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The scoop on the fly brain:

glial engulfment functions in *Drosophila*

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Abstract

Glial cells provide support and protection for neurons in the embryonic and adult brain, mediated in part through the phagocytic activity of glia. Glial cells engulf apoptotic cells and pruned neurites from the developing nervous system, and also clear degenerating neuronal debris from the adult brain after neural trauma. Studies indicate that *Drosophila melanogaster* is an ideal model system to elucidate the mechanisms of engulfment by glia. The recent studies reviewed here show that many features of glial engulfment are conserved across species and argue that work in *Drosophila* will provide valuable cellular and molecular insight into glial engulfment activity in mammals.

Keywords

Phagocyte; phagocytosis; *Drosophila* glia

INTRODUCTION

Though dismissed originally as ‘brain glue’, glial cells are now considered to be essential participants in the development and function of the nervous system. During CNS assembly, glia exert significant control over neuronal morphogenesis, axon outgrowth, synapse formation and synapse maturation (reviewed in Chotard and Salecker, 2004; Allen and Barres, 2005; Edenfeld *et al.*, 2005; Freeman and Doherty, 2006). Glia are equally important in regulating the function of the mature CNS. For example, glia modulate synaptic signaling by releasing ‘gliotransmitters’ (reviewed in Halassa *et al.*, 2007), and they nourish neurons by secreting an array of trophic factors and amino acids and supplying neurons with energy sources (reviewed in Kahlert and Reiser, 2004; Magistretti, 2006). Glia are also the primary CNS immune cell type responsible for maintaining brain health; they survey the CNS constantly for signs of neuronal trauma or infection, modulate inflammatory responses in the brain after neural insult, and clear potentially harmful degenerating debris from the CNS (reviewed in Vilhardt, 2005; Seifert *et al.*, 2006; Farina *et al.*, 2007).

Many of the beneficial effects of glial immune function are mediated through glial phagocytic activity. During nervous system development many neurons are destroyed through apoptosis as a means of reducing neuronal cell numbers. Glia have a central role in this event both by promoting the destruction of certain subsets of neurons and, later, by engulfing neuronal ‘cell corpses’ after apoptosis (Sonnenfeld and Jacobs, 1995a; Marin-Teva *et al.*, 2004; Mallat *et al.*, 2005). Glial cells also help to sculpt neural circuits by

engulfing pruned axons and dendrites as neuronal connections are remodeled and refined during development (Awasaki and Ito, 2004; Bishop *et al.*, 2004; Watts *et al.*, 2004). In the adult brain, engulfment by glia is a crucial defense tactic to destroy invading pathogens rapidly and remove neurons that are damaged as a result of either trauma or disease (Pekny and Nilsson, 2005; Farina *et al.*, 2007). Alterations to glial immune responses, including phagocytosis, are associated with (and might promote the progression of) several human pathologies including multiple sclerosis and Alzheimer's disease (Fiala *et al.*, 2005; Simard *et al.*, 2006; Farina *et al.*, 2007). Thus, a thorough understanding of the engulfment mechanisms used by glial cells is essential as we try to understand the process of nervous system morphogenesis and develop treatment for brain trauma and neurodegenerative disorders.

A key challenge is to identify the molecular signaling pathways that allow glia to recognize and destroy diverse engulfment targets such as neuronal cell corpses and degenerating axons. Mammalian glia express numerous molecules that are implicated in immune signaling pathways, but it remains unclear precisely how these factors contribute to glial engulfment activity (Husemann *et al.*, 2001; Smith, 2001; Husemann *et al.*, 2002; Farina *et al.*, 2007). How do glia distinguish between healthy and apoptotic neurons *in vivo*? How do glia recognize and dispose of degenerating axons after brain injury or during disease? What are the 'eat-me' cues that are presented by dead or dying neurons and how are they recognized? How do glia internalize and destroy engulfed neuronal corpses and neural debris? Here, we review our current understanding of the cellular and molecular details of glial phagocytic function in developing and adult *Drosophila*. Several recent studies provide new insights into how glia recognize engulfment targets, and the molecular pathways that are involved appear to be well conserved from *Caenorhabditis elegans* to *Drosophila* to mammals.

Nervous system engulfment basics: what gets engulfed, why, and how?

