# 4

# Constructing by Disposing: Regulation of Neuronal Morphogenesis by Phagocytosis

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## Abstract

The construction of a functional nervous system involves not only the addition of new neurites or synapses but also the elimination of excessive or temporary structures. Being responsible for the trimming work, diverse phagocytes engulf apoptotic neurons, dissolve large pieces of axons or dendrites, or nibble away specific synapses. In these processes, phagocytes do not merely clean up the mess resulting from neuronal destruction. Instead, they are often responsible for killing the neurons or dismantling the excessive neurites/synapses. Thus, failure of phagocytosis can result in defects in neuronal morphology and connectivity. Phagocytosis of neurons is triggered by "eat-me" signals exposed on the neuronal surface. Engulfment receptors on phagocytes recognize these signals and promote cytoskeletal and transcriptional changes necessary for phagocytosis and other responses. Here, we review the experimental systems used to study phagocytosis during neuronal remodeling in both invertebrates and vertebrates and recent progress that sheds light on the molecular pathways underlying phagocytosis in the developing nervous system.

## Main Text

To build functional neural circuits, neurons need to constantly interact with the surrounding tissues. Many steps in neuronal morphogenesis involve removing neurons or neuronal compartments that are no longer needed. The

removal of unnecessary neuronal material is primarily carried out by resident phagocytes in both the central nervous system (CNS) and the peripheral nervous system (PNS). These resident phagocytes may perform other functions but can turn into phagocytic cells when they are presented with the material to be engulfed. Phagocytosis sculpts the nervous system at multiple scales – from elimination of dying neurons, large-scale remodeling of axons or dendritic arbors, to pruning of axonal/dendritic tips or synapses. Abnormal phagocytosis can have a profound impact on the development and homeostasis of the nervous system (Faust et al., 2021; Galloway et al., 2019; Neniskyte and Gross, 2017). In this chapter, we survey the neuronal morphogenic processes in which phagocytosis of neuronal structures has been observed and discuss the role of phagocytosis in the construction of mature neurons or neural circuits. We also review the molecular mechanisms underlying phagocytosis of neuronal structures. One focus is on a surface signal that labels degenerative compartments of neurons to trigger phagocytosis. The second focus is on the phagocytic receptors involved in recognizing the signal in diverse systems and the signaling downstream of receptor activation. Other topics that have been extensively reviewed elsewhere, such as the neuronal-intrinsic processes of prime neurons for degeneration, are not discussed in detail here.

## 4.1 Phagocytosis in Diverse Contexts of Neuronal Morphogenesis

### 4.1.1 Elimination of whole neurons

#### 4.1.1.1 Elimination of neuronal corpses resulting from programmed cell death (PCD)

PCD, or apoptosis, is a common phenomenon in the development of the nervous system in both invertebrates and vertebrates (reviewed by Buss et al., 2006). By estimate, about half of the motoneurons produced during the embryonic development of the mouse (Lance-Jones, 1982), rat (Oppenheim, 1986), and chicken (Oppenheim et al., 1997) are lost. PCD serves a variety of adaptive functions in the nervous system, including adjusting cell numbers to control organ and tissue sizes (Cecconi et al., 1998; Rogulja-Ortmann et al., 2007), matching the numbers of neurons with their efferent targets and afferent inputs (reviewed by Buss et al., 2006), and eliminating defective cells resulting from developmental errors and noise (Baek et al., 2013; Clarke, 1992; Jiang and Reichert, 2012; Rogulja-Ortmann et al., 2007). During early morphogenesis of the neural tube and the brain in vertebrates, PCD regulates

dynamic movement of cells and erases signaling centers (Nonomura et al., 2013; Offner et al., 2005). During metamorphosis in insects (Choi et al., 2006; Togane et al., 2012; Winbush and Weeks, 2011) and puberty in mammals (Forger, 2009), PCD removes certain anatomical structures of the post-embryonic CNS before the formation of the final adult structures. Apoptotic cells resulting from PCD are usually efficiently engulfed by tissue-resident phagocytes before rupture, preventing leakage of toxic materials and inflammatory damage to neighboring cells (Figure 4.1(A)) (reviewed by Fricker et al., 2018).

In *Drosophila*, three resident glial subtypes remove cell corpses in the CNS at both distinct and overlapping developmental stages. Cortex glia engulf apoptotic neurons from early embryogenesis to larval and pupal stages (Etchegaray et al., 2016; Kurant et al., 2008; McLaughlin et al., 2019; Nakano et al., 2019). Astrocyte-like glia and ensheathing glia engulf apoptotic neurons during metamorphosis (Hilu-Dadia et al., 2018). In vertebrates, microglia are the major phagocytes for clearing neuronal corpses. Microglia have been reported to engulf apoptotic neurons in the embryonic brain of zebrafish (Mazaheri et al., 2014) and in the developing and the mature CNS of mammals (Dalmau et al., 2003; Sierra et al., 2010). Other glial types can engulf apoptotic cells during development. For example, in the mouse PNS, satellite glial cell precursors remove apoptotic corpses in developing dorsal root ganglia (DRG) (Wu et al., 2009).

#### 4.1.1.2 Phagocytosis of live neurons

Phagocytes can sometimes engulf stressed cells that are still alive. This type of cell execution, termed "phagoptosis" (Brown and Neher, 2012), is distinct from PCD, in that inhibiting phagocytosis in phagoptosis prevents cell death, while inhibiting phagocytosis in PCD results in accumulation of dead cells. Studies on *Caenorhabditis elegans* showed that loss-of-function (LOF) mutations in genes encoding engulfment receptors cause survival of neuronal precursors that are normally lost during development (Darland-Ransom et al., 2008; Hoeppner et al., 2001; Reddien et al., 2001). Similarly, studies in rats and monkeys showed that microglia engulf neural precursors to regulate the size of the precursor pool in the developing cerebral cortex (Cunningham et al., 2013). In addition, microglia kill and engulf differentiating neurons in the developing cerebellum and hippocampus of mice (Marin-Teva et al., 2004; Wakselman et al., 2008). Thus, killing extra neural precursors or neurons by phagocytosis is a conserved developmental mechanism to achieve the desired number of neurons in the mature nervous system.

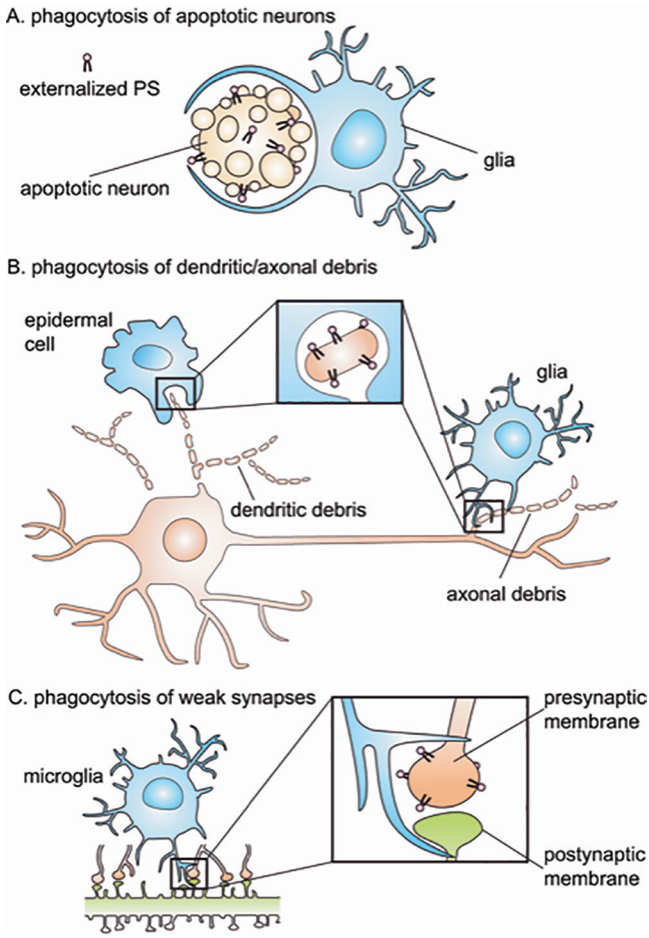


**Figure 4.1 Phagocytosis in the developing nervous system. (A)** In the CNS, resident phagocytes (blue), such as astrocytes and microglia, engulf apoptotic neurons (orange). **(B)** Glia also engulf damaged or pruned neurites. In the PNS, amateur phagocytes that normally contact neuronal processes, such as epidermal cells in *Drosophila* and zebrafish skins, break down and clear degenerating neurites. **(C)** In the CNS, glia eliminate weak synapses by engulfing pre- and/or post-synaptic membranes. In all scenarios, phosphatidylserine (PS, pink) is externalized on the surface of the degenerative compartment of neurons to serve as an "eat-me" signal.

### 4.1.2 Large-scale remodeling of axons/dendrites during development

During development, neurons often make inappropriate, excessive, or transient connections that must be eliminated as circuits mature. Pruning of selective axonal and dendritic branches is an important aspect of neural circuit

refinement (reviewed by Corty and Freeman, 2013; Luo and O'Leary, 2005). In some animals, the same neuron can belong to two different functional circuits at the juvenile and adult stages. The axonal and dendritic connections established for the juvenile stage need to be pruned before the construction of the adult circuits. For instance, holometabolous insects, such as *Drosophila*, undergo dramatic remodeling of many neuronal structures when transitioning from the larval stage to the adult stage (Watts et al., 2003; Williams and Truman, 2005a, b). In mammals, large-scale axon elimination precedes the generation of adult patterns of callosal, intracortical, and subcortical projections due to the reduced numbers of collaterals and targets in the adults (reviewed by O'Leary, 1992; O'Leary and Koester, 1993). Thus, phagocytotic removal of the pruned axons and dendrites is a shared mechanism in circuit remodeling in both invertebrates and vertebrates.

