Microglial phagocytosis of neurons in neurodegeneration, and its regulation

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Abbreviations: Aβ, amyloid beta; ABCA7, ATP-binding cassette sub-family A member 7; ABI3, ABI family member 3; AD, Alzheimer's disease; ADP, adenosine diphosphate; ALS, amyotrophic lateral sclerosis; AMD, Age-related macular degeneration; ApoE, apolipoprotein E; APP, amyloid precursor protein; arp2/3, actin-related protein 2/3; ATP, adenosine triphosphate; CD2AP, CD2 Associated Protein; CD33/siglec-3, sialic acid binding Ig-like lectin 3; CFB, complement factor B, CFH; complement factor H; CNS, central nervous system; CR1, complement receptor 1; CR3, complement receptor 3; C3aR, complement 3a receptor; CR4, complement receptor 4; CSF, cerebral spinal fluid; DAM, disease associated microglia; EMAP II, endothelial monocyte-activating polypeptide II; FTD, frontotemporal dementia; FTDP-17, frontotemporal dementia and parkinsonism linked to chromosome 17; FTLD, frontotemporal lobar degeneration; GPR56, G protein-coupled receptor 56; GWAS, genome wide association studies; ITIM, immunoreceptor tyrosine-based inhibition motif; iNOS, inducible nitric oxide synthetase; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; IL-4, interleukin-4; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; i.p, intraperitoneal injection; Jmjd3, Jumonji domain containing 3; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; LRP1, low density lipoprotein receptor-related protein 1; LRRK2, leucine-rich repeat kinase 2; MAC, membrane attack complex; MerTK, proto-oncogene tyrosine-protein kinase MER; MFG-E8, milk fat globule-EGF factor 8 protein; MGnD, microglial neurodegenerative phenotype; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; P2Y6R, P2Y6 receptor; P2Y12R, P2Y12 receptor; PD, Parkinson's disease; PILRA, paired immunoglobin like type 2 receptor Alpha; PLCG2, phospholipase C, gamma 2; PLD3, phospholipase D3; PLSCR1, phospholipid scramblase 1; PRC2, polycomb repressive complex 2; PS1, presenilin 1; PS2, presenilin 2; PTEN, phosphatase and tensin homolog; RP, Retinal pigmentosa; RPS19, dimerised ribosomal protein S19; S1P, sphingosine-1-phosphate; SHP-1, src homology 2 domain-containing protein tyrosine phosphatase 1; SHP-2, src homology 2 domain-containing protein tyrosine phosphatase 2; Siglec, sialic acid-binding immunoglobulin-type lectin; SIRPα, signal-regulatory protein alpha ; SIRPβ1, signal-regulatory protein beta 1; TDP-43, TAR DNA-binding protein 43; TET2, Ten-eleven translocation 2; TGFβ, transforming growth factor beta; TMEM16F, transmembrane protein 16F; TNFα, tumor necrosis factor Alpha; TREM2,

triggering receptor expressed on myeloid cells 2; TyrRS, tyrosyl tRNA synthetase; UDP, uridine diphosphate; UTP, uridine triphosphate; VR, vitronectin receptor; XKR8, scramblase Xk-related protein 8; ZYX, zyxin

Abstract

There is growing evidence that excessive microglial phagocytosis of neurons and synapses contributes to multiple brain pathologies. RNA-seq and genome wide association (GWAS) studies have linked multiple phagocytic genes to neurodegenerative diseases, and knock-out of phagocytic genes has been found to protect against neurodegeneration in animal models, suggesting that excessive microglial phagocytosis contributes to neurodegeneration. Here, we review recent evidence that microglial phagocytosis of live neurons and synapses causes neurodegeneration in animal models of Alzheimer's disease and other tauopathies, Parkinson's disease, frontotemporal dementias, multiple sclerosis, retinal degeneration, and neurodegeneration induced by ischaemia, infection or ageing. We also review factors regulating microglial phagocytosis of neurons, including: nucleotides, frackalkine, phosphatidylserine, calreticulin, UDP, CD47, sialylation, complement, galectin-3, Apolipoprotein E, phagocytic receptors, Siglec receptors, cytokines, microglial epigenetics and expression profile. Some of these factors may be potential treatment targets to prevent neurodegeneration mediated by excessive microglial phagocytosis of live neurons and synapses.

1. INTRODUCTION

The neurodegeneration of neurodegenerative diseases is accompanied by progressive loss of neurons, synapses, dendrites, myelin and brain tissue. However, there are very few dead or dying cells in the neurodegenerating brain, and there is little evidence that neurodegeneration is caused by neuronal death, i.e. that blocking neuronal death prevents neurodegeneration (Fricker et al., 2018; Yang & Wang, 2018). This raises the possibility that brain tissue is removed alive by phagocytes such as microglia during neurodegenerative disease (Rajendran & Paolicelli, 2018; Vilalta & Brown, 2018). In effect, the brain may eat itself. If so, this changes our whole concept of neurodegeneration, and suggests radically different ways of tackling these devastating diseases.

Microglia are the brain's main phagocytes (cells capable of engulfing and digesting large extracellular particles), and protect the brain by phagocytosing bacteria, aggregated proteins and cellular debris (Wolf et al., 2017; Vilalta & Brown, 2018; Galloway et al., 2019). Microglia derived from the yolk sac, invade the brain prior to birth and are maintained by self-renewal within the adult brain (Ginhoux et al., 2010; Hashimoto et al., 2013). During normal brain development, microglia shape neuronal circuits by phagocytosing excess synapses, dendrites, axons, myelin, neurons and neuronal precursors (Vilalta & Brown, 2018). The developmental loss of synapses is known as 'synaptic pruning', and this is partly mediated by microglial phagocytosis of such synapses (Paolicelli et al., 2011). Microglia also phagocytose live neuronal precursors during development to regulate neuronal numbers (Cunningham et al., 2013; Anderson et al., 2019).

After development, microglia are normally sessile and ramified within brain parenchyma, but their long processes are constantly moving to scan synapses, neurons and other cells for any changes, damage or pathogens (Nimmerjahn et al., 2005). Signs of substantial damage or pathogens, result in inflammatory activation of microglia, including: chemotaxis of microglial processes and the whole microglia to the site of activation, retraction of other processes to the cell body to form more-or-less amoeboid microglia, NF-κB-dependent expression of inflammatory genes including phagocytic receptors, and release of opsonins and cytokines.

Activated microglia can kill neurons by releasing TNF-α, glutamate, cathepsin B, superoxide or nitric oxide (Brown & Vilalta, 2015). Proinflammatory cytokines such as IL-1β and TNF-α

can induce neuronal cell death in culture and *in vivo* (McCoy & Tansey, 2008; Glass et al., 2010), but in general, this is indirect toxicity mediated by activation of glia (Taylor et al., 2005; Neniskyte et al., 2014). TNF-α can induce glutaminase release from neurons, generating glutamate from glutamine extracellularly, resulting in excitotoxicity (Ye et al., 2013). Similarly, activated microglia can release glutaminase to induce excitotoxicity (Huang et al., 2011). Cathepsin B can be released by activated microglia to mediate the neurotoxicity of Aβ (Gan et al., 2004) and chromogranin A (Kingham & Pocock, 2001). The microglial NADPH oxidase (PHOX) generates superoxide and hydrogen peroxide, which may mediate neuronal death induced by LPS (Cheret et al., 2008), 6-hydroxy-dopamine (Hernandes et al., 2013), transient ischemia (Yoshioka et al., 2010) and retinal degeneration (Zeng et al., 2014). Activated microglia can express iNOS (inducible nitric oxide synthetase) producing nitric oxide (NO) that: can kill neurons under hypoxic conditions (Mander et al., 2005), or reacts with superoxide to produce neurotoxic peroxynitrite (Mander & Brown, 2005). High doses of TNF-α, glutamate, superoxide, nitric oxide or peroxynitrite may cause direct death of neurons in culture, however, low doses of each of these agents can stress neurons such that they are phagocytosed by microglia (Neher et al., 2011; Neniskyte et al., 2014; Neniskyte et al., 2016; Hornik et al., 2016), as reviewed below.