In all animals, phagocytes are responsible for removing foreign matter and unwanted cells through engulfment, a process by which either target cells or debris are recognized, internalized and destroyed. The best known phagocytes are 'professional' immune cells such as macrophages, which circulate throughout the body, survey the health of various tissues and organs, and remove cells that are either dying or damaged (Stuart and Ezekowitz, 2005; Krysko *et al.*, 2006). Typically, however, circulating professional phagocytes are excluded from the healthy CNS (Galea *et al.*, 2007). Instead, glia assume the role of phagocytes within the CNS to remove dead cells, defend the brain from invading pathogens and maintain tissue homeostasis (Sonnenfeld and Jacobs, 1995a; Cantera and Technau, 1996; Granucci *et al.*, 2003; Wyss-Coray *et al.*, 2003; Awasaki and Ito, 2004; Block *et al.*, 2004; Gomez *et al.*, 2004; Kaur *et al.*, 2004; Koenigsknecht and Landreth, 2004; Laporte *et al.*, 2004; Marin-Teva *et al.*, 2004; Song *et al.*, 2004; Watts *et al.*, 2004; MacDonald *et al.*, 2006). Matter that can be engulfed by glia either *in vivo* or *in vitro* fall into two general categories: 'non-self' and 'modified self'. Bacteria, yeast and environmental toxins fall into the category of non-self because they are all foreign to the body. Modified self includes dead cells (e.g. apoptotic cell corpses), cellular debris (e.g. degenerating axons) and endogenously produced toxic materials (e.g. β -amyloid plaques).

Frequently, glia engulf modified self during nervous system development. For example, many embryonic neurons are destroyed through programmed cell death (PCD) (Bangs and White, 2000; Buss *et al.*, 2006) and glial cells clear their corpses from the CNS (Sonnenfeld and Jacobs, 1995a; Marin-Teva *et al.*, 2004). Similarly, during development many superfluous neuronal connections are generated initially and are pruned later as mature neural circuits are refined. Short axonal extensions can be pruned by retraction, but during large-scale pruning of either long projections or large bundles of axons branches are severed

from the parent arbor and degenerate subsequently (reviewed in Luo and O'Leary, 2005). Such axonal pruning events are widespread and have been observed in various regions of the mammalian and *Drosophila* CNS (Truman, 1990; O'Leary and Koester, 1993; Lee *et al.*, 1999; Lee *et al.*, 2000; Kantor and Kolodkin, 2003; Watts *et al.*, 2003). In *Drosophila*, glial cells rapidly engulf pruned, degenerating axons to clear them from the CNS (Cantera and Technau, 1996; Awasaki and Ito, 2004; Watts *et al.*, 2004; Hoopfer *et al.*, 2006). Although it has not been demonstrated definitively that glia engulf pruned axons in the developing mammalian CNS, it is likely that microglia and/or astrocytes clear degenerating pruned axons from the CNS because these glial subtypes actively engulf axons that are degenerating as a result of injury (Bechmann and Nitsch, 1997).

Together, glial phagocytic functions help sculpt the morphology and connectivity of the developing neural circuits, and stop degenerating neuronal material accumulating in the forming nervous system. Non-engulfed apoptotic cells can leak toxic intracellular contents or antigenic molecules into the extracellular environment (Savill *et al.*, 2002). Therefore, ineffective removal of neuronal debris might either damage healthy neurons or trigger secondary inflammatory immune responses that potentially contribute to autoimmune disease.

How do glia recognize dead or damaged neurons? Recognition and internalization of engulfment targets by phagocytes is a multi-step process (Fig. 1). Cells that are destined for destruction are thought to present an 'eat-me' cue on their surface, which is recognized by receptors on phagocytes. If the phagocyte is adjacent to the engulfment target, immediate recognition can ensue. However, when the phagocyte is not nearby, dying cells can recruit phagocytes by secreting 'come-get-me' chemoattractant signals (Grimsley and Ravichandran, 2003; Lauber *et al.*, 2004). Activation of recognition receptors on the phagocyte by 'eat-me' signals promotes cytoskeletal reorganization in the phagocyte, pseudopodial extension around the engulfment target and, finally, internalization of the target cell into a phagosome that fuses with lysosomal compartments to digest the internalized cellular debris completely (Krysko *et al.*, 2006). Although the cellular steps that drive these complex engulfment events are well described, little is known about the molecular pathways that underlie glial recognition and phagocytosis of engulfment targets.

***Drosophila* as a model to understand glial phagocytic function**

The fruit fly *Drosophila* is an excellent model for investigating the cellular and molecular mechanisms of engulfment by glial cells. Similar to the mammalian brain, the *Drosophila* CNS is inaccessible to hematopoietically derived immune cell types such as macrophages, and glia are the primary endogenous phagocytic cell type within the nervous system. As outlined below, *Drosophila* glia, like their mammalian counterparts, act to clear a range of engulfment targets from the developing and mature nervous system, including apoptotic neurons and degenerating axon debris (Sonnenfeld and Jacobs, 1995a; Cantera and Technau, 1996; Awasaki and Ito, 2004; Awasaki *et al.*, 2006; MacDonald *et al.*, 2006).