#### 4.1.2.1 Axon pruning of *Drosophila* mushroom body (MB) γ-neurons

The *Drosophila* MB γ-neurons have been a popular model for studying developmental axon pruning. MBs in the *Drosophila* brain play an essential role in olfactory learning and memory (de Belle and Heisenberg, 1994; Heisenberg et al., 1985). Axons of MB γ neurons initially bifurcate into dorsal and medial lobes at the larval stage. Shortly after puparium formation, γ axons in the lobes are selectively pruned by local degeneration to retain only the main processes, which then project into the adult medial γ lobe without bifurcation (Lee et al., 1999; Watts et al., 2003). Astrocyte-like glia selectively invade MB axon lobes at the onset of metamorphosis and engulf degenerating axon fragments during pruning (Awasaki and Ito, 2004; Awasaki et al., 2006; Hakim et al., 2014; Tasdemir-Yilmaz and Freeman, 2014; Watts et al., 2004). In addition to clearing axonal debris, astrocyte-like glia also actively promote fragmentation of pruned axons (Figure 4.1(B)), as inhibition of glia-mediated phagocytosis suppresses axon breakdown (Awasaki and Ito, 2004; Awasaki et al., 2006; Hakim et al., 2014).

#### 4.1.2.2 Dendrite pruning of *Drosophila* dendritic arborization (da) neurons

Dendritic arborization (da) neurons are somatosensory neurons that innervate the larval epidermis with free-ending dendrites. A subset of these neurons dies during metamorphosis, while others persist and remodel their dendritic arbors for the adult stage (Shimono et al., 2009). Among those that survive are dorsal class IV da (C4da) neurons (Shimono et al., 2009; Williams and Truman, 2005a). Because these neurons are close to the

translucent body wall, they are particularly suitable for living imaging in the immobile pupa.

Soon after metamorphosis starts, C4da neurons undergo a series of degenerative events, including severing of proximal dendrites, thinning and beading of low-order branches, and dendrite fragmentation. Dendrite degeneration and clearance complete by 16–18 hours (hrs) after puparium formation (APF), while the cell bodies and axons remain (Han et al., 2014; Kuo et al., 2005; Lee et al., 2009; Williams and Truman, 2005a). The epidermal cells that directly contact da sensory dendrites function as the primary phagocytes responsible for clearing the pruned dendrites (Figure 4.1(B)) (Han et al., 2014). Rather than merely internalizing disintegrated dendrite pieces, epidermal cells also wrap degenerating dendrites to facilitate dendrite fragmentation (Han et al., 2014). Circulating phagocytic blood cells called plasmatocytes can also attack dendritic branches and contribute to dendrite fragmentation (Williams and Truman, 2005a); however, they are not required for the clearance of degenerating dendrites (Han et al., 2014). Phagocytic clearance of sensory neurites seems to be a conserved function of epidermal cells from insects to vertebrates, as zebrafish epidermal cells are also responsible for clearing degenerating sensory axons after injury (Rasmussen et al., 2015).

#### 4.1.2.3 Axon removal at the mammalian neuromuscular junction (NMJ)

During development of the mammalian motor system, one motor neuron initially projects axonal branches to multiple muscle fibers, and each muscle fiber is innervated by axons from multiple motor neurons. Within the first several postnatal weeks, through a process of activity-dependent intercellular competition, only one "winning" motor input is left to innervate one muscle fiber, with "loser" axons and pre-synaptic terminals being eliminated (Colman et al., 1997; Walsh and Lichtman, 2003). The "losing" axonal branches withdraw by shedding membrane vesicles termed axosomes, resulting in large-scale elimination of axon branches (Bishop et al., 2004; Song et al., 2008). Special Schwann cells that ensheath the junctions play important roles in eliminating the "loser" synapses and axons. They phagocytose nerve terminals contacting the muscle fiber, thus promoting synaptic turnover (Smith et al., 2013) and engulf the axosomes shed by retracting axons (Song et al., 2008).

### 4.1.3 Activity-dependent elimination of short axon/dendrite segments or synapses

In addition to developmentally programmed large-scale pruning of axons and dendrites, neuronal remodeling may occur at restricted compartments, such

as specific synapses and axon or dendrite tips. In this type of remodeling, neuronal activity usually plays an important role in defining the neuronal compartments to be eliminated (reviewed by Neniskyte and Gross, 2017).

#### 4.1.3.1 Membrane shedding during *Drosophila* NMJ expansion

In the *Drosophila* larva, a motor neuron innervates a muscle fiber by elaborating a single axon arbor onto the muscle. At the NMJ, the axon arbor sheds presynaptic debris or ghost boutons (undifferentiated synaptic boutons) in an activity-dependent manner during normal synaptic growth (Fuentes-Medel et al., 2009). However, instead of eliminating arbors, as seen in the axonal removal of mammalian NMJs, this membrane shedding in *Drosophila* occurs while new arbors are being added at the NMJ. The shed material is cleared by both the glia and muscle cells that directly contact the axons: Glia invade the NMJ and mainly engulf small presynaptic debris, while post-synaptic muscles mainly engulf larger ghost boutons. The presynaptic debris is inhibitory to synaptic growth and expansion if left unengulfed (Fuentes-Medel et al., 2009).

#### 4.1.3.2 Engulfment of worm sensory microvilli

The AFD neuron is one of the major thermosensory neurons in adult *C. elegans*. The neuron-receptive ending (NRE), the sensory structure of AFD, comprises actin-based microvilli and a single microtubule-based cilium, both embedded in the AMsh glial cell (Singhvi et al., 2016). In healthy adult worms, AFD neurons constantly shed fragments of the NRE, leaving pieces disconnected from the rest of the neuron (Raiders et al., 2021; Singhvi et al., 2016). A recent study found that AMsh glia actively engulf the NRE fragments. Perturbations of the glial engulfment led to defects in the NRE shape and the associated thermosensory behaviors of the animals (Raiders et al., 2021). Thus, constant glial engulfment of the NRE maintains tissue homeostasis and physiological functions of the AFD neuron.

#### 4.1.3.3 Shedding of mammalian photoreceptor outer segments (POS)

In the mammalian retina, the light-sensitive outer segments of photoreceptor rods and cones are each composed of a stack of many hundreds of densely packed discs, which are formed by invaginated plasma membrane. The photoreceptors continuously renew their outer segments by shedding their aged tips (Young, 1967, 1971). Rods shed their membranes each morning at the onset of light, whereas cones shed their membranes at the onset of night (LaVail, 1976; Young, 1977). The membrane shedding precedes a burst of phagocytosis by the adjacent retinal pigment epithelium (RPE), which rapidly clears shed POS. Daily phagocytosis of aging POS is essential for the

function and longevity of photoreceptor neurons. Failure of this process causes accumulation of photoreceptor cell debris that compromises the normal vision (Nandrot et al., 2004; Young and Bok, 1969).

#### 4.1.3.4 Synaptic pruning in the developing mammalian CNS

Maturation of neuronal circuits requires selective elimination of excessive synaptic connections generated during early development (reviewed by Chung and Barres, 2012; Wilton et al., 2019). The disruption of synaptic remodeling has been suggested to play a role in neurodevelopmental disorders such as autism and schizophrenia (reviewed by Salter and Stevens, 2017; Wilton et al., 2019). Synaptic pruning often involves glial engulfment of synaptic material (Figure 4.1(C)). Microglia and astrocytes, tissue-resident phagocytes of the vertebrate CNS, play important roles in removing redundant synapses (reviewed by Riccomagno and Kolodkin, 2015; Wilton et al., 2019). The dorsolateral geniculate nucleus (dLGN) of the thalamus and the hippocampus are two regions of the developing mammalian brain where synaptic pruning has been extensively studied.

During early postnatal stages, the axons of retinal ganglion cells (RGCs) extend into the mouse dLGN and form excessive synaptic connections with relay neurons. Overlapping RGC inputs from both eyes undergo a process of remodeling called eye-specific segregation, resulting in the separation of ipsilateral and contralateral inputs into distinct non-overlapping domains in the mature dLGN (Katz and Shatz, 1996). Defective synaptic pruning of RGC inputs to the relay neurons in the dLGN leads to incomplete eye-specific segregation (Huberman, 2007; Jaubert-Miazza et al., 2005). Microglia participate in the pruning of excess RGC synaptic inputs during peak retinogeniculate pruning at around postnatal day (P) 5 in mouse (Lehrman et al., 2018; Li et al., 2020; Schafer et al., 2012; Scott-Hewitt et al., 2020; Stevens et al., 2007). This microglia-mediated engulfment of presynaptic terminals is regulated by RGC activity such that microglia preferentially engulf inputs from the "weaker" eye (Schafer et al., 2012). Astrocytes also actively engulf excessive synapses in the dLGN during the same developmental period (Chung et al., 2013). Disrupting microglia-mediated or astrocyte-mediated phagocytosis results in sustained deficits in synaptic connectivity (Chung et al., 2013; Schafer et al., 2012).

Synaptic pruning continues in the dLGN from eye opening to later developmental stages (P8–P30 in mouse). Initially, each dLGN neuron is innervated by multiple RGC axons. Between P11 and P16, spontaneous retinal activity drives bulk elimination of excess connections while strengthening the remaining synapses. Later, visual experience is required for synaptic plasticity

(Hooks and Chen, 2006). Microglia are involved in eliminating excessive synapses during both the early and later stages (Vainchtein et al., 2018).

Synaptic elimination in the hippocampus happens around P8–P18 as neural circuits mature (Filipello et al., 2018; Paolicelli et al., 2011; Scott-Hewitt et al., 2020; Weinhard et al., 2018) and in the adult during memory formation (Attardo et al., 2015). Again, microglia are involved in the elimination of excessive synapses in this brain region during development (Filipello et al., 2018; Paolicelli et al., 2011; Scott-Hewitt et al., 2020; Weinhard et al., 2018). Disruptions in microglia function result in delayed maturation of hippocampal synaptic circuits (Paolicelli et al., 2011). In the adult hippocampus, astrocytes phagocytose synapses to maintain proper hippocampal synaptic connectivity and plasticity (Lee et al., 2021).