Under inflammatory conditions, neurons may be damaged/stressed such that they expose eat-me signals, lose don't-eat-me signals or bind opsonins, which leads to increased microglial phagocytosis of stressed-but-viable neurons or synapses (Neher et al., 2011; Fricker et al., 2012; Fricker et al., 2012; Hornik et al., 2016). However, phagocytosis of live neurons results in death of the neuron, and such cell death by phagocytosis is known as primary phagocytosis or phagoptosis. Whereas phagoptosis during brain development contributes to neuronal network development, the aberrant removal of live neurons during pathological conditions such as chronic inflammation can be detrimental and result in neuronal loss and neurodegeneration.

How can we determine whether microglial phagocytosis of neurons contributes to neuronal death? One way is to look for microglia phagocytosing neurons; however: this provides only correlational data, is difficult to do *in vivo*, and cannot distinguish between phagocytosis of live, dying and dead neurons. More useful conclusions can be drawn from blocking microglial phagocytosis, for example by inhibition or knock-out of phagocytic receptors, and then determining whether neuronal death or loss is prevented. If microglia are phagocytosing only dead or dying neurons, then blocking phagocytosis of these will not prevent any neuronal death,

and just cause an accumulation of dead neurons. Whereas, if microglia are phagocytosing stressed-but-viable neurons, then blocking phagocytosis of these will prevent their death, resulting in reduced neuronal loss. This is the critical test to distinguish between phagocytosis of dead and dying neurons versus phagocytosis of live and viable neurons.

By "stressed-but-viable neurons" we mean here neurons that have been perturbed by neurodegeneration sufficiently to signal (or be targeted) for phagocytosis, but not sufficiently to induce neuronal death in the absence of phagocytosis. Examples of such stressed neurons might include: i) neurons exposed to sub-toxic doses of glutamate (Neher et al., 2013), and ii) neurons with tau aggregates (Brelstaff et al., 2018). By "live neurons" we simply mean neurons that are not dead. Microglial phagocytosis of degenerating/dying neurons could be beneficial by removing debris or dysfunctional neurons, but it could be detrimental by prematurely removing otherwise functional neurons, even-though they are destined to die. In contrast to degenerating/dying neuron, "stressed-but-viable neurons" are destined to live as long as they are not phagocytosed, and thus it is likely to be beneficial to prevent this phagocytosis.

In this review, we will focus on the new evidence that phagocytosis contributes to neurodegeneration, and new insights into the mechanisms involved. Below, we start by reviewing the signalling between neurons and microglia that determines whether a neuron, neurite or synapse is phagocytosed or not. Dysfunctional signalling could cause excessive phagocytosis in pathological conditions, providing us with new potential therapeutic targets to prevent neurodegeneration.

2. PHAGOCYTIC SIGNALLING

Whether one cell eats another depends on signals expressed or released by the target cell (in this case neurons). These signals include find-me signals, eat-me signals, don't-eat-me signals and opsonins (Ravichandran, 2010; Figure 1).

2.1 Find-me signals: fractalkine and nucleotides (ATP and ADP)

CX3CL1 (fractalkine) is a protein released from neurons or synapses, which chemoattracts microglia via the CX3CR1 receptor (Truman et al., 2008). Several groups have shown that in CX3CR1 knock-out mice, the microglia migrate less, resulting in reduced or delayed phagocytosis of synapses during development (Fuhrmann et al., 2010; Pagani et al., 2015).

This delayed synaptic pruning can result in autism-like behaviour in mice, suggesting the possibility that autism results from insufficient phagocytosis of synapses (Paolicelli et al., 2011; Peça et al., 2011; Tang et al., 2014). CX3CL1 is expressed on cortical neurons and the metalloprotease ADAM10 cleaves CX3CL1 into a secreted form that chemoattracts microglia. Interestingly, it has been shown that inhibition of ADAM10, knock-out of CX3CL1 or knock-out of CX3CR1 prevent synaptic pruning by microglia induced by reduced sensory and synaptic activity (Gunner et al., 2019). One could speculate that synaptic loss in neurodegeneration is a pathological extension of developmental synaptic pruning, and one stimulus for this might be loss of synaptic activity.

Nucleotides, including adenosine triphosphate (ATP) and uridine triphosphate (UTP) can be released by: i) active neuronal synapses as co-transmitters, or ii) apoptotic or stressed cells via Pannexin-1 channels (Chekeni et al., 2010; Yamaguchi et al., 2014). ATP and UTP can chemoattract macrophages by activating P2Y2 receptors (Elliott et al., 2009). However, extracellular ATP is rapidly converted to adenosine diphosphate (ADP), which induces microglial migration and chemotaxis towards neural injury *in vivo* via activating P2Y12 receptors (Haynes et al., 2006). The P2Y12 receptor (P2Y12R) also appears to mediate microglial recruitment to synapses, so that knock-out of P2Y12R delays activity-dependent synaptic pruning by microglia during development (Sipe et al., 2016). P2Y12R also seems to regulate microglial phagocytosis of myelinated axons in the spinal cord *in vivo* (Maeda et al., 2010). More recently, it was found that microglial processes spend most of their time on neuronal cell bodies, recruited by ATP release from the neurons via activating microglial P2Y12R (Cserép et al., 2020).

A variety of other find-me signals have been identified outside the central nervous system (CNS), including: lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), dimerised ribosomal protein S19 (RP S19), endothelial monocyte-activating polypeptide II (EMAP II), tyrosyl tRNA synthetase (TyrRS), and formyl peptides (Fond & Ravichandran, 2016). However, these have generally been identified as find-me signals released by apoptotic cells recruiting macrophages, and their roles in recruiting microglia to neurons, if any, is unknown.

2.2 Eat-me signals: phosphatidylserine, calreticulin and UDP

The best characterised eat-me signal is phosphatidylserine, which is usually present on the inner leaflet of the plasma membrane, because ATP-driven aminophospholipid translocases

ATP8A1 and ATP8A2 pump phosphatidylserine from the outer to inner side of the membrane (Sapar et al., 2018). However, phosphatidylserine can be exposed on the cell surface, either: i) reversibly on live cells due to calcium-activated phosphatidylserine scramblases, such as phospholipid scramblase 1 (PLSCR1) and transmembrane protein 16F (TMEM16F) (Shin & Takatsu, 2020; Zhang et al., 2020), or ii) irreversibly on apoptotic cells due to caspase-activated scramblase Xk-related protein 8 (XKR8) (Suzuki et al., 2013). Glutamate or oxidants can induce reversible phosphatidylserine exposure on live neurons, which induces their phagocytosis by microglia (Neher et al., 2011; Sapar et al., 2018). Exposed phosphatidylserine can be re-internalised by ATP-driven translocases, and inactivating mutations or knock-out of such phosphatidylserine translocases can cause neurodegeneration in animals via phosphatidylserine exposure on live neurons (Zhu et al., 2012; Sapar et al., 2018). Phosphatidylserine exposed on neurons induces microglial phagocytosis either directly by microglial receptors triggering receptor expressed on myeloid cells 2 (TREM2) or G-protein coupled receptor 56 (GPR56), or indirectly by binding opsonins Gas6 or milk fat globule-EGF factor 8 protein (MFG-E8), which induce phagocytosis via the microglial phagocytic receptors proto-oncogene tyrosine-protein kinase MER (MerTK) or the vitronectin receptor (VR, integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$), respectively (Fricker et al., 2012; Wang et al., 2015; Wijeyesakere et al., 2016; Kasikara et al., 2017; Li et al., 2020). Phosphatidylserine is also exposed on synapses during developmental synaptic pruning and induces microglial phagocytosis of such synapses by activating microglial GPR56 (Li et al., 2020).