The *Drosophila* nervous system contains well-defined glial subtypes that are reminiscent of the major glial subtypes found in mammals (Ito *et al.*, 1995; Klambt *et al.*, 1996). 'Cell body glia' are located among neuronal cell bodies of the cortex and probably perform general, astrocyte-like, support functions. 'Neuropil glia', which are similar to mammalian oligodendrocytes, wrap axon fascicles in the CNS. 'Surface glia' cover the entire surface of the *Drosophila* CNS to form a protective barrier that is comparable to the blood-brain barrier of mammals (Fig. 2A). 'Peripheral glia' ensheath peripheral axon projections, as Schwann cells do in vertebrates (Ito *et al.*, 1995). Finally, 'midline glia', which are analogous to floorplate cells in vertebrates, occur only at the midline of the CNS and influence axon guidance during embryogenesis. Relative to mammalian model systems, the

Drosophila nervous system is significantly less complex, develops in a much shorter time and is highly accessible experimentally. Most embryonic glial lineages can be identified precisely and traced from precursor to post-mitotic glia, excellent markers are available to label specific glial subtypes, and a myriad of powerful molecular genetic tools are available in *Drosophila* to manipulate glial gene function *in vivo*.

Glia engulf neuronal cell corpses in the embryonic nervous system of *Drosophila*

In all organisms with a complex nervous system, significant numbers of newly-born neurons commit suicide through well-conserved apoptotic pathways during neurogenesis (Bangs and White, 2000; Buss *et al.*, 2006). How many cells die in the developing nervous system? The spatial and temporal pattern of PCD has been characterized exceptionally well in the *Drosophila* embryonic nervous system. Recently, Rogulja-Ortmann and colleagues (Rogulja-Ortmann *et al.*, 2007) used DiI lineage tracing of uniquely identifiable, fully defined neuroblast (NB) lineages to compare the progeny made by NBs in control and PCD-deficient embryos. Strikingly, it was found that PCD is likely to be responsible for the destruction of ~30% of the roughly 500 neurons produced per hemisegment (~180 cells, on average, per hemisegment) during normal embryonic CNS development (Rogulja-Ortmann *et al.*, 2007). In some lineages the destruction of neurons is extreme. For example, the NB lineage referred to as NB7-3 (based on its position in the CNS) consists normally of four neurons, but in PCD-deficient embryos ~nine neurons are present, which indicates that under normal developmental circumstances ~five out of nine neurons within this lineage undergo PCD (Rogulja-Ortmann *et al.*, 2007).

Impressively, glial cells (of which there are only ~32 per hemisegment) engulf this large population of neuronal cell corpses efficiently within the 20-hour period of embryonic nervous system development. Sonnenfeld and Jacobs (1995a) provided the first evidence that glia are capable of engulfing apoptotic neurons in the *Drosophila* embryo. Using electron microscopy (EM), they found that glia (but not neurons) in both the CNS and PNS often contain internalized cell corpses. The engulfment of neuronal cell corpses by glia is likely to be rapid because nearly all corpses were found within glia and only a few unengulfed cell corpses were observed inside the CNS. Whereas all glial subtypes were found to contain cell corpses, it appears that one particular class of *Drosophila* glia, surface glia, contain most of the CNS cell corpses observed in these EM studies (Sonnenfeld and Jacobs, 1995a). Surface glia ensheath the entire CNS but also send projections into the cortex of the nervous system (Ito *et al.*, 1995). This unique morphology allows them to associate intimately with the developing CNS cell cortex (where neuronal cell corpses are generated) and with peripheral macrophages that migrate throughout the non-CNS tissues of the embryo (Fig. 2A) (Sonnenfeld and Jacobs, 1995a; Sonnenfeld and Jacobs, 1995b; Zhou *et al.*, 1995). The observed abundance of cell corpses in surface glia indicates that engulfment and/or destruction of neuronal cell corpses might be a function that is assigned specifically to surface glia. This raises the possibility that surface glia function as the major phagocytic cell type and/or act as a sink for cell corpses in the embryonic CNS.

In addition to clearing dead neurons from the CNS, glia might also promote PCD in specific populations of neurons. For example, during normal cerebellum development in mammals, a significant number of developing Purkinje cell neurons undergo PCD and are engulfed by microglia. Because microglia actively engulf caspase-3-positive, apoptotic Purkinje cells, they secrete reactive oxygen species to facilitate neuronal destruction (Marin-Teva *et al.*, 2004). This approach is reminiscent of those used by professional macrophages to rapidly destroy engulfment targets, such as invading pathogens (Halliwell, 2006). If engulfing microglia are prevented from invading the cerebellum, far fewer Purkinje cells undergo apoptotic death, which indicates that microglia are able to reduce Purkinje cell populations by actively promoting the destruction of a subset of these neurons. These results, coupled

with the observation that apoptotic cell death is suppressed partially in *C. elegans* when cell corpse engulfment is blocked genetically (Hoepfner *et al.*, 2001; Reddien *et al.*, 2001), have led to the suggestion that the final execution of PCD in neuronal cells requires input from glia in the form of phagocytic activity. Such a mechanism would allow glia to modulate directly which neurons survive in the nervous system. However, it remains to be determined whether glial phagocytic activity enhances neuronal PCD in the developing *Drosophila* nervous system.