## 4.2 Induction of Phagocytosis in Neuronal Remodeling

Phagocytosis of neurons during remodeling results from both neuronal-intrinsic and neuronal-extrinsic mechanisms. Intrinsically, molecular events that occur globally in the cell or locally in specific neurites or compartments lead to changes in cell surface properties, which ultimately induce engulfment by phagocytes. The molecular pathways leading to these changes are diverse and context-specific. For instance, caspase-mediated self-destruction underlies both neuronal PCD and at least some cases of neurite pruning; transcriptional control mediated by ecdysone receptor (EcR) is essential for large-scale axon and dendrite pruning during *Drosophila* metamorphosis; neuronal activity is an important regulator of synaptic pruning in mammals. Many cell-intrinsic pathways have been extensively discussed in previous reviews (Boulanger and Dura, 2015; Faust et al., 2021; Fricker et al., 2018; Riccomagno and Kolodkin, 2015; Yu and Schuldiner, 2014) or in other chapters of this book, and thus are not discussed here.

Extracellularly, phagocytosis is induced by the so-called "eat-me" signals that are exposed on the surface of neurons. "Eat-me" signals tag the neurons and neuronal compartments destined for phagocytosis so that they are distinguishable from other healthy neurons or healthy parts of neurons. Several membrane-anchored "eat-me" signals, including phosphatidylserine (PS), Calreticulin (Calr), Pretaporter (Prtp), and *Drosophila* calcium-binding protein 1 (DmCaBP1), have been identified from earlier work on the clearance of apoptotic cells (Gardai et al., 2005; Kuraishi et al., 2009; Okada et al., 2012; Ravichandran, 2010). Among these, PS is the best studied and its involvement in the clearance of neuronal materials is well-documented across species. In the last couple of decades, much has been learned regarding

the roles of PS in phagocytosis of neuronal materials, recognition of PS by phagocytic receptors, and the signaling downstream of phagocytic receptor activation.

### 4.2.1 Phosphatidylserine (PS) as a major "eat-me" signal

PS is a negatively charged phospholipid that is normally confined to the cytoplasmic leaflet of the plasma membrane in healthy cells (reviewed by Leventis and Grinstein, 2010). However, during apoptosis, PS is externalized to the exoplasmic leaflet of dying cells and is recognized by phagocytes (Segawa and Nagata, 2015). Recent studies show that PS also functions as a potent "eat-me" signal exposed on neurons during developmental remodeling of the nervous system (Figure 4.1), clearance after neurite injury, and in neurological disease conditions. Below, we focus on the role of PS as an "eat-me" signal in the nervous system.

### 4.2.2 PS exposure marks degenerating neurites and synapses during nervous system remodeling

Given that PS is an "eat-me" signal for apoptotic cells (Mapes et al., 2012; Segawa and Nagata, 2015), PS is expected to be exposed globally on the surface of dying neurons. Interestingly, during neuronal remodeling, PS also specifically tags the compartments of neurons (axons or dendrites) that are destined to be phagocytosed. Compartmentalized PS exposure on degenerating axons was first shown using a PS sensor based on Annexin V in axotomy-treated mouse dorsal root ganglia (DRG) explants (Sievers et al., 2003). This finding was later corroborated by experiments using another PS sensor MFG-E8$^{D89E}$, a mutant version of milk fat globule-EGF factor 8 (MFG-E8, also called lactadherin) that cannot interact with integrin receptors, to label mouse DRG explants treated by vincristine or axotomy (Shacham-Silverberg et al., 2018). The first *in vivo* evidence that PS is specifically exposed on degenerative parts of neurons came from a study with injured rat sciatic nerves (Kim et al., 2010). PS exposure on injured, but not intact, nerves was shown with a polarity-sensitive annexin-based biosensor (pSIVA) that fluoresces only when binding to PS on membranes. Sapar et al. provided further *in vivo* evidence in *Drosophila* indicating that PS is an "eat-me" signal on neurites in intact live animals. Using genetically encoded PS binding proteins, Annexin V and the Lactadherin C1C2 domain, local PS exposure was found on dendrites undergoing injury-induced (or Wallerian) degeneration (Sapar et al., 2018) and dendrites of neurons deficient in $NAD^+$ biosynthesis, which molecularly

resembles Wallerian degeneration (Ji et al., 2022). Long-term time-lapse live imaging revealed that PS exposure occurs prior to dendrite fragmentation and phagocytic engulfment of neuronal debris, supporting the role of PS as an "eat-me" signal (Ji et al., 2022; Sapar et al., 2018).

PS is also exposed on degenerating neurites during developmental remodeling. In an *in vitro* model for axon pruning, cultured mouse DRG explants under nerve growth factor (NGF) deprivation exposed PS on subaxonal segments during degeneration (Kim et al., 2010; Shacham-Silverberg et al., 2018). The *in vivo* evidence came from experiments examining dendrite pruning during *Drosophila* metamorphosis, in which PS was exposed specifically on pruned dendrites that were severed from the cell body of C4da neurons (Sapar et al., 2018).

Besides neurites that have been detached from the cell body, PS exposure is also observed on locally restricted neuronal compartments that are degenerative. For example, in freshly dissected mouse retina, PS externalization was found to be restricted to POS tips with discrete boundaries using pSIVA (Ruggiero et al., 2012). At the synaptic level, PS exposure was detected at synapses in mouse dLGN and hippocampus with the fluorescent probe PSVue during the periods when microglial-mediated developmental pruning takes place (Li et al., 2020; Scott-Hewitt et al., 2020). In these contexts, PS-flagged pre-synaptic material was engulfed by microglia (Li et al., 2020; Scott-Hewitt et al., 2020), suggesting that local PS exposure instructs engulfment of synapses. Consistent with this idea, in brains of juvenile mice, PS was found preferentially exposed on inhibitory post-synapses, which are frequently engulfed by microglia (Park et al., 2021).

In all the above examples, PS exposure marks the compartments destined to be engulfed. The fact that PS can be exposed transiently on a restricted portion of the plasma membrane suggests that PS exposure can be regulated locally within the neuron.

### 4.2.3 Exposed PS dominantly triggers phagocytosis of neurons or neuronal processes

The PS asymmetry in the plasma membrane is established and maintained by transmembrane aminophospholipid flippases encoded by the P4-ATPase family. Several members in the P4-ATPase family have been demonstrated to be PS-specific flippases that unidirectionally transport PS from the outer leaflet of the plasma membrane to the inner leaflet (reviewed by Bevers and Williamson, 2016; Leventis and Grinstein, 2010). The PS-specific flippases involved in nervous system remodeling include mammalian ATP8A1/2, their

*Drosophila* homolog ATP8A, and their *C. elegans* homolog TAT-1 (Darland-Ransom et al., 2008; Sapar et al., 2018; Zhu et al., 2012). These PS-flippases require a chaperone protein called CDC50A for proper subcellular localization and function (Takatsu et al., 2011; Tanaka et al., 2011; van der Velden et al., 2010). PS asymmetry on the plasma membrane can be disrupted by the activity of lipid scramblases, enzymes that bi-directionally translocate PS between the two leaflets of biomembranes (reviewed by Bevers and Williamson, 2016; Leventis and Grinstein, 2010). One of the scramblases, Xk-related protein 8 (Xkr8), is activated by caspase-mediated cleavage and is responsible for PS exposure on apoptotic cells (Suzuki et al., 2013). Members of TMEM16 family are $Ca^{2+}$-activated scramblases in non-apoptotic cells (Falzone et al., 2018). For example, TMEM16F activation by $Ca^{2+}$ induces PS exposure on platelets during blood clotting (Fujii et al., 2015; Suzuki et al., 2010).

The loss of PS flippases induces ectopic PS exposure on otherwise normal cells, offering an opportunity for investigating the consequence of non-apoptotic PS exposure. *TAT-1* LOF in living neurons caused PS exposure, and these cells were removed by neighboring phagocytes in *C. elegans* (Darland-Ransom et al., 2008), demonstrating an example of phagoptosis. Studies in *Drosophila* larval PNS further demonstrated the sufficiency of PS exposure in inducing neurite degeneration of sensory neurons. LOF of *CDC50A* or *ATP8A* in C4da neurons resulted in low levels of ectopic PS exposure at distal terminal dendrites, which were phagocytosed by surrounding epidermal cells (Sapar et al., 2018). This PS-induced neurite loss was exacerbated by combining *CDC50* knockout (KO) with the overexpression (OE) of a hypersensitive mutant of TMEM16F that is known to elevate PS exposure (Segawa et al., 2011). These results suggest that PS exposure is sufficient to induce neuronal membrane loss and that distal dendrites are more sensitive to disruptions of the PS asymmetry than proximal dendrites and the cell body.

The approaches of inducing ectopic PS exposure with flippase KO and scramblase OE also helped to reveal the role of PS-mediated phagocytosis in promoting dendrite fragmentation during Wallerian degeneration. PS exposure and fragmentation of injured dendrites is strongly blocked if the neuron overexpresses a chimeric protein called Wallerian degeneration slow ($Wld^S$) (Ji et al., 2022; Sapar et al., 2018). Reintroducing PS exposure by knocking out *CDC50* or overexpressing TMEM16F in $Wld^S$-expressing neurons restored fragmentation of injured dendrites (Ji et al., 2022). Considering that *CDC50* KO and TMEM16F OE cause milder PS exposure than that induced by injury (Sapar et al., 2018), PS-mediated phagocytosis should be sufficient to drive dendrite fragmentation after injury.

Dysregulation of PS asymmetry shows conserved effects in mammals. Mice with *wabbler-lethal* (*wl*) mutations develop progressive ataxia due to axonal degeneration in both the CNS and the PNS. The disease-causing *wl* mutations are in *ATP8A2*, the gene encoding the ATP8A2 flippase. Although spontaneous PS exposure on axons has not been demonstrated in *wl* mutant mice, the neurodegeneration phenotype discovered in *wl* mutant mice suggests that PS exposure due to flippase LOF can cause axonal degeneration in mammals (Zhu et al., 2012). In the context of synaptic pruning in mice, neuronal-specific deletion of *CDC50A* led to PS exposure on neuronal outer membranes, which caused specific loss of inhibitory post-synapses and audiogenic seizures (Park et al., 2021). These results collectively suggest that LOF of PS flippases induces axonal or synaptic degeneration in mammalian brains.