In primary mixed neuronal-glial cultures, addition of amyloid beta (Aβ) induces phosphatidylserine exposure on live neurons, and the consequent microglial phagocytosis of live neurons can be prevented by blocking phosphatidylserine, MFG-E8, MerTK or VR (Neher et al., 2011, 2013; Neniskyte et al., 2011; Fricker et al., 2012; Hornik et al., 2016; Neniskyte et al., 2016). Extracellular tau can also induce phosphatidylserine exposure on live neurons, inducing neuronal loss by microglial phagocytosis, which can be prevented by inhibiting the phagocytic receptor MerTK, or by eliminating microglia (Pampuscenko et al., 2019).

In Drosophila, phosphatidylserine exposure has been imaged on dendrites during developmental dendritic pruning or neuronal injury (Sapar et al., 2018). Forced phosphatidylserine exposure induced by knock-out of phosphatidylserine translocase or overexpression of the phosphatidylserine scramblase resulted in microglial phagocytosis of

dendrites and axons *in vivo* (Sapar et al., 2018). Similarly, overexpression of the phagocytic glial receptors, Simu and Drpr, in Drosophila caused loss of dopaminergic and GABAergic neurons. Interestingly, only the GABAergic neurons exposed phosphatidylserine *in vivo*, and masking the exposed phosphatidylserine prevented the loss of GABAergic neurons, but not the dopaminergic neurons (Hakim-Mishnaevski et al., 2019), suggesting that the GABAergic neuronal loss was due to microglial phagocytosis of live phosphatidylserine-exposed neurons, but phagocytosis of dopaminergic neurons was mediated by other signals.

Cell surface calreticulin can act as an eat-me signal inducing phagocytes to phagocytose such cells via the low-density lipoprotein receptor-related protein 1 (LRP1) (Gardai et al., 2005). Calreticulin is normally confined to the endoplasmic reticulum but can be released to the surface by endoplasmic reticulum stress or inflammatory signalling (Feng et al., 2015). Interestingly, blocking calreticulin on the surface of neurons and/or its receptor LRP1 on the surface of microglia was sufficient to block lipopolysaccharide (LPS) and Aβ-induced phagocytosis of live neurons by microglia (Fricker et al., 2012). This suggests that calreticulin can act as an eat-me signal for neurons. However, calreticulin can also be regarded as an opsonin that binds cell-surface galactose and other sugar residues exposed by desialylation (Feng et al., 2018) and gram-negative bacteria (Cockram et al., 2019). In this context, cell-surface exposed galactose residues can be regarded as an eat-me signal, which can bind opsonins including calreticulin, C1q, and galectin-3.

Uridine diphosphate (UDP) can act as a soluble eat-me signal, when released by damaged or stressed neurons, activating P2Y6 receptors (P2Y6R) on microglia which triggers phagocytosis of the neurons (Koizumi et al., 2007). Pharmacological inhibition of P2Y6 receptor was sufficient to prevent microglial phagocytosis of live neurons *in vitro* and *in vivo* (Neher et al., 2014; Emmrich et al., 2013; Neniskyte et al., 2014), suggesting that its inhibition may prevent neurodegeneration. However, activation of P2Y6R may be protective in ischemia and radiation-induced brain injury, and the role of P2Y6R may vary with brain pathology (Anwar et al., 2020).

2.3 Don’t-eat-me signals: CD47 and sialic acid

CD47 is a transmembrane protein expressed on most mammalian cells, including neurons, and inhibits phagocytosis of such cells via engaging signal-regulatory protein alpha (SIRPα) on

phagocytes to inhibit phagocytosis (Brown & Frazier, 2001; Gardai et al., 2005). CD47 was also found to be expressed on synapses during development, where it inhibits microglia-mediated synapse removal (Lehrman et al., 2018). CD47 expression on myelin debris has also been shown to inhibit its phagocytosis via SIRPα (Elberg et al., 2019).

The surface of neurons is highly sialylated, i.e. there is a high density of sialic acid residues on its glycoproteins and glycolipids. These sialic acid residues prevent microglial phagocytosis of such neurons by i) activating Siglec (sialic acid-binding immunoglobulin-type lectin) receptors on microglia that inhibit microglial phagocytosis, and ii) blocking the binding of opsonins C1q, C3b and galectin-3 (reviewed by Puigdellivol et al., 2020). Similar to SIRPα, most microglial Siglec receptors signal via immunoreceptor tyrosine-based inhibition motif (ITIM) domains, which activate src homology 2 domain-containing protein tyrosine phosphatases 1 and 2 (SHP-1/SHP-2), inhibiting microglial phagocytosis (Ulyanova et al., 1999; Crocker & Varki, 2001). Thus, for example, murine Siglec-E and human Siglec-11 were found to recognise sialic acid residues on the neuronal glycocalyx and inhibit phagocytosis of such sialylated neurons (Claude et al., 2013; Wang & Neumann, 2010). Activated microglia released a sialidase that desialylated co-cultured PC12 cells, enabling their phagocytosis by microglia (Nomura et al., 2017). Sialylated PC12 cells did not bind the opsonin galectin-3, but desialylated PC12 cells bound galectin-3, enabling galectin-3 mediated phagocytosis of live cells (Nomura et al., 2017). Heterozygous knock-out of a sialylating enzyme in mice caused a mild (~20-30 %) reduction in sialylation, followed by progressive loss of synapses and neurons, prevented by complement C3 knock-out (Klaus et al., 2020). This all indicates that neuronal sialylation inhibits microglial phagocytosis of neurons.

2.4 Opsonins: complement, galectin-3 and Apolipoprotein E

Complement proteins can be deposited on dendrites and synapses to promote their removal by microglial cells (Stevens et al., 2007; Schafer et al., 2012). The key step in complement activation is proteolytic cleavage of C3 to C3a and C3b, where C3a can recruit and activate microglia, while C3b opsonises synapses and neurons (complement roles in the brain reviewed in Lee et al., 2019). The classical pathway of complement activation starts with complement protein C1 (C1q, r and s) being deposited on a cell surface and enzymatically cleaving C2 to C2a and C2b, and cleaving C4 to C4a and C4b, then C2a binds to C4b to form a ‘classical C3 convertase’ that cleaves C3 to C3a and C3b (reviewed in Bajic et al., 2015). In the alternative

pathway, C3 spontaneously hydrolyses and binds factor B (which is cleaved by factor D to Bb) to form an alternative C3 convertase that cleaves C3. In addition, C3b can bind factor B and properdin to form an amplifying C3 convertase cleaving more C3 to C3a and C3b. C3b and C4b covalently attach to cell surface hydroxyl groups, typically on sugars, opsonising such surfaces for phagocytosis. Sialylation has been shown to inhibit C1q and C3b deposition on dendrites *in vitro* (Linnartz et al., 2012). In addition, C1q can bind to exposed phosphatidylserine (Paidassi et al., 2008), and is required to remove apoptotic neurons by microglia *in vitro* (Fraser et al., 2010). C1q-opsonized targets can be removed by LRP1 plus calreticulin (Ogden et al., 2001), C4b-opsonized targets by CR1, and C3b- or iC3b-opsonized targets by complement receptor 1 (CR1), complement receptor 3 (CR3) or complement receptor (CR4) (Reviewed in Ricklin et al., 2010) (Figure 2).

Knock-out of C1q, C3 or CR3 in mice reduces synaptic pruning during development, resulting in excess synapses, indicating that complement proteins mediate microglial phagocytosis of synapses (Stevens et al., 2007; Schafer et al., 2012; Hong et al., 2016). Complement also mediates microglial phagocytosis of weak synapses in adults, for example during forgetting of memories (Wang et al., 2020), suggesting the possibility that excessive phagocytosis may disrupt cognition (Miyanishi et al., 2020).

One of the main genetic risk factors for schizophrenia is variants of C4, and there is evidence that C4 may drive excessive microglial phagocytosis of synapses in schizophrenia (Sekar et al., 2016). New-born retinal ganglion neurons in mouse retina were found to be tagged with C1q and selectively phagocytosed alive by microglia, so retinal ganglion cell numbers were increased by microglial depletion or CR3 knock-out (Anderson et al., 2019). This indicates that complement contributes to phagocytosis of live neurons as well as synapses.

