of AD, PD and FTD by unknown mechanisms, but these are likely to involve phagocytosis and/or inflammation[65]. Loss-of-function mutations in *TREM2* or *DAP12* are sufficient to induce Nasu-Hakola disease, a neurodegenerative disorder. DAP12 and microglial phagocytosis have also been implicated as key factors in AD using an integrated systems approach[69].

PD is characterized by motor dysfunction, which results from progressive loss of dopaminergic neurons of the substantia nigra that project into the striatum. Substantia nigra neurons are black because they contain the pigment neuromelanin, which can be released from dying neurons[70]. Neuromelanin, when recognized by microglia, causes their inflammatory activation, and this leads to dopaminergic neuronal loss in culture and *in vivo*. Importantly, this neuronal loss is prevented if the microglial phagocytic receptor CR3 is genetically deleted[71]. The inflammatory response that accompanies PD can also be modelled by LPS injection into the substantia nigra or the striatum. As outlined above, we found that LPS injection into the rodent striatum caused microglial activation and subsequent neuronal loss, which was strongly decreased in *Mfge8*-knockout mice or by co-injection of a VNR inhibitor in rats[10].

PD can also be experimentally induced by the exposure of cells or animals to mitochondrial inhibitors or 6-hydroxydopamine[58,72]. We found that the mitochondrial complex I inhibitor rotenone stimulated microglial

phagocytosis of neurons *in vitro*, and blocking microglial phagocytosis prevented rotenone-induced neuronal loss and death[73]. Others have shown that *in vivo* PD can be induced in rodents by the mitochondrial complex I inhibitor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and the subsequent loss of dopaminergic neurons can be prevented by blocking microglial phagocytosis at either VNR[72] or RHO-associated protein kinase (ROCK)[74]. Furthermore, in a murine model of PD induced by intrastriatal injection of 6-hydroxydopamine, it was found that loss of substantia nigra neurons was accompanied by microglial processes penetrating into live neurons, and genetic inactivation of the phagocytic adaptor protein DAP12 decreased the loss of dopaminergic neurons[75,76]. Note, however, that VNR, ROCK and DAP12 may regulate processes other than phagocytosis, so the role of phagocytosis in PD neurodegeneration remains equivocal.

Perspectives

In conclusion, there is accumulating evidence for microglial phagocytosis of live neurons in various circumstances. But what is the function of this phagoptosis? Phagocytosis of whole, live neurons might be beneficial when there is an excess of neurons or precursors during development or adult neurogenesis, or during neuronal infection, neuronal senescence or neuronal damage that is sufficient to disrupt networks or induce seizures. In addition, microglial phagoptosis of non-neuronal cells may protect the brain by removing viable, activated neutrophils that invade the brain[18] or living glioma cells[19]. In other circumstances, particularly during inflammation, phagoptosis of whole, live neurons may be a dysfunctional consequence of excessive or inaccurate removal of dead, dying, damaged or infected neurons, neuronal processes and/or synapses, or pathogens. In such circumstances, inhibition of phagocytosis or specific phagocytic receptors, such as VNRs, MERTK or CR3, may be therapeutically beneficial.

Guy C. Brown is at the University of Cambridge, Department of Biochemistry, Hopkins Building, Tennis Court Road, Cambridge, CB2 1QW, UK.

Jonas J. Neher is at the Department of Cellular Neurology, Hertie Institute for Clinical Brain Research, University of Tübingen, D-72076 Tübingen, Germany; and the German Center for Neurodegenerative Diseases (DZNE), D-72076 Tübingen, Germany.

Correspondence to G.C.B.
e-mail: gcb3@cam.ac.uk

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Competing interests statement

The authors declare no competing interests.