Interestingly, PS exposure induced by flippase LOF and scramblase GOF can cause distinct degeneration patterns in the fly CNS. In *Drosophila* OR22a olfactory receptor neurons (ORNs), *CDC50* KO led to a progressive age-dependent axon degeneration, suggesting that a low level of PS externalization can have a cumulative effect in causing axon loss over time. Overexpression of TMEM16F in OR22a neurons, in contrast, led to a rapid, neuronal activity-dependent axon loss (Sapar et al., 2018), consistent with TMEM16F being a $Ca^{2+}$-activated scramblase.

The above studies involving ectopic PS exposure in multiple species show that PS is a conserved neuronal "eat-me" signal that can dominantly induce dendrite and axon loss in both PNS and CNS due to attacks by phagocytes. These findings provide a potential mechanistic base for understanding neurodegeneration associated with aging and disease conditions.

### 4.2.4 PS exposure is required for phagocytosis of neurites or synapses in some contexts

The requirement of PS exposure in phagocytosis of neurons has been studied by masking PS signals using PS binding proteins. Blocking exposed PS using PS antibody or Annexin V reduced phagocytosis of POS by RPE in culture (Ruggiero et al., 2012). Direct masking of PS using MFG-$E8^{D89E}$ reduced engulfment of axonal debris in NGF-deprived neuronal culture (Shacham-Silverberg et al., 2018). Masking of PS using Annexin V also partially prevented synapse elimination in hippocampal neuron and microglia co-cultures (Scott-Hewitt et al., 2020). Corroborating this *in vitro* evidence, a more recent *in vivo* study showed that Annexin V injection in juvenile brains was sufficient to increase the number of inhibitory post-synapses and to prevent

the loss of inhibitory post-synapses in *CDC50* conditional knockout (cKO) mice, presumably by masking the exposed PS (Park et al., 2021).

The above data are consistent with the idea that PS contributes to phagocytosis during neuronal remodeling. A more recent study on the degeneration of *Nmnat* KO neurons provides evidence for the requirement of PS exposure in phagocytosis of neurites (Ji et al., 2022). The *Drosophila Nmnat* gene encodes the nicotinamide mononucleotide adenylyltransferase required for $NAD^+$ production; *Nmnat* KO in C4da neurons resulted in spontaneous dendrite degeneration. This $NAD^+$ loss-induced neurodegeneration was previously thought to result from neuronal self-destruction (Zhai et al., 2006). Surprisingly, the dendrite degeneration of *Nmnat* KO neurons was completely rescued by either overexpressing ATP8A in neurons or suppressing epidermal phagocytosis, suggesting that PS-induced phagocytosis drives the degeneration of *Nmnat* KO neurons in *vivo* (Ji et al., 2022). These results also suggest that the maintenance of PS asymmetry on the plasma membrane requires sufficient levels of $NAD^+$ in the cell, a conclusion also supported by the observation that $NAD^+$ supplementation inhibited PS exposure on degenerating axons of cultured DRG neurons (Shacham-Silverberg et al., 2018).

Given that multiple "eat-me" signals can contribute to phagocytosis of apoptotic cells, it remains unclear whether PS exposure is required for phagocytosis in all developmental remodeling contexts.

## 4.3 Engulfment Receptors Mediating Phagocytosis during Neuronal Morphogenesis

Phagocytes rely on transmembrane engulfment receptors on their surface to detect and engulf neuronal material (Figure 4.2). Given the importance of PS exposure in phagocytosis, animals have evolved complex systems to recognize PS exposed on the surface of engulfment targets. While some of these systems are conserved from worms to flies and to humans, many others are new inventions of the evolution that are found only in vertebrates. Many engulfment receptors involved in neuronal morphogenesis have been shown to mediate PS recognition: some of them interact with PS directly while others recognize PS through bridging molecules. Here, we review the roles of these engulfment receptors in neuronal remodeling (see Table 4.1 for a summary) and the molecular pathways they activate to drive phagocytosis. We elaborate more on the Draper pathway in *Drosophila* and the complement system in mammals, as their mechanisms are better characterized, while summarizing work related to other receptors.

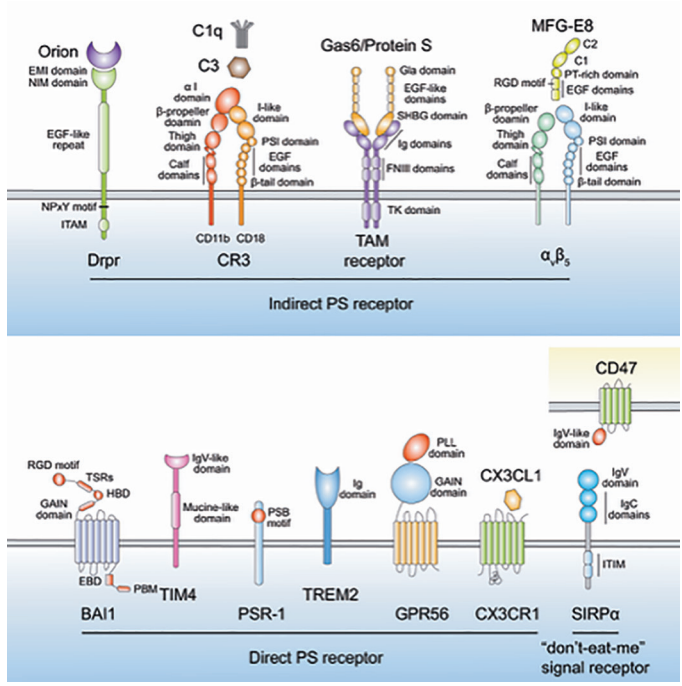


**Figure 4.2 Engulfment receptors involved in phagocytosis of neurons.** Engulfment receptors on phagocytes can recognize targets through a variety of mechanisms. These mechanisms include indirect recognition of neuronal PS through soluble bridging molecules, direct interactions with neuronal PS, and PS-independent ligand–receptor interactions. Recognition of neuronal PS by the indirect PS receptors Drpr, CR3, TAM receptors, and integrin $\alpha_v\beta_5$ requires specific PS-binding bridging molecules, Orion, C1q/C3, Gas6/Protein S, and MFG-E8, respectively. Direct PS receptors include BAI1, TIM4, PSR-1, TREM2, and GPR56. Drpr can also bind to PS directly *in vitro* and may do so *in vivo* when overexpressed. In addition to PS receptors, the chemokine receptor CX3CR1 is involved in synaptic pruning by interacting with its ligand CX3CL1. Lastly, the receptor SIPRα prevents phagocytosis of synapses by interacting with its ligand, the "don't-eat-me" signal CD47, on neuronal membranes. Domain/motif abbreviations: EGF, epidermal growth factor; ITAM, immunoreceptor tyrosine-based activation motif; PSI, plexin/semaphorin/integrin; Gla, gamma-carboxyglutamic acid-rich; SHBG, sex hormone binding globulin; Ig, immunoglobulin; FNIII, fibronectin type III; TK, tyrosine kinase; RGD, arginine-glycine-aspartate; PT, proline/threonine; TSR, thrombospondin type 1 repeat; HBD, hormone-binding domain; GAIN, GPCR autoproteolysis-inducing; EBD, ELMO-binding domain; PBM, PDZ domain-binding motif; IgV, immunoglobulin variable; PSB, PS-binding; PLL, pentraxin/laminin/neurexin/sex-hormone-binding-globulin-like; IgC, immunoglobulin constant; ITIM, immunoreceptor tyrosine-based inhibitory motif. Protein domain structures are based on (Behrens et al., 2022; Boulanger et al., 2021; Druart and Le Magueresse, 2019; Hanayama et al., 2002; Lamers et al., 2021; Lemke and Rothlin, 2008; Miyanishi et al., 2007; Park et al., 2020; Salzman et al., 2016; Ulland and Colonna, 2018; Weng et al., 2019; Wojdasiewicz et al., 2014; Wu et al., 2009; Yang et al., 2015; Ziegenfuss et al., 2008).

**Table 4.1 Models of phagocytosis in the developing nervous system and the corresponding engulfment pathways**

| Morphogenesis type | Species | Phagocyte | "eat-me" signal | Bridging molecule | Engulfment receptor |
|---|---|---|---|---|---|
| Elimination of dead neurons | Fly | Cortex glia (Etchegaray et al., 2016; Kurant et al., 2008; McLaughlin et al., 2019; Nakano et al., 2019), astrocyte-like glia (Hilu-Dadia et al., 2018), and ensheathing glia (Hilu-Dadia et al., 2018) | PS? (Segawa and Nagata, 2015) | | Drpr (Freeman et al., 2003; Tung et al., 2013) |
| | Zebrafish | Microglia (Mazaheri et al., 2014) | | | BAI1, and TIM4 (Mazaheri et al., 2014) |
| | Mammal | Microglia in CNS (Dalmau et al., 2003; Sierra et al., 2013; Sierra et al., 2010) | | Gas6, and Pros1 (Fourgeaud et al., 2016; Grommes et al., 2008) | Mer, and Axl (Fourgeaud et al., 2016; Ji et al., 2013) |
| | Mammal | Satellite glial cell precursors in DRG (Wu et al., 2009) | | | Jedi-1, and MEGF10 (Scheib et al., 2012; Wu et al., 2009) |
| Developmental phagocytosis of live neuronal precursors | Worm | | PS? (Darland-Ransom et al., 2008) | | PSR-1, and CED-1 (Darland-Ransom et al., 2008; Hoeppner et al., 2001; Reddien et al., 2001) |
| | Mammal | Microglia (Cunningham et al., 2013; Marin-Teva et al., 2004; Wakselman et al., 2008) | | | CR3 subunit CD11b (Wakselman et al., 2008) |
| Pruning of mushroom body γ-neuron axons | Fly | Astrocyte-like glia (Awasaki and Ito, 2004; Awasaki et al., 2006; Hakim et al., 2014; Tasdemir-Yilmaz and Freeman, 2014; Watts et al., 2004) | | Orion? (Boulanger et al., 2021) | Drpr (Awasaki et al., 2006; Hakim et al., 2014; Hoopfer et al., 2006; Tasdemir-Yilmaz and Freeman, 2014) |