Cleavage of C3 generates both C3a and C3b, and C3a apparently activates microglia via C3a receptors (C3aR), which acutely stimulates phagocytosis (Lian et al., 2016) and may be chemotactic for microglia (Surugiu et al., 2019). C3aR antagonists prevented microglial phagocytosis of neurons *in vivo* (Surugiu et al., 2019), and C3aR knock-out prevented microglial phagocytosis of synapses (Vasek et al., 2016). C3aR antagonists or knock-out were beneficial in mouse models of CNS lupus (Jacob et al., 2010), multiple sclerosis (MS) (Boos et al., 2004), ischemia/reperfusion injury (Ducruet et al., 2008), amyloid Alzheimer's disease

(AD) models (Lian et al., 2015, 2016), a tau AD model (Litvinchuk et al., 2018) and vascular white matter disease (Zhang et al., 2020).

Galectin-3 can act as an opsonin by binding to galactose residues on the cell surface and MerTK on phagocytes (Caberoy et al., 2012; Nomura et al., 2017). Galectin-3 was found to be released by activated microglia and bound to desialylated PC12 neurons and promoted their phagocytosis by microglia via microglial MerTK receptor *in vitro* (Nomura et al., 2017). Traumatic brain injury in mice induced galectin-3 release into cerebral spinal fluid (CSF) and neuronal loss was prevented by galectin-3 knock-out or antibodies, consistent with galectin-3 role in mediating neuronal loss (Yip et al., 2017).

Apolipoprotein E (ApoE) can opsonise apoptotic cells (Grainger et al., 2004), probably by binding phosphatidyserine on apoptotic cells and a variety of ApoE receptors on phagocytes. ApoE has been shown to bind and opsonise apoptotic (N2a) neurons for phagocytosis via the microglial TREM2 receptor, which directly binds to ApoE (Atagi et al., 2015). Expressing ApoE4 (but not ApoE2 or ApoE3) in a microglial cell line increased phagocytosis of apoptotic neurons (Muth et al., 2019). ApoE can bind to C1q to block complement activation (Yin et al., 2019). ApoE has also been implicated in synapse removal by astrocytes *in vitro* and in rodent brain development (Chung et al., 2016). However, it is still unclear whether ApoE can directly opsonise live synapses or neurons for microglial phagocytosis.

3. MICROGLIAL STATES REGULATING PHAGOCYTOSIS

Microglial phagocytosis of neurons does not just depend on the state of the neurons, but also the state of the microglia, as microglia in different states express different phagocytic genes and have different phagocytic capacity (Figure 3).

3.1 Cytokines

Michelucci et al (2009) reported that IFNγ inhibited microglial phagocytosis, while IL-10 increased phagocytosis, and IL-4 did little or nothing to phagocytosis. However, it should be noted that cytokine effects on phagocytosis are often time-dependent, and Haga et al (2016), using somewhat more physiological conditions, found that IFNγ increased microglial phagocytosis, and IL-4 inhibited microglial phagocytosis. We found that the pro-inflammatory cytokine TNFα stimulated microglial phagocytosis of beads and live neurons, resulting in

neuronal loss in mixed glial-neuronal cultures, which was prevented by inhibiting phagocytosis (Neniskyte et al., 2014). Interestingly, the anti-inflammatory cytokine TGF-β was found to stimulate neuronal expression of C1q and C1q tagging of synapses, promoting microglial synaptic pruning during development (Bialas & Stevens, 2013). Type 1 interferons (IFN), such as IFN-β, increased microglial phagocytosis (Chan et al., 2003), and mediated the expression of phagocytic genes in prion-infected mouse brain, such that blocking this response reduced neuronal and synaptic loss in this model (Nazmi et al., 2019). IFN-β and IFN-γ were found to increase expression of SIRP-β1, which increased microglial phagocytosis of neuronal debris, beads and Aβ through binding an unknown ligand on neurons (Gaikwad et al., 2009). Thus, a variety of cytokines can regulate microglial phagocytosis.

3.2 Sialylation of microglia

The microglial cell surface is sialylated, but LPS, Aβ and tau induce desialylation (Allendorf et al., 2020a), mediated by the cell surface expression and release of neuraminidase 1 (Sumida et al., 2015; Allendorf et al., 2020b). Microglial desialylation increases microglial phagocytosis, partly via reduced activation of Siglec-2 (CD22) (Pluvinage et al., 2019). Microglial desialylation also increased microglial phagocytosis via activating CR3, and induced microglia to phagocytose healthy neurons (Allendorf et al., 2020a). Addition of LPS or Aβ to glial-neuronal cultures induced neuronal loss that could be blocked by inhibiting sialidases or CR3 (Allendorf et al., 2020a). Thus, inflammatory stimuli can induce desialylation of both microglia and neurons, which stimulates microglial phagocytosis of neurons, which might contribute to neurodegeneration (reviewed in Puigdellivol et al., 2020).

3.3 Disease-Associated Microglia (DAM)

Single-cell transcriptional profiling of microglia identified common transcriptional changes in microglia from mouse models of AD (APP/PS1), amyotrophic lateral sclerosis (ALS) (SOD1) and aging (Holtman et al., 2015). Other research groups found a similar microglial transcriptional profile from mouse models of AD (APP/PS1 and 5xFAD) (Krasemann et al., 2017; Keren-Shaul et al., 2017), tauopathy (Tau P301L), and ageing (Kang et al., 2018). Microglia with this expression profile have been called various names, including ‘disease-associated microglia’ (DAM) and ‘microglial neurodegenerative phenotype’ (MGnD). This common transcriptional profile included upregulated expression of *Itgax, Clec7a, Axl, TREM2* and *Apoe*. The *Itgax* gene codes for CD11c, a component of the phagocytic receptor CR4,

which mediates phagocytosis of iC3b-opsonised cells. The *Clec7a* gene codes for a phagocytic receptor Dectin-1, mediating phagocytosis of cells exposing the glucose polymer beta-glucan. The *Axl* gene codes for the phagocytic receptor Axl, which mediates phagocytosis of phosphatidylserine-exposed cells (Tondo et al., 2019). The *TREM2* gene codes for the phagocytic receptor TREM2, which mediates phagocytosis of phosphatidylserine-exposed cells (Takahashi et al., 2005). The *Lgals3* gene codes for galectin-3, an opsonin mediating phagocytosis of galactose-exposing cells (see Opsonin section). The *Apoe* gene codes for ApoE, which regulates phagocytosis of synapses and neurons, and inhibits C1q opsonisation, but also helps transport of lipids and cholesterol, necessary for digestion of targets (see Opsonin section). This up-regulation of phagocytosis genes in DAM microglia is likely to increase their phagocytic capacity, and indeed DAM microglia have been shown to cluster around amyloid plaques and to contain an increased amyloid load, consistent with upregulated phagocytosis (Keren-Shaul et al., 2017). Unfortunately, we do not know whether DAM microglia are beneficial, detrimental, both or neutral for neurodegeneration.

3.4 Microglial phagocytosis of neurons can change microglial state

Microglia that have phagocytosed apoptotic neurons have an altered transcriptional profile that may resemble the DAM profile (Krasemann et al., 2017). Knock-out of the phagocytic receptor TREM2 prevents part of the DAM profile (Krasemann et al., 2017), suggesting that phagocytosis itself could partially trigger this switch into a microglial DAM profile. Microglial phagocytosis of dying cells causes multiple transcriptional changes, including up-regulation of phagocytic genes, mediated by epigenetic changes (Ayata et al., 2018). Microglia that have phagocytosed apoptotic neurons also secrete different cytokines, chemokines and other factors, and one consequence of this is an inhibition of neurogenesis (Diaz-Aparicio et al., 2020). Microglial phagocytosis of apoptotic T cells supressed microglial activation and antigen presentation (Magnus et al., 2001). It is unclear whether microglial phagocytosis of live-but-stressed neurons or synapses is pro- or anti-inflammatory, and whether microglia can present antigens from phagocytosed live neurons, synapses or myelin – but this is important to know, as it could contribute to disease.