Glia engulf cell corpses and pruned axons during *Drosophila* metamorphosis

During insect metamorphosis, the larval nervous system undergoes extensive remodeling to establish the mature circuitry of the adult nervous system. During this transformation, many larval neurons are destroyed by PCD and are cleared from the CNS, and the projections of many larval neurons are pruned so that they can extend new, adult-specific neurites (Technau and Heisenberg, 1982; Truman, 1990; Lee *et al.*, 1999). *Drosophila* glia have an important role during this larva-to-adult transition by engulfing these apoptotic neurons and degenerating neurites, thereby assisting in the morphogenesis of adult-specific cell populations and their patterns of connectivity (Cantera and Technau, 1996; Watts *et al.*, 2003; Awasaki and Ito, 2004; Awasaki *et al.*, 2006; Hoopfer *et al.*, 2006).

Cantera and Technau (1996) were the first to observe glial phagocytosis of cell corpses during metamorphosis by performing EM analysis on *Drosophila* pupae. Unlike in the embryo, where most cell corpses are found in surface glia (Sonnenfeld and Jacobs, 1995a), corpses are internalized in virtually all glial subtypes of the pupae (Cantera and Technau, 1996). Intriguingly, individual glial cells that are wrapped around healthy neurons can also contain an engulfed corpse (Cantera and Technau, 1996). This observation suggests that glia can recognize and destroy an apoptotic neuronal corpse while simultaneously nourishing and ensheathing healthy neurons. However, EM studies do not allow one to follow the fates of individual cells over time and it is possible that these seemingly healthy neurons in contact with phagocytic glia are actually on the verge of either death or pruning themselves.

More recent studies have addressed the role of glia in clearing pruned neurites during metamorphosis. In the larval mushroom body (MB) a subset of neurons referred to as gamma neurons extend dendritic projections close to their cell bodies and a single, long axon that bifurcates into two branches that project into the dorsal lobe and the medial lobe of the MB (Fig. 2B). During metamorphosis, the dorsal and medial branches of gamma neurons are pruned back, and these neurons then send out more elaborate adult-specific projections (Lee *et al.*, 1999; Lee *et al.*, 2000). Pruning of gamma neuron projections is marked by the breakdown of the microtubule cytoskeleton, separation of axon fragments from the parent arbor, fragmentation of pruned axon fibers and, finally, clearance of cellular debris from the nervous system (Fig. 2B) (Lee *et al.*, 2000; Watts *et al.*, 2003; Awasaki and Ito, 2004). Coordinate with the onset of gamma neuron axon degeneration in *Drosophila*, glial cells accumulate around the MB, extend cellular processes into the dorsal and medial lobes, and begin engulfing fragmenting axons and dendrites (Fig. 2B) (Awasaki and Ito, 2004; Watts *et al.*, 2004). By genetically labeling glia and gamma neuron membranes before EM analysis, Watts *et al.* (2004) showed that degenerating axonal debris accumulates in invading glial cells. Moreover, Awasaki and colleagues (2004) demonstrated that genetically blocking glial endocytic function significantly suppresses the clearance of both the dorsal and medial branches of gamma neuron axons. Thus, glial engulfment activity is essential for the timely removal of pruned axon fibers in the *Drosophila* MB.

It remains unclear whether glia actively promote neurite pruning via engulfment or whether they are simply cleaning up CNS cellular debris. Awasaki *et al.* have shown that

microtubule cytoskeleton disruption (the first hallmark of pruning) is still detectable in gamma neuron axons when glial invasion into the MB lobes is suppressed (Awasaki *et al.*, 2006). This indicates that glial engulfment is not essential for initiation of the axon pruning program. However, it is possible that glial phagocytic function promotes destruction of gamma neuron axons because larval gamma neuron axon fibers persist in the adult MB lobes when glial invasion is suppressed, but the extent of fragmentation of the remaining axons is unclear (Awasaki and Ito, 2004; Awasaki *et al.*, 2006; Hoopfer *et al.*, 2006).

Glia respond to axon injury and engulf degenerating axonal debris in the brain of adult *Drosophila*

Many forms of trauma, including anoxia, bacterial invasion and mechanical injury elicit potent immune responses from mammalian astrocytes and microglia. These responses, which are referred to collectively as ‘reactive gliosis’, include glial hypertrophy (increased size), proliferation, migration towards severed axons, and phagocytosis of degenerating neurons and neuronal debris (Pekny and Nilsson, 2005; Binder and Steinhauser, 2006). Do *Drosophila* glia undergo any type of reactive gliosis in response