| | | | | | |
|---|---|---|---|---|---|
| Pruning of da neuron dendrites | Fly | Epidermal cells (Han et al., 2014) | PS (Han et al., 2014; Ji et al., 2022; Sapar et al., 2018) | Orion (Ji, bioRxiv 2022) | Drpr (Han et al., 2014; Williams et al., 2006) |
| Elimination of axons at NMJ | Mammal | Schwann cells (Song et al., 2008) | | | |
| Growth of axons at NMJ | Fly | Glia, and muscles (Fuentes-Medel et al., 2009) | | | Drpr (Fuentes-Medel et al., 2009) |
| Elimination of AFP neuron sensory microvilli | Worm | AMsh glia (Raiders et al., 2021) | PS? (Raiders et al., 2021) | | PSR-1, and PAT-2 (Raiders et al., 2021) |
| Phagocytosis of POS in retina | Mammal | RPE (Nandrot et al., 2004; Young and Bok, 1969) | PS (Ruggiero et al., 2012) | MFG-E8 (Nandrot et al., 2007)<br>Gas6, and Protein S (Burstyn-Cohen et al., 2012) | $\alpha_v\beta_5$ (Nandrot et al., 2007; Nandrot et al., 2004)<br>Mer (D'Cruz et al., 2000; Duncan et al., 2003; Prasad et al., 2006; Vollrath et al., 2001) |
| Synaptic pruning in dLGN | Mammal | Microglia (Lehrman et al., 2018; Li et al., 2020; Schafer et al., 2012; Scott-Hewitt et al., 2020; Stevens et al., 2007; Vainchtein et al., 2018), and astrocytes (Chung et al., 2013) | PS (Li et al., 2020; Scott-Hewitt et al., 2020) | C1q (and C3?) (Stevens et al., 2007) | CR3 (Schafer et al., 2012)<br>MEGF10 (Chung et al., 2013), Mer (Chung et al., 2013), and GPR56 (Li et al., 2020) |
| Synaptic pruning in hippocampus | Mammal | Microglia (Filipello et al., 2018; Paolicelli et al., 2011; Scott-Hewitt et al., 2020; Weinhard et al., 2018), and astrocytes (Lee et al., 2021) | PS (Li et al., 2020) | | MEGF10 (Lee et al., 2021), TREM2 (Filipello et al., 2018; Scott-Hewitt et al., 2020), GPR56 (Li et al., 2020), and CX3CR1 (Paolicelli et al., 2011) |

### 4.3.1 Draper (Drpr), an important engulfment receptor in phagocytosis of neurons in *Drosophila*

*C. elegans* CED-1, *Drosophila* Drpr, and mammalian MEGF10 and Jedi-1 represent a conserved MEGF family of engulfment receptors that are important in the recognition and removal of apoptotic cells (Wu et al., 2009). The founding member CED-1 was identified for its role in the engulfment of cell corpses in the worm (Hedgecock et al., 1983; Zhou et al., 2001b), and later CED-1 was also found to be required for phagocytosis of *TAT-1*-deficient neurons (Darland-Ransom et al., 2008) and the debris of injured axons (Chiu et al., 2018). Drpr (Freeman et al., 2003) is involved in many contexts of phagocytosis in and outside the nervous system in *Drosophila*. Jedi-1 and MEGF10 also function as engulfment receptors in the clearance of apoptotic neurons in developing DRG (Scheib et al., 2012; Wu et al., 2009). In addition, MEGF10 is partially required for astrocyte-mediated synapse elimination in the developing dLGN (Chung et al., 2013) and the adult hippocampus (Lee et al., 2021). Numerous studies on this family have contributed to the understanding of phagocytosis of neurons and the pathways involved. Here, we provide more details of *Drosophila* Drpr as its roles in the nervous system are best understood (Figure 4.3).

#### 4.3.1.1 The involvement of Drpr in phagocytosis during neuronal remodeling

During embryonic development, Drpr is expressed in *Drosophila* glia to promote the clearance of apoptotic neurons (Freeman et al., 2003; Tung et al., 2013). Later in metamorphosis, astrocyte-like glia acquire phagocytic activity, and Drpr is localized on the membrane of astrocyte-like glia infiltrating MB axon bundles (Awasaki and Ito, 2004; Tasdemir-Yilmaz and Freeman, 2014). *drpr* mutations and knockdown of *drpr* in all glia strongly suppressed debris clearance of pruned MB axons (Awasaki et al., 2006; Hoopfer et al., 2006). Similarly, astrocyte-specific knockdown of *drpr* also produced clearance defects (Hakim et al., 2014).

The involvement of Drpr in the clearance of degenerating axons has also been demonstrated in axon injury models. After surgical removal of adult ORNs, ensheathing glia in the antenna lobe extend membrane processes to the injured axons and engulf axonal debris (Doherty et al., 2009; MacDonald et al., 2006). Drpr expression in the ensheathing glia is upregulated upon axonal injury (Doherty et al., 2009; MacDonald et al., 2006; Macdonald et al., 2013). In *drpr* mutants or when glial *drpr* expression is knocked down, glia fail to respond morphologically to axon injury, and severed axons are not cleared from the CNS (Doherty et al., 2009; MacDonald et al., 2006).

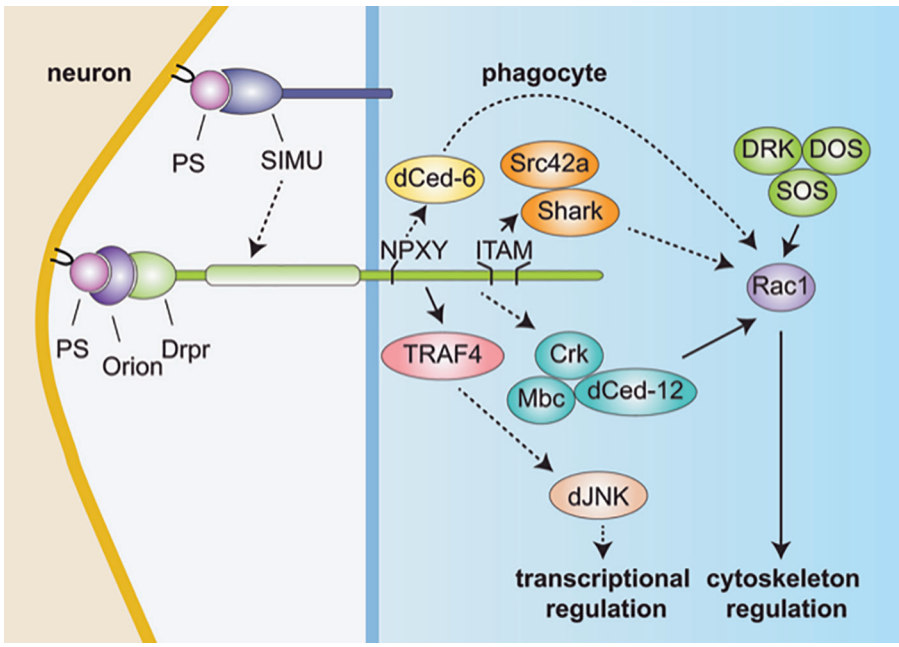


**Figure 4.3 Drpr-mediated phagocytosis in the nervous system of *Drosophila*.** Drpr is an engulfment receptor expressed by phagocytes in *Drosophila*. *In vivo*, Drpr recognizes PS exposed on neurites through the PS-binding bridging molecule Orion. A tethering receptor SIMU acts upstream of Drpr in the phagocytosis of apoptotic neurons in embryos. Drpr can interact directly with PS *in vitro* and may do so *in vivo* when both the levels of PS exposure and Drpr expression are high. Downstream of Drpr activation, the adaptor protein dCed-6 and the kinases Src42a and Shark bind to the intracellular tail of Drpr and promote activation of the small GTPase Rac1, which regulates the subsequent cytoskeleton rearrangement. Rac1 can be activated by two GEF complexes, Crk/Mbc/dCed-12 and DRK/DOS/SOS, in *Drosophila* phagocytes. In another pathway downstream of Drpr, TRAF4 binds to Drpr and activates the dJNK pathway to regulate transcription.

Similarly, Drpr is required for the clearance of axons of gustatory receptor neurons in the ventral nerve cord (VNC) after axotomy (Purice et al., 2017).

The role of Drpr in dendrite clearance has been mainly investigated in the PNS, where C4da neurons serve as excellent models of dendrite pruning (Williams and Truman, 2005a) and dendrite injury (Tao and Rolls, 2011). When *drpr* is knocked down in epidermal cells, the resident phagocytes that engulf degenerating dendrites, the clearance of pruned C4da dendrites is strongly delayed at the pupal stage (Han et al., 2014). In the larva, Drpr-mediated phagocytosis promotes the fragmentation of injured C4da dendrites (Ji et al., 2022) and is required for the clearance of dendrite fragments (Han et al., 2014).

Drpr has also been reported to be involved in other scenarios of neuronal degeneration. Drpr is required for the clearance of destabilized boutons

at growing NMJs at the larval stage (Fuentes-Medel et al., 2009). In aged flies, reduced Drpr activity in nearby glia results in delayed clearance of injured ORN axons (Purice et al., 2016). Furthermore, glial clearance of neurotoxic Aβ peptides in a *Drosophila* Alzheimer's disease (AD) model is Drpr-dependent, suggesting that Drpr can be protective in neurogenerative disease models (Ray et al., 2017).

Taken together, studies conducted in both the CNS and the PNS demonstrated that Drpr plays an important role in a variety of neuronal degeneration contexts. Interestingly, Drpr seems to be absolutely required for clearing injured neurites but is only partially required for clearing neurite debris resulting from developmental pruning. This suggests that other engulfment receptors and pathways likely work in parallel with Drpr during developmental pruning.

#### 4.3.1.2 Ligands for Drpr

Since Drpr and its homologs play important roles in phagocytosis in the nervous system, whether Drpr recognizes PS exposed on neuronal membranes became an interesting question. Recent studies reveal the requirement of Drpr in PS-induced phagocytosis. In the *Drosophila* PNS, PS-exposing dendrites of *CDC50* KO and TEME16F OE C4da neurons shed membranes in a Drpr-dependent manner (Sapar et al., 2018), suggesting that Drpr is involved in PS recognition. However, whether PS is a direct ligand of Drpr *in vivo* had been elusive.