3.5 Epigenetics

Microglia from cortex and striatum were found to be less phagocytic than microglia from cerebellum, apparently due to expression of polycomb repressive complex 2 (PRC2), which methylates histones (H3K27me3) repressing phagocytic genes (Ayata et al., 2018). Knock-out

of PRC2 in microglia upregulated phagocytic genes in cortical and striatal microglia, and resulted in synaptic loss and behavioural deficits, attributed to excessive microglial phagocytosis (Ayata et al., 2018). Similarly, Jumonji domain containing 3 (Jmjd3) is a histone H3K27 demethylase, which suppresses microglial activation and neuronal loss in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-model of Parkinson's disease (Tang et al., 2014). Systemic inflammation induced by repeated LPS i.p. injection into mice induced sustained epigenetic and transcriptional changes in brain microglia, including increased expression of complement and phagosome genes (Bodea et al., 2014; Wendeln et al., 2018). This resulted in loss of dopaminergic neurons in the *substantia nigra* that was prevented in complement C3 knock-out mice (Bodea et al., 2014), suggesting that the activated microglia had phagocytosed live C3-tagged neurons. Microglial activation induced by LPS and other stimuli can also be mediated by DNA methylation, and the methylcytosine dioxygenase Ten-eleven translocation 2 (TET2) (Carrillo-Jimenez et al., 2019). Altogether, these studies indicate that epigenetics regulates microglial phagocytosis, and this may contribute to brain-region-specific differences in microglial phagocytosis.

4. PHAGOCYTOSIS OF NEURONS AND SYNAPSES IN NEURODEGENERATION

4.1 Alzheimer's disease (AD) and tauopathies

AD is the most common cause of dementia, characterised by extracellular plaques of aggregated Aβ, intracellular neurofibrillary tau tangles, synapse loss, neuronal loss, brain atrophy, microglial activation and memory loss (Hamos et al., 1989; Nestor et al., 2008; Bondi et al., 2017).

Early-onset AD is caused by mutations in the genes encoding APP (amyloid precursor protein) or presenilin 1 or 2 (PS1, PS2), which cleave APP to generate Aβ (O'Brien & Wong, 2011). Thus, early-onset AD is thought to be driven by the accumulation and aggregation of Aβ with age. In an amyloid model of AD, expression of human mutant APP and PS1 results in progressive loss of synapses, neurons and memory, which are all prevented by complement C3 knock-out, apparently due to reduced microglial phagocytosis of synapses and neurons (Shi et al., 2017). Similarly, in amyloid models of AD, C1q tagged synapses for phagocytosis by microglia, and synaptic loss was prevented by knock-out of C1q, C3 or CR3 (Hong et al., 2016; Wu et al., 2019). Thus, complement seems to be central to synaptic loss in amyloid models of

neurodegeneration (Figure 2). Note, however, that complement factors can induce inflammation and cell death, independent of phagocytosis, although the requirement for CR3 favours phagocytosis being required in this case.

Two-photon imaging of intact brains of mice with APP, PS1 and tau mutations revealed that neuronal loss was accompanied by microglial recruitment and process mobility prior to neuronal loss, and preventing microglial recruitment by knock-out of Cx3cr1 prevented neuronal loss (Fuhrmann et al., 2010). In a similar 5xFAD mouse model, microglia were found to preferentially interact with amyloid-laden neurons, and microglia exposed to amyloid *in vivo* phagocytosed neurites, even before plaque formation (Von Saucken et al., 2020).

Insoluble intracellular inclusions of hyperphosphorylated tau, known as neurofibrillary tangles are a hallmark of AD, frontotemporal dementia (FTD) and several other neurodegenerative diseases, known as tauopathies (Crowther & Goedert, 2000), and tau variants are associated with AD and FTD by genome wide association studies (GWAS), implicating tau as causal in disease. Expression of FTD mutant tau (P301S or P301L) in mice results in synaptic and neuronal loss, prevented by C1q antibodies (Dejanovic et al., 2018), CR3 knock-out (Litvinchuk et al., 2018), and C3 knock-out (Wu et al., 2019), suggesting that tau-induced synaptic and neuronal loss is via complement-mediated microglial phagocytosis. Synapses and neurons in P301L tau mice exposed phosphatidylserine prior to phagocytosis by microglia, and a phosphatase and tensin homolog (PTEN) inhibitor prevented this phagocytosis (Benetatos et al., 2020). Apparently tau normally supresses PTEN, but aggregated tau does not, resulting in phosphatidylserine exposure, which induces phagocytosis by microglia (Benetatos et al., 2020). Consistent with this, only neurons with tau aggregates from P301S tau mice exposed to phosphatidylserine in culture, were specifically phagocytosed by co-cultured microglia. This phagocytosis could be prevented by blocking phosphatidylserine (Brelstaff et al., 2018). Similarly, application of extracellular tau caused phosphatidylserine exposure on the neurons and microglial phagocytosis of such neurons, and blocking microglial phagocytosis prevented the tau-induced neuronal loss (Pampuscenko et al., 2019,2020).

Some FTD can be caused by mutations of the GRN gene encoding progranulin and granulin. Progranulin can inhibit phagocytosis of apoptotic cells, synapses and axons (Kao et al., 2011; Petoukhov et al., 2013). Thus, it is possible that loss-of-function mutations of GRN promote excessive microglial phagocytosis of neurons and synapses.

In late-onset AD most of the genetic inheritability is linked to variants of ApoE (Lambert et al., 2013). Using ApoE variant specific knock-in mice there is *in vivo* and *in vitro* evidence for differential phagocytic capability of astrocytes to phagocytose synapses (Chung et al, 2016). ApoE also inhibits C1q-mediated phagocytosis (Yin et al., 2019). Variants of the phagocytic receptor TREM2 are also linked to AD risk, and in a tau mouse model, TREM2 knock-out prevented neuronal loss (Leyns et al., 2017), which is compatible with the idea that neuronal loss may be due to TREM2-mediated phagocytosis.

Aggregates of TDP-43 (TAR DNA-binding protein 43) are the main constituent of glial and neuronal inclusions in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) patients, and TDP-43 has pathological roles in other neurodegenerative diseases including AD (Gao et al., 2018). Interestingly, Paolicelli et al (2017) demonstrated that knock-out of TDP-43 in mouse microglia increased microglial phagocytosis of both Aβ and synapses, resulting in reduced amyloid plaques but increased synaptic loss in an amyloid mouse model, illustrating the protective and detrimental roles of microglial phagocytosis.

Note, however, that TDP-43, GRN, APOE, TREM2 and complement affect functions other than phagocytosis, so it can be difficult to disentangle the mechanisms by which they affect neurodegeneration.

4.2 Parkinson's disease (PD)

PD is a neurodegenerative disease characterised by: motor dysfunction, the presence of Lewy bodies and the loss of dopaminergic neurons in the *substantia nigra* (Jankovic, 2008). PD-risk genes known to affect microglial phagocytosis include leucine-rich repeat kinase 2 (LRRK2). LRRK2 is a cytoplasmic protein, with GTPase and protein kinase domains, that regulates microglial phagocytosis and other processes (Marker et al., 2012; Kim et al., 2018; Lee et al., 2020). Activation of LRRK2 in BV-2 microglia increased phagocytosis of live neuronal axons and dendrites, which was prevented by LRRK2 knock-down or blocking exposed phosphatidylserine (Marker et al., 2012). LRRK2 directly phosphorylates the WAVE2 complex that mediates the actin remodelling of phagocytosis. The LRRK2-G2019S variant that increases PD risk also increased microglial phagocytosis of live dopaminergic neurons, and this was prevented by blocking phagocytosis at WAVE2 or Arp2/3 (Kim et al., 2018). LRRK2-

G2019S knock-in mice had increased dopaminergic neuronal loss in the *substantia nigra* in response to LPS injection, prevented by WAVE2 knock-down (Kim et al*.,* 2018). Similar results were found in a Drosophila model (Kim et al., 2018; Maksoud et al., 2019). This indicates that the PD-risk variant of LRRK2 (G2019S) increases inflammatory microglial phagocytosis of dopaminergic neurons, directly identifying the mechanism of neurodegeneration.

Another important PD risk gene codes for α-synuclein. α-synuclein is the main component of Lewy bodies and may mediate spreading of the disease (Spillantini et al., 1997). Extracellular α-synuclein can activate microglia and induces complex changes in phagocytosis, dependent of time and aggregation state of α-synuclein (Janda et al., 2018). However, soluble and fibrillar α-synuclein can stimulate microglial phagocytosis (Fellner et al., 2013), and microglia from α-synuclein gene-ablated mice have reduced phagocytosis (Austin et al., 2006). Transgenic mice expressing aggregate-prone A53T α-synuclein had increased microglial expression of the phagocytic receptors Axl and MerTK, and knock-out of these receptors extended survival (Fourgeaud et al., 2016), suggesting that microglial phagocytosis of neurons contributed to the pathology.

It is well established that degeneration of dopaminergic neurons of the *substantia nigra* plays a key role in the pathogenesis of PD and those neurons contain high levels of the protein neuromelanin (Sulzer et al., 2000). Extracellular neuromelanin activates microglia *in vitro* and injection into the *substantia nigra* caused neuroinflammation and loss of neurons *in vivo* (Zhang et al., 2011; Zecca et al., 2008). In culture, neuromelanin resulted in neuronal loss prevented by knock-out of the phagocytic receptor CR3 (Zhang et al., 2011), suggesting that microglial phagocytosis of live neurons may cause this neuronal loss. Accordingly, iC3b was found on melanized neurons in the *substantia nigra* of PD patients (Loeffler et al., 2006), supporting the idea that complement activation may contribute to PD neuronal loss.

Gut dysfunction occurs early in PD, which may result in elevated serum endotoxin (LPS) (Brown, 2019). In mice, chronic peripheral LPS causes activation of microglia in the *substantia nigra*, up-regulation of complement factors and neuronal loss, prevented by complement C3 knock-out (Bodea et al., 2014). MPTP and rotenone are environmental toxins that can induce microglial activation and degeneration of dopaminergic neurons *in vivo* (Członkowska et al.,

1996, Sherer et al., 2003). Rotenone has been shown to induce loss of neurons in neuronal-glial co-cultures, and this neuronal loss was blocked by inhibition of microglial phagocytic receptors (Emmrich et al*.,* 2013). Similarly, MPTP induced microglia to phagocytose whole degenerating dopaminergic neurons *in vivo*, implicating phagocytosis in dopaminergic degeneration (Barcia et al., 2011, 2012). Injection of 6-hydroxydopamine into mouse striatum induced microglial phagocytosis of dopaminergic neurons in the *substantia nigra*, consistent with the neuronal loss being due to microglial phagocytosis (Marinova-Mutafchieva et al., 2009; Virgone-Carlotta et al., 2013).

Thus, there is accumulating evidence that microglial phagocytosis of neurons may be responsible for neuronal loss in PD. However, the evidence remains circumstantial, and microglial phagocytosis may also have protective roles in PD (Janda et al., 2018; Tremblay et al., 2019).

4.3 Multiple sclerosis (MS)

MS is a neuroinflammatory disease with characteristic demyelinated lesions in cortical grey and subcortical white matter, and neurodegeneration at chronic stages (Pinto & Fernandes, 2020). Microglial phagocytosis of myelin classically activates and makes them neurotoxic in culture (Pinteaux-Jones et al., 2008). On the other hand, there is abundant evidence that increasing microglial phagocytosis of myelin debris is beneficial during remyelination, as myelin debris inhibits oligodendrocyte differentiation and remyelination (Pinto & Fernandes, 2020). Whether microglia could strip myelin off axons from live neurons is unclear, but microglia do appear to survey myelin sheath *in vivo* (Zhang et al., 2019), and anti-myelin antibodies or complement might promote such myelin stripping (Vargas et al., 2010; DeJong & Smith, 1997). Complement is activated in MS (Watkins et al., 2016), and C1q knock-out mice were protected against white matter loss in a mouse obesity model, suggesting that C1q may mediate microglial phagocytosis of live myelin (Graham et al., 2020). Recently, it has been shown that C3, but not C1q, localise with synapses in several models of MS (Werneburg et al., 2020). Following C3 deposition, microglia have been shown to eat presynaptic inputs in these models. Overexpression of the complement inhibitor Crry at synapses successfully reduced microglial engulfment of synapses (Werneburg et al., 2020). This study focused on synapse loss, but suggests that myelin and neuronal loss in MS might result from excessive microglial phagocytosis. Mouse models of MS also have "neuronophagia" i.e. evidence of glia phagocytosing neurons (Guo et al., 2004; Lee et al., 2007).

4.4 Retinal degeneration

Age-related macular degeneration (AMD) is a degenerative disease of the retina, and a leading cause of blindness. Complement components C3, complement factor B (CFB) and complement factor H (CFH) are elevated in AMD, and variants of complement genes (CFH, CFB, C2, SERPING1, and C3) have been shown to increase the risk of AMD (Geerlings et al., 2019). Inhibition of complement components C3a and C5a (Nozaki et al., 2006), or complement regulators CFB and membrane attack complex (MAC) (Lipo et al., 2013), or administration of complement regulators CD59 (Bora et al., 2010) and CFH (Kim et al., 2013) can reduce pathology in animal models of AMD. A C3 inhibitor, APL-2, reduced retinal atrophy in a phase 2 clinical trial for AMD (Kassa et al., 2019). Another retinal degenerative disease, retinitis pigmentosa (RP), is characterised by progressive loss of retinal rod cells and consequently peripheral and night vision. In a mouse model of RP, the loss of rod cells was found to be due to microglial phagocytosis of live rod cells, and inhibition of this phagocytosis prevented retinal degeneration (Zhao et al., 2015). This evidence supports the concept of microglia and complement mediating phagocytosis of live cells.

4.5 Ischaemia and stroke

Ischaemia causes acute and delayed neuronal loss in stroke, and chronic or intermittent ischaemia is central to vascular dementias. Following stroke or cerebral ischemia, activated microglia rapidly phagocytose dead or degenerating neurons which is beneficial for recovery after ischemic stroke (reviewed in Li et al., 2020). However, in the penumbra and the peri-infarct regions, loss of stressed-but-viable neurons is delayed, and inhibition of microglial phagocytosis can be beneficial. Thus, knock-out of the phagocytic receptor MerTK or opsonin MFG-E8 (that binds exposed phosphatidylserine) prevented neuronal loss and long-term functional deficits after cerebral ischemia (Neher et al., 2013), suggesting that microglial-mediated phagocytosis contributes to neuronal loss after transient cerebral ischemia. Such transient ischemia induces reversible phosphatidylserine exposure on neurons in mice (Mari et al., 2004). Similarly, neurons exposed to ischaemia were shown to reversibly expose phosphatidylserine via the calcium-activated phosphatidylserine scramblase TMEM16F, and TMEM16F knock-down prevented microglial phagocytosis of stressed neurons and functional deficits after ischaemia-reperfusion in mice (Zhang et al., 2020). Complement component C3 is also an opsonin guiding phagocytosis of neurons and synapses by microglia. Thus, a targeted inhibitor of C3 activation, B4Crry, prevented phagocytosis of stressed-but-viable neurons in

the ischaemic penumbra area (Alawieh et al., 2018), reduced phagocytosis of synapses and improved overall cognitive function in a model of embolic stroke in mice (Alawieh et al., 2020).