On the one hand, the Drpr extracellular EMI and NIM domains have been shown to bind to PS *in vitro* (Tung et al., 2013). Consistent with this finding, PS exposed on lipid bilayer-coated beads is sufficient to induce engulfment of the beads by Drpr-transfected *Drosophila* S2 cells (Williamson and Vale, 2018). In this system, PS is sufficient to locally trigger Drpr phosphorylation at the intracellular ITAM motif and to activate downstream engulfment signaling (Williamson and Vale, 2018). On the other hand, evidence suggests that Drpr may need bridging molecules (or opsonins) to interact with PS. Drpr has been reported to work along with a tethering receptor called six-microns-under (SIMU) in clearing apoptotic neurons during embryogenesis (Kurant et al., 2008). SIMU can bind to PS with its extracellular domains (Shklyar et al., 2013) and appears to function upstream of Drpr (Kurant et al., 2008). In addition, CED-1, the *C. elegans* homolog of Drpr, requires a secreted bridging molecule TTR-52 to recognize PS during phagocytosis of apoptotic cells (Wang et al., 2010).

Two recent studies provided new insights into the mechanisms of Drpr-mediated PS sensing. In a forward genetic screen for factors involved in MB axon remodeling, a secreted protein called Orion was identified to be

required for astrocyte infiltration and clearance of axonal debris (Boulanger et al., 2021). Orion was further analyzed in the larval PNS, where distributions of Orion-GFP fusion proteins can be examined in live animals (Ji et al., 2023). *In vivo* assays showed that Orion binds to both PS and Drpr and is required for Drpr-mediated phagocytosis of C4da neurons (Ji et al., 2023). Furthermore, a membrane-tethered version of Orion triggers PS-independent but Drpr-dependent phagocytosis of healthy dendrites when it is expressed in neurons, while its expression in epidermal cells suppresses engulfment of injured dendrites. These results provide strong evidence that Drpr normally requires Orion as a PS-binding bridging molecule to sense PS on neurons (Figure 4.3). The same study also shows that Drpr overexpression in epidermal cells can bypass the requirement of Orion in engulfing injured dendrites, presumably through direct Drpr–PS interaction (Figure 4.3). However, whether endogenous Drpr directly senses PS exposure under physiological conditions *in vivo* is unknown.

Drpr has also been reported to recognize Prtp and DmCaBP1 during the clearance of apoptotic cells in *Drosophila* embryos (Kuraishi et al., 2009; Okada et al., 2012). Prtp and DmCaBP1 are ER proteins that are exposed on the cell surface upon apoptosis, but these ligands are not involved in axonal pruning during metamorphosis (Kuraishi et al., 2009; Okada et al., 2012). Whether they function in other contexts of neuronal remodeling remains to be determined.

#### 4.3.1.3 Upstream regulation of Drpr

Besides being activated by PS on the target cell surface, Drpr expression in phagocytes can be regulated by various degeneration cues and developmental signals. In the larval CNS, dying neurons signal to phagocytic cortex glia via releasing a toll receptor ligand, Spätzle5. This cue activates a Toll-6 transcriptional pathway, which upregulates the expression of the Drpr in glia (McLaughlin et al., 2019). During metamorphosis, Drpr transcription in glia is upregulated. Expression of a dominant-negative mutant of ecdysone receptor (EcR-DN) in glia suppresses this upregulation (Awasaki et al., 2006; Hakim et al., 2014), suggesting that Drpr expression is regulated by hormonal signaling at the time of large-scale remodeling. In contrast, upregulation of Drpr expression in glia in response to axonal injury is mediated through c-Jun N-terminal kinase (dJNK) signaling (Losada-Perez et al., 2021; Macdonald et al., 2013).

#### 4.3.1.4 Signaling downstream of Drpr

Drpr and its homologs are large single-pass transmembrane proteins with many extracellular EGF-repeats and a short intracellular domain containing

tyrosine (Tyr) phosphorylation sites (Scheib et al., 2012; Zhou et al., 2001b; Ziegenfuss et al., 2008). They share some common downstream effectors to trigger phagocytosis of neuronal debris.

The initial molecular insights into the signaling downstream of these engulfment receptors came from studies of *cell death abnormal* (*ced*) mutants in *C. elegans*. Two partially redundant pathways activate engulfment of cell corpses during development and in the germline (reviewed by Reddien and Horvitz, 2004). In one pathway, the CED-6 adaptor protein transduces signals from CED-1, through possible binding between the phosphotyrosine-binding-domain protein (PTB) in CED-6 and the conserved NPXY (Asn-Pro-any amino acid-Tyr) motif in the intracellular region of CED-1, to promote removal of cell corpses (Kavanaugh et al., 1995; Liu and Hengartner, 1999; Su et al., 2000). CED-1 and CED-6 act upstream of the small GTPase CED-10 (Rac1 in mammals) to regulate cytoskeleton changes in phagocytes (Kinchen et al., 2005). The second pathway involves CED-2 (CrkII in mammals), CED-5 (DOCK180 in humans and Myoblast City (Mbc) in *Drosophila*), and CED-12 (ELMO in mammals). CED-2, CED-5, and CED-12 appear to form a ternary guanine nucleotide exchange factor (GEF) complex in response to upstream engulfment signals and activate CED-10 to promote cytoskeletal reorganization and corpse engulfment (Brugnera et al., 2002; Gumienny et al., 2001; Reddien and Horvitz, 2000; Wu and Horvitz, 1998; Wu et al., 2001; Zhou et al., 2001a). Both pathways contribute to the removal of necrotic neurons in *C. elegans* (Yang et al., 2015).

dCed-6, the *Drosophila* homolog of CED-6, is involved in Drpr signaling during phagocytosis in the nervous system (Figure 4.3). Glia-specific knockdown of *dCed-6* by RNAi partially suppresses glial engulfment of MB axons during metamorphosis (Awasaki et al., 2006). A combination of *dCed-6* RNAi and heterozygous *drpr* mutation in the pupa resulted in significantly reduced glial action compared with *dCed-6* RNAi alone or heterozygous *drpr* mutation alone, suggesting that *drpr* and *dCed-6* interact genetically in the glial engulfment of the MB axons (Awasaki et al., 2006). During NMJ development, RNAi knockdown of *dCed-6* in muscles or glia phenocopies *drpr* RNAi knockdown in these tissues (Fuentes-Medel et al., 2009). In the adult brain, dCed-6 is expressed in ensheathing glia but not in astrocyte-like glia, indicating that it may play a role in clearance of injured ORN axons (Doherty et al., 2009).

Srk family proteins that share similar domains with components of the CED-2/CED-5/CED12 pathway are also involved in Drpr signal transduction (Figure 4.3). Studies on clearance of injured ORN axons reveal that Drpr activation initiates signals through its immunoreceptor tyrosine-based activation motif (ITAM) domain to recruit downstream effectors, the Src family kinase

Src42a and Syk-related Tyr kinase Shark (containing dual SH2 domains) (Ziegenfuss et al., 2008). The involvement of Shark in glial phagocytosis has been shown in the clearance of neuronal cell corpses in embryos and injured ORN axons in adult flies, and engulfment of apoptotic cell corpses by Drpr-transfected S2 cells *in vitro* (Doherty et al., 2009; Williamson and Vale, 2018; Ziegenfuss et al., 2008). Mammalian Drpr homologs, Jedi-1 and MEGF10, recruit Shark homolog, Syk, through their ITAM domains as well, suggesting that this engulfment pathway is conserved (Scheib et al., 2012).

Consistent with the CED-1-mediated engulfment pathway, the small GTPase Rac1 that regulates the actin cytoskeleton appears to be an important downstream effector of Drpr (Figure 4.3). Glia-specific knockdown of *rac1* potently suppressed clearance of axonal debris after ORN injury, phenocopying *drpr*-null mutants (Ziegenfuss et al., 2012). Rac1 activation requires two redundant sets of GEF complexes, the Crk/Mbc/dCed-12 complex and a complex composed of downstream of receptor kinase (DRK) (Grb2 in mammals)/daughter of sevenless (DOS) (Gab2 in mammals)/son of sevenless (SOS) (mSOS in mammals) (Lu et al., 2014; Ziegenfuss et al., 2012). Rac1 activity promotes the activation of glia after axon injury and internalization of axonal debris by glia (Lu et al., 2014; Ziegenfuss et al., 2012). Cytoskeleton changes that are potentially downstream of Rac1 have been studied in the *Drosophila* PNS. The association of actin-rich epidermal membranes with degenerating dendrites was reduced when *drpr* was knocked down in the epidermis, confirming that Drpr activation results in actin polymerization surrounding the engulfment target (Han et al., 2014).

The recent identification of tissue necrosis factor receptor associated factor 4 (TRAF4) as a Drpr binding partner links Drpr to the JNK pathway (Figure 4.3). TRAF4 acts downstream of Drpr to activate the JNK pathway, resulting in changes of gene expression in glia after ORN axon injury (Lu et al., 2017). JNK signaling is also important for neuronal remodeling in multiple contexts, including glial phagocytosis of apoptotic neurons downstream of Drpr during *Drosophila* metamorphosis (Hilu-Dadia et al., 2018), glia-mediated functional regeneration of the CNS after crush injury in adult *Drosophila* (Losada-Perez et al., 2021), CED-1-mediated axon regrowth in *C. elegans* (Chiu et al., 2018), and astrocyte proliferation in mammals (Gadea et al., 2008).

### 4.3.2 CR3 and the complement system in mammalian synaptic pruning

In mammals, CR3 and the complement system are a better-characterized pathway in phagocytosis of neuronal material. As a part of the innate immune

system, the classical complement pathway contributes to the recognition and phagocytosis of invading pathogens and stimulates other components of the immune system (reviewed by van Lookeren Campagne et al., 2007). A simplified complement pathway involves soluble proteins C1q, C4, C3, and complement receptor 3 (CR3, also named as Mac-1, integrin $\alpha_M\beta_2$, or CD11b/CD18) expressed by macrophages. C1q, the first component of the pathway, binds to the engulfment target and activates the complement cascade through a series of proteolytic events, ending in activation of C3. Activated C3 is recruited to the target and triggers phagocytosis by interacting with its receptor CR3 (reviewed by Presumey et al., 2017).