4.6 Brain viral infections

Brain infection with West Nile Virus caused complement deposition on synapses, microglial phagocytosis of synapses and memory loss, which was prevented by eliminating microglia, C3 or C3aR (Vasek et al., 2016). Viral infection of neurons by the hand-foot-and-mouth disease enterovirus 71 caused exposure of calreticulin on neurons that were apparently eaten alive by glia (Hu et al., 2017). Virally-infected cells can also expose phosphatidylserine and be phagocytosed alive by microglia via the phagocytic receptor MerTK (Miner et al., 2015; Tufail et al., 2017; Chua et al., 2018). This suggests the possibility that infected neurons (or synapses) signal to be phagocytosed in order to limit further infection, but if too many neurons become infected this phagocytosis may be responsible for the neuronal loss.

4.7 Brain ageing

Aging is one of the main risk factors for developing neurodegenerative diseases such as Alzheimer's disease, but in the absence of such diseases, ageing itself may cause neurodegeneration. During aging of mice and rats, there is a slow but progressive loss of synapses and neurons (Shi et al., 2015, 2017; Morterá & Herculano-Houzel, 2012). In aged mice, there is loss of synapses and neurons in the CA3 region of the hippocampus, but this loss is prevented in C3-deficient mice, implicating complement-mediated phagocytosis in such loss (Shi et al., 2015). Similarly, mice lacking an enzyme required for cell surface sialylation had accelerated loss of synapses and neurons in CA3, prevented in C3-deficient mice (Klaus et al., 2020), suggesting that sialylation can protect against this loss, potentially by inhibiting phagocytosis. Ageing-induced neuronal loss in mouse hippocampus and *substantia nigra* was also prevented in TREM2 knock-out mice, supporting a central role of microglial phagocytosis in this loss (Linnartz-Gerlach et al., 2019). However, the anti-phagocytic receptor CD22 (Siglec-2) is upregulated in aged microglia, and CD22-blocking antibodies improved cognitive performance in aged mice, possibly due to increased microglial phagocytosis of debris and protein aggregates (Pluvinage et al., 2019). This reminds us that microglial phagocytosis has both beneficial and detrimental roles in brain pathology. Moreover, it highlights the need for a better understanding of the role that microglial receptors have in phagocytosing particular

ligands, as this could be crucial to design therapeutic strategies that promote phagocytic clearance of apoptotic cells, abnormal protein aggregates and debris, while avoiding phagocytosis of stressed-but-viable neurons and/or synapses.

4.8 Human data

Evidence for microglial phagocytosis of live neurons contributing to neurodegeneration is more limited in humans, compared to the mouse models and culture systems described above, but a variety of supporting evidence is reported below.

The general idea of glial phagocytosis of neurons contributing to human pathology originates in 1890's Paris from the research of Georges Marinesco on human neuropathology (Marinesco, 1907). Marinesco observed glial cells phagocytosing neurons in fixed brain sections from neurology patients, and referred to this phenomena as "neuronophagia". Neuropathologists have since reported observing neuronophagia in many different pathologies, including PD (Kremer & Bots, 1993), ALS (Troost et al., 1993), ageing (Rath-Wolfson et al., 2017), Gaucher disease (Pàmpols et al., 1999), epilepsy (Boyd et al., 2010), stroke with diabetes (Li et al., 2011), and most recently SARS-CoV2 (Al-Dalahmah et al., 2020). This is consistent with microglial phagocytosis of neurons, but does not tell us whether the neurons were eaten dead or alive. Note, however, that there is no evidence in AD of neuronophagia or microglia clustering around tau neurofibrillary tangles. This may be because AD is a slow disease and therefore neuronal removal is a rare event, or it may be that different processes are involved in neuronal loss in AD.

There is an increase in phagocytic (CD68-positive) microglia in FTD and AD (Woollacott et al., 2020); and expression profiling of plaque-associated microglia from AD brains showed up-regulation of genes for phagocytosis and immune response (Yin et al., 2017). Microglia freshly isolated from AD brains, release more C1q and nitric oxide derivatives potentially causing more phagocytosis (Lue et al., 2001). Furthermore, there is evidence that: microglia from AD brains contain more synaptic material than microglia from healthy brains, and synapses from AD brains are more readily phagocytosed by microglia (Tzioras et al., 2019). Ohm et al (2020) reported that in stereological analysis of brain sections from AD patients, activated microglia were positively associated with neuronal loss, consistent with activated microglia being responsible for the neuronal loss. Intriguingly, in post-mortem brains from MS patients, there was increased microglial phagocytosis and evidence for myelin mRNA transcripts inside

microglia, suggesting microglial phagocytosis of myelin and transfer of mRNA (Schirmer et al., 2019).

As outlined previously, genome wide association studies (GWAS) have linked a number of genes regulating microglial phagocytosis to risk of AD, including opsonins APOE and CLU/APOJ, phagocytic receptors TREM2, CR1, CD33 and PILRA, and downstream signalling PLCG2, CD2AP, ZYX and ABI3 (Hollingworth et al., 2011; Naj et al., 2011; Malik et al., 2013; Ramanan et al., 2015; Podleśny-Drabiniok et al., 2020) . LRRK2 variants, such as G2019S, increase PD risk, and increase microglial phagocytosis (Kim et al., 2018), and reactive microglia are found in the *substantia nigra* of PD patients (McGeer et al., 1988). Variants of the TDP-43 gene affect ALS risk, and TDP-43 is known to regulate microglial phagocytosis (Paolicelli et al 2017). Variants of the GRN gene affect FTD risk, and the gene product progranulin regulates microglial phagocytosis (Kao et al., 2011; Petoukhov et al., 2013). Thus, GWAS indicates that microglial phagocytosis is important in neurodegenerative disease, but in general does not tell us whether it is beneficial, detrimental or both in pathology.

5. Knowledge gaps, challenges and potential therapeutic targets

The hypothesis that microglial phagocytosis of live neurons contributes to neurodegeneration in human brain pathologies remains a hypothesis, and below we outline some of the key issues that need to be resolved to test this hypothesis.

1. Observation of microglial phagocytosis of neurons *in vivo*. Current methods for imaging microglia dynamics *in vivo* (such as two-photon imaging) are poor at imaging phagocytosis, and cannot distinguish between phagocytosis of live and dead neurons *in vivo*. Clearly, better methods are required, as well as markers for healthy, stressed, degenerating, dying and dead neurons *in vivo*.
2. Neuropathology of human disease. We need markers of microglial phagocytosis of neurons or synapses that can be used on fixed sections of human brain, and last for days or weeks after the phagocytic event, and ideally distinguish between phagocytosis of live and dead neurons.
3. Inflammation and phagocytosis are linked in that inflammatory activation of microglia generally increases microglial phagocytosis. As both inflammation and phagocytosis may independently contribute to neurodegeneration, it can be difficult to distinguish these contributions. Thus, we need drugs, knock-outs and markers that clearly distinguish microglial inflammation and phagocytosis.

It appears that microglial phagocytosis has multiple beneficial and detrimental roles in neurodegeneration, and these may change during the course of neurodegeneration. Microglial phagocytosis may be beneficial by clearing debris and protein aggregates, but may be detrimental by clearing live synapses and neurons. Hence, it is inadvisable to simply block or boost microglial phagocytosis. Rather it may be necessary to block the phagocytosis of specific targets (synapses and neurons) at specific stages of disease, which presents significant challenges. Potential therapeutic targets to prevent excessive microglial phagocytosis of neurons and synapses may include: complement components C1 (Williams et al., 2016), C3 (Werneburg et al., 2020; Alawieh et al., 2018) and C3aR (Jacob et al., 2010; Litvinchuk et al., 2018; Ahmad et al., 2019;), the opsonin Galectin-3 (Yip et al., 2017; Boza-Serrano et al., 2019; Srejovic et al., 2020; Puigdellívol et al., 2020), the phagocytic receptors P2Y6 (Neher et al., 2014; Anwar et al., 2020), MerTK (Neher et al., 2013) and TREM2 (Deczkowska et al., 2020), and the desialylating enzyme neuraminadase 1 (Allendorf et al., 2020a; Allendorf et al., 2020b; Puigdellívol et al., 2020).