Intriguingly, components of the complement system are also found in the brain, and they are involved in neuronal death in the mouse hippocampus (Wakselman et al., 2008) and activity-dependent synapse elimination by microglia in the developing mouse visual system (Stevens et al., 2007). Complement-dependent phagocytosis of synapses is also dysregulated in developmental disorders, such as schizophrenia (Sekar et al., 2016), and neurodegenerative disorders, such as glaucoma (Howell et al., 2011; Stevens et al., 2007), aging-related cognitive decline (Stephan et al., 2013), Alzheimer's disease (Hong et al., 2016), and frontotemporal dementia (Lui et al., 2016). Here, we review the molecular mechanisms of complement-dependent phagocytosis in the development of healthy dLGN.

#### 4.3.2.1 CR3 as an engulfment receptor required for synaptic pruning in the developing dLGN

In the developing dLGN, the complement receptor CR3 is exclusively expressed in microglia, which phagocytose "weak" RGC inputs during the peak synaptic pruning period (Schafer et al., 2012). CR3 is highly expressed at P5 and its expression decreases over time (Schafer et al., 2012). CR3 on cultured microglia mediates the removal of desialylated neurites of co-cultured neurons (Linnartz et al., 2012). Consistent with these *in vitro* results, microglia in brain slices of CR3 KO mice had decreased capacity to engulf RGC inputs (Schafer et al., 2012), suggesting that phagocytosis by microglia is mediated by CR3. Furthermore, CR3 KO resulted in sustained deficits in eye-specific segregation and excessive immature synapses in the adult dLGN (Schafer et al., 2012), suggesting that CR3-mediated engulfment is important for synaptic pruning.

#### 4.3.2.2 Components of the complement system upstream of CR3

CR3 is a receptor for activated C3 (Figure 4.2) (reviewed by Vorup-Jensen and Jensen, 2018). Before CR3's role was identified in synaptic pruning, C3

had been shown to tag subsets of synapses and to be required for synapse elimination in the developing brain (Stevens et al., 2007). C3 is enriched in synaptic regions of the postnatal dLGN but is downregulated when pruning is largely completed (Schafer et al., 2012; Stephan et al., 2013; Stevens et al., 2007). C3 KO mice displayed defects in eye-specific segregation and synapse elimination, which was phenocopied by CR3 KO mice (Schafer et al., 2012; Stevens et al., 2007). Together, these results suggest that C3/CR3 signaling is required for synaptic pruning.

Being required for C3 activation, C4 is also involved in synaptic refinement in the developing brain. In *C4*-deficient mice, C3 immunostaining in the dLGN was greatly reduced and fewer synaptic inputs were tagged by C3 (Sekar et al., 2016). Furthermore, similar to C3/CR3 KOs, mice lacking C4 had deficits in eye-segregation (Sekar et al., 2016).

As the initiator of the complement cascade, C1q has a similar distribution to C3 on synapses in the developing retina and brain (Stevens et al., 2007). Interestingly, C1q predominantly binds to presynaptic membranes of the synaptosomes isolated from the adult cerebral cortex tissue (Gyorffy et al., 2018). *In vitro*, C1q binds to desialylated neurons (Linnartz et al., 2012), suggesting that C1q recognizes neurons with altered glycocalyx. C1q KO resulted in significant defects in eye-specific segregation in the dLGN, consistent with C1q being upstream of C3 and CR3 (Stevens et al., 2007). Due to their ability to tag synapses during developmental pruning, C1q and C3 function like bridging molecules for CR3 (Figure 4.2).

#### 4.3.2.3 PS exposure specifies the synapses to be eliminated by complement-mediated phagocytosis

What is unique about the synapses tagged by C1q and C3? Recent studies revealed that PS exposure on neuronal membranes acts upstream of the complement cascade in synapse elimination. Cell culture studies suggest that C1q can recognize PS directly (Paidassi et al., 2008) or indirectly (Martin et al., 2012) to facilitate phagocytic removal of apoptotic cells. Consistent with these results, C1q co-localizes with the PS marker Annexin V *ex vivo* on isolated synaptosomes (Gyorffy et al., 2018). Furthermore, in the developing dLGN, C1q and PSVue co-localize at a subset of retinogeniculate presynaptic inputs, and the loss of C1q resulted in an increase in PS-positive presynaptic inputs due to reduced microglial engulfment (Scott-Hewitt et al., 2020). These findings support a model in which locally exposed PS interacts with complement factors (and perhaps other proteins) to promote microglial engulfment during the critical periods of synaptic refinement.

So far, the complement signaling has been mainly studied in the visual system. Given that the loss of C1q, C3, or CR3 greatly interfered with eye-specific segregation but did not completely prevent it (Schafer et al., 2012; Stevens et al., 2007), other engulfment receptors are likely involved in this process as well.

### 4.3.3 Other engulfment receptors requiring PS-binding bridging molecules for phagocytosis of neurons

Besides CR3 and MEGF receptors, TAM receptors and integrins are also known to mediate PS-induced phagocytosis through extracellular bridging molecules. Their roles in the nervous system are mostly characterized in mammals.

#### 4.3.3.1 Tyro3, Axl, and Mer (TAM) receptors

TAM receptors (Figure 4.2) are receptor tyrosine kinases that play important roles in several scenarios of phagocytosis in the nervous system, including synaptic pruning (Chung et al., 2013; Park et al., 2021), engulfment of retinal POS (Duncan et al., 2003), and clearance of apoptotic neurons (Fourgeaud et al., 2016). TAM receptors do not bind to PS directly but instead recognize PS via the bridging molecules Growth arrest-specific-6 (Gas6) and Protein S (Dransfield et al., 2015; Stitt et al., 1995; Zagorska et al., 2014). Gas6 and Protein S bridge TAM receptors on phagocytes to PS exposed on phagocytic targets (Dransfield et al., 2015; Lemke, 2017; Lew et al., 2014; Zagorska et al., 2014). Gas6 binds to and activates all three TAMs, whereas Protein S binds to and activates only Tyro3 and Mer (Lew et al., 2014). Although TAM receptors have also been extensively studied in the immune system (reviewed by Lemke, 2013), here, we focus only on the role of TAM receptors and their bridging molecules in the context of the developing nervous system.

The requirement of Mer in the phagocytosis of distal POS in the retina was demonstrated by two rodent models. In a retinal degeneration rat model called Royal College of Surgeons (RCS), photoreceptors are lost due to an LOF deletion within the rat *Mertk* gene (which encodes Mer) (D'Cruz et al., 2000). Gene transfer of *Mertk* to the RPEs in RCS rat retina resulted in correction of the RPE phagocytosis defect and preservation of photoreceptors (Vollrath et al., 2001). Similarly, in mice deficient of *Mertk*, photoreceptors undergo progressive degeneration due to the lack of phagocytosis of POS by RPEs (Duncan et al., 2003). Consistent with these findings, Mer and Tyro3 were found to be expressed in RPE cells in the mouse retina (Prasad et al., 2006). Later, the bridging molecules of Mer, Gas6, and Protein S were found

to act redundantly in mediating phagocytosis of retinal POS (Burstyn-Cohen et al., 2012).

In the mouse brain, Mer is expressed by both astrocytes and microglia (Cahoy et al., 2008; Chung et al., 2013; Gautier et al., 2012; Grommes et al., 2008; Ji et al., 2013). In the developing dLGN, Mer is required for synaptic elimination mediated by phagocytic astrocytes but is dispensable for the phagocytic function of microglia (Chung et al., 2013). Evidence suggests that Mer mediates engulfment of PS-exposing synapses: deleting microglial *Mertk* saved the loss of inhibitory post-synapses and seizure phenotype in *CDC50A* cKO mice and increased the number of PS-exposing inhibitory post-synapses in the wild-type juvenile brains (Park et al., 2021).

The signaling downstream of TAM receptors has been mostly studied using *in vitro* assays in the context of phagocytosis of apoptotic cells (reviewed by Lemke, 2013, 2019). In brief, the activation of the TAM kinase activity is necessary for phagocytosis (Zagorska et al., 2014). Mer also works with the integrin pathway to regulate CrkII/DOCK180/Rac1 modules in controlling rearrangements of the actin cytoskeleton in phagocytes (Wu et al., 2005).

#### 4.3.3.2 Integrin receptors

Integrin receptors (Figure 4.2) are another major player in the phagocytosis of retinal POS in mammals. In rodent and human retinas, the integrin receptor $\alpha_v\beta_5$ localizes specifically to the apical surface of RPE cells (Anderson et al., 1995; Finnemann et al., 1997) and is required for diurnal bursts of RPE phagocytosis of POS (Nandrot et al., 2007; Nandrot et al., 2004). $\alpha_v\beta_5$ has been shown to recognize PS indirectly through the bridging molecule MFG-E8 (Akakura et al., 2004). A discoidin-like domain at the carboxyl terminus of MFG-E8 recognizes PS exposed on apoptotic cells (Hanayama et al., 2002). In the retina, extracellular MFG-E8 promotes phagocytosis of shed POS by ligating $\alpha_v\beta_5$ on RPE cells (Nandrot et al., 2007). These results suggest that the phagocytosis of POS by RPEs is mediated by PS-MFG-E8-$\alpha_v\beta_5$ signaling. Intracellularly, the engagement of $\alpha_v\beta_5$ activates focal adhesion kinase, at the same time recruiting CrkII-Dock180 complex to activate Rac1, the common effector involved in phagocytosis (Akakura et al., 2004; Albert et al., 2000; Finnemann, 2003; Wu et al., 2005).

### 4.3.4 Direct PS receptors involved in phagocytosis of neurons

Several engulfment receptors, including brain-specific angiogenesis inhibitor 1 (BAI1), T-cell immunoglobulin- and mucin-domain-containing 4 (TIM4), triggering receptor expressed on myeloid cells 2 (TREM2), adhesion G

protein-coupled receptor G1 (ADGRG1), and PSR-1 are known to directly interact with PS (Figure 4.2). With the exception of PSR-1, which is conserved from worms to humans, all of the above are microglial surface receptors that exist only in vertebrates. Compared to indirect PS receptors discussed above, relatively less is known about the roles of these receptors in the nervous system. Here, we summarize the main findings pointing to their involvement in phagocytosis of neurons.

BAI1 is a seven-transmembrane protein belonging to the adhesion-type G-protein-coupled receptor family (Park et al., 2007). TIM4 is a type I transmembrane protein (Miyanishi et al., 2007). Both BAI1 and TIM4 can directly bind to PS on apoptotic cells (Kobayashi et al., 2007; Miyanishi et al., 2007; Park et al., 2007; Sokolowski et al., 2011). Microglia lacking BAI1 and TIM-4 display clearance defects in removing dying neurons in the embryonic zebrafish brain (Mazaheri et al., 2014).

TREM2 is known for its association with AD (reviewed by Colonna and Wang, 2016). It can bind to PS directly (Wang et al., 2015) and is found to regulate microglial function in response to PS exposed on apoptotic cells *in vitro* (Shirotani et al., 2019). Recent studies revealed that TREM2 is essential for microglia-mediated synaptic refinement during brain development in mice (Filipello et al., 2018; Scott-Hewitt et al., 2020).

ADGRG1 (also called GPR56) is another adhesion G protein-coupled receptor expressed by microglia (Bennett et al., 2016; Singer et al., 2013). A recent study shows that one of its splicing isoforms is involved in microglia-mediated synaptic pruning in the mouse dLGN via direct PS binding (Li et al., 2020).

Lastly, PSR-1 is an engulfment receptor whose roles are not limited to the nervous system. In *C. elegans*, loss of RSP-1 (Yang et al., 2015) causes a mild delay in the clearance of apoptotic cells (Wang et al., 2003) and necrotic neuronal corpses (Yang et al., 2015). PSR-1 acts in the CED-2 phagocytosis pathway, in parallel with CED-1, to promote phagocytosis (Yang et al., 2015). A recent study revealed that the glial PSR-1 is required for the engulfment of sensory endings of the AFD neuron (Raiders et al., 2021), possibly by recognizing PS on the dendrites. In the same study, PAT-2, an α-integrin subunit implicated in apoptotic cell phagocytosis in *C. elegans* (Hsieh et al., 2012), was found to coordinate with PSR-1 to regulate glial engulfment of neuronal debris (Raiders et al., 2021).

### 4.3.5 Other immune molecules involved in phagocytosis of neurons

In addition to known PS receptors, several immune molecules that are not known to recognize PS have been shown to be involved in phagocytosis of

neurons. CX3CR1, the receptor for the chemokine CX3CL1 (fractalkine) (Figure 4.2), is expressed by microglia in the mammalian brain (Harrison et al., 1998; Jung et al., 2000). Paolicelli et al. first identified a role for CX3CR1 in synaptic pruning in the mouse hippocampus (Paolicelli et al., 2011). CX3CR1 was also found to be involved in dendritic spine elimination and formation in the motor cortex (Parkhurst et al., 2013), reduced functional connectivity in the hippocampus (Zhan et al., 2014), and activity-dependent synaptic pruning in barrel cortex (Gunner et al., 2019). Even though CXCR1 has not been reported as a PS receptor, the sequence and functional similarity between CX3CL1 and the *Drosophila* chemokine-like Orion (Boulanger et al., 2021; Ji et al., 2023) points to a potential link between PS and CXC3L1-CXCR1 signaling.

Besides CX3CR1, some other immune molecules have also been described in the contexts of synaptic pruning. The major histocompatibility complex (MHC) class I proteins (Figure 4.2) are required for synaptic elimination in the mouse dLGN (Lee et al., 2014). Astrocyte-derived cytokine, IL-33, is also required for synaptic development in the thalamus and the spinal cord by signaling to microglia to promote synaptic engulfment (Vainchtein et al., 2018). To counteract the effect of "eat-me" signal, the so-called "don't-eat-me" signals have been proposed to protect synapses from being engulfed. The best studied "don't-eat-me" signal is the transmembrane immunoglobulin-related cell surface protein CD47 (Figure 4.2). The expression patterns of CD47 and its receptor, SIRPα, correlate with peak pruning in the dLGN. Supporting a role for CD47-SIRPα signaling in preventing excess microglial phagocytosis, *CD47* KO or *SIRPα* KO mice have fewer synapses and increased pruning in the dLGN (Lehrman et al., 2018). It remains unclear how CD47 as a "don't-eat-me" signal and PS as an "eat-me" signal coordinate in synaptic pruning.

## 4.4 Perspectives

The contribution of phagocytosis in sculpting the nervous system during morphogenesis has been increasingly appreciated. In the last two decades, the field of phagocytosis in the nervous system has made important advances. First, evidence suggests that phagocytosis not only clears neuronal corpses or degenerating neurites but also actively breaks and removes non-fragmenting neurites or synapses in many cases, refuting the conception that phagocytes merely passively clear neuronal debris after apoptosis or neurite fragmentation. Second, recent work deciphering the identity of the "eat-me" signal for degenerating neurites and weak synapses in diverse contexts has filled important gaps in understanding the trigger and specificity of phagocytosis.

Third, multiple engulfment receptors and pathways have been identified as being involved in sculpting neural circuits, providing a more complete picture of the major players in phagocytosis. Moreover, many studies took advantage of *in vivo* model systems to elucidate molecular mechanisms of phagocytosis in more physiologically relevant settings. These findings greatly expanded our understanding of how phagocytosis contributes to the morphogenesis of the nervous system.

However, several important questions in the field remain unanswered. Most crucially, since PS has been shown as an "eat-me" signal in most of the phagocytosis scenarios, including elimination of dying neurons, fragmented neurites, and intact neurites and synapses destined to be pruned, how does this same molecule trigger various degrees of phagocytosis in different scenarios? Interestingly, PS has been shown to play a role in axon fusion instead of triggering phagocytosis in *C. elegans* (Neumann et al., 2015). What properties of the PS exposed on neuronal surface instruct surround phagocytes to engulf the entire neuron, engulf a local branch, or leave the neuron alone? One possibility could be that the levels, the rates, and the regions of PS exposure on neuronal surfaces are distinct among different scenarios. The spatiotemporal pattern of PS exposure may dictate, to some extent, the bridging molecules or engulfment receptors recruited to trigger a specific behavior of phagocytes. In this model, PS exposure must be tightly regulated at a local scale for a controlled phagocytosis. How PS exposure is regulated, especially by the neuronal activity, is thus an important question to answer in coming years. The second possibility is that the exposed PS coordinates with other surface molecules, such as other "eat-me" signals or "don't-eat-me" signals, to generate a precise phagocytosis trigger. The involvement of the "don't-eat-me" signal CD47 in synaptic pruning (Lehrman et al., 2018) has provided some evidence for this possibility. The interesting cases of PS-exposing cells without being phagocytosed (Bevers and Williamson, 2016) could be good models to study the interplay between "eat-me" signals and "don't-eat-me" signals.

Yet another possibility is that the consequence of phagocytosis is determined by the status of the phagocytes. Some phagocyte types are known to be more potent in engulfing neuronal debris than others. For example, microglia are professional phagocytes, while other glial types are considered amateur phagocytes, because the latter normally fulfill other functions but turn into phagocytes when needed. So a cell could be programmed to execute a certain phagocytosis task in a given developmental window. Although cytoskeletal changes seem to be the convergent point of many phagocytosis behaviors, a lot is still unknown about how signaling is transduced in each type of phagocyte to regulate cytoskeletal changes. It would be interesting to investigate

what factors determine the baseline potency of phagocytes and how cell signaling modulates this potency. Some phagocytes also maintain the homeostasis of the nervous system. For example, besides eliminating pruned synapses, microglia play an important role in regulating synapse growth and plasticity in the CNS (Wilton et al., 2019). How phagocytes balance their phagocytosis functions and other functions is an interesting question worth exploring.

Another interesting observation is that a number of engulfment receptors and pathways are involved in synaptic pruning in the mammalian CNS, including CR3, CX3CR1, Mer, TREM2, GPR56, and MEGF10. Why are so many receptors involved in synaptic pruning? One possibility is that they may function redundantly to back each other up. For instance, the complement system, Mer, and GPR56 all seem to be involved in synaptic elimination in the developing dLGN (Chung et al., 2013; Li et al., 2020; Schafer et al., 2012; Stevens et al., 2007). They may coordinate to ensure synaptic pruning happens at the right time and location. Alternatively, each engulfment receptor is only responsible for phagocytosis in certain brain regions and/or at certain developmental stages. A piece of evidence is that complement system and CX3CR1 are involved in synaptic pruning in non-overlapping brain regions (Gunner et al., 2019; Paolicelli et al., 2011; Parkhurst et al., 2013; Schafer et al., 2012; Stevens et al., 2007). Thus, restricted expressions of receptors and PS bridging molecules may provide specificity in synaptic pruning. A systematic survey of these receptors in different brain regions and developmental stages could provide a more complete picture of the contribution of each pathway.

Finally, many of the players involved in phagocytosis in the developing nervous system are implicated in neurodevelopmental and neurodegenerative disorders (Chung et al., 2015; Colonna and Wang, 2016; Galloway et al., 2019; Neniskyte and Gross, 2017; Salter and Stevens, 2017; Sierra et al., 2014; Stephan et al., 2012). The fact that phagocytes actively engulf non-dying neurons and break neurites during development (Brown and Neher, 2014; Hakim et al., 2014; Han et al., 2014; Ji et al., 2022; Raiders et al., 2021) further implies possible contributions of active phagocytosis in pathogenesis. The research on how phagocytosis contributes to neuronal remodeling during development could thus help us to understand the causes of neurological disorders and to explore potential phagocytosis-dependent therapies.

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## Conflict of Interest Statement

Nothing declared.

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