Conflict of Interest

The authors declare there was no conflict of interest in writing this review. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The authors contributed equally to writing this review.

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Supporting materials

None

Figures

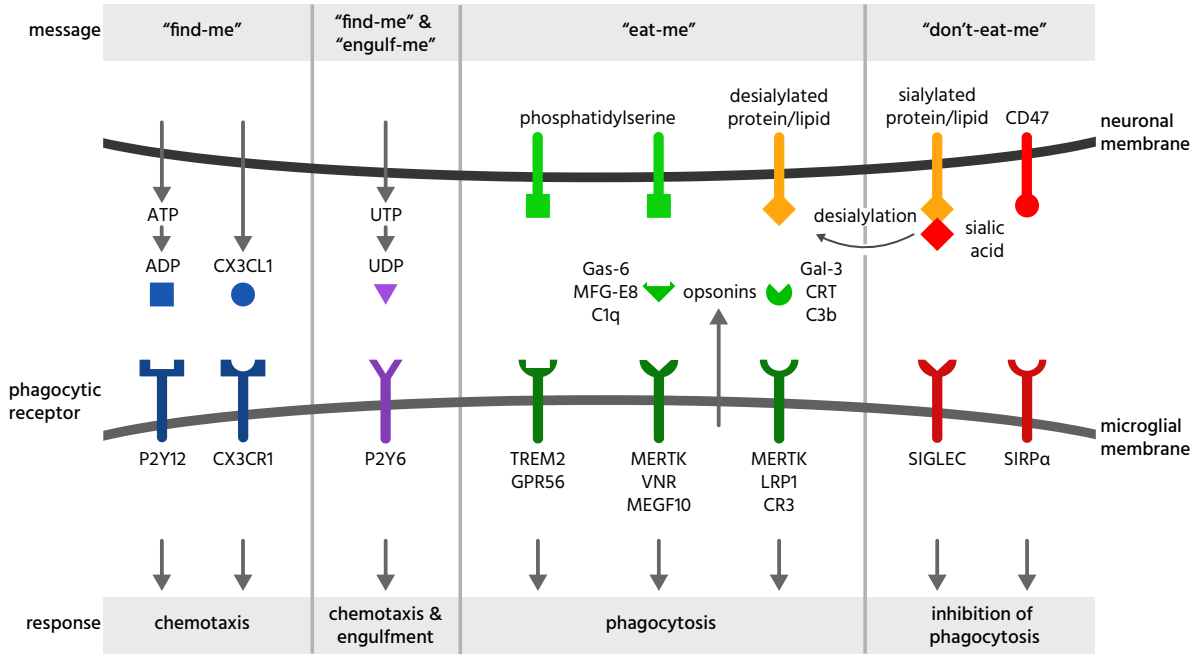

Figure 1. Overview of find-me, eat-me, don't-eat-me signals and opsonins, that regulate microglia phagocytosis of neurons. Find-me signals are chemotactic signals, such as ADP and CX3CL1 (fractalkine) released from neurons and binding to microglial P2Y12 and CX3CR1 receptors respectively, resulting in chemotaxis of microglia to these neurons. Eat-me signals are released by stressed or dying neurons, and induce phagocytosis of neurons expressing them. Such signals include UDP, which activates the P2Y6 receptor on microglia. Phosphatidylserine, when exposed on the surface of neurons, can either bind directly to microglia receptors triggering receptor expressed on myeloid cells 2 (TREM2) and G-protein coupled receptor 56 (GPR56), or indirectly via binding opsonins, Gas-6, apolipoprotein E (APOE), milk fat globule-EGF factor 8 (MFG-E8) and complement component C1q (C1q), which then bind to microglial receptors: receptor tyrosine kinase (MerTK), TREM2, vitronectin (VNR) and multiple EGF-like-domains 10 (MEGF10), respectively. Glycosylated proteins and lipids which have been desialylated (had the terminal sialic acid residues removed) can bind opsonins galectin-3 (Gal-3), calreticulin (CRT), C1q and complement protein 3b (C3b), which bind to microglial receptors MERTK, low density lipoprotein receptor related protein 1 (LRP1) and complement receptors 1/3/4, respectively. Don't-eat-me signals inhibit phagocytosis, and include sialylated glycoproteins and lipids (with terminal sialic acid residue present, recognised by sialic acid binding immunoglobulin-type lectin (Siglecs) receptors) and the protein CD47, which can bind the microglial receptor signal-regulatory protein alpha (SIRPα) to inhibit phagocytosis. Together these signals regulate microglial phagocytosis of neurons.

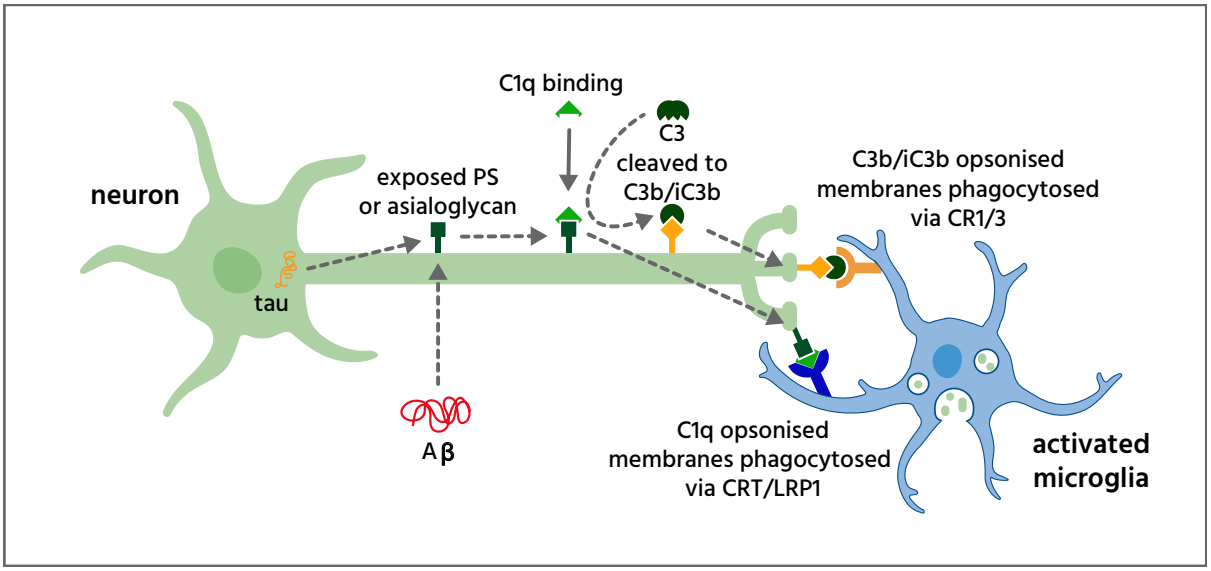

Figure 2. Complement mediated phagocytosis of neurons and synapses. Aggregates of extracellular amyloid beta (Aβ) or intracellular tau may stress neurons, causing exposure of phosphatidylserine (PS) or asialoglycans that bind complement C1q. Bound C1q can (indirectly) cause deposition of complement C3 as opsonins C3b and iC3b, which can induce phagocytosis of synapses or neurons by microglia via complement receptors 1 and 3 (CR1 and CR3). Alternatively, membranes opsonised with C1q can bind calreticulin and be phagocytosed via microglial LRP1. Exposure of phosphatidylserine on stressed neurons may also induce microglial phagocytosis of stressed neurons via other opsonins and receptors.

Figure 3. Factors regulating microglial phagocytosis. Microglial phagocytosis is activated by: A) binding of cytokines TGFβ, TNFα, IFNβ and IFNγ, B) desialylation of the microglial cell surface, C) up-regulation of phagocytic genes, such as *Itgax, Clec7a, Axl, TREM2* and *Apoe*, or D) epigenetic changes regulating transcription of phagocytic genes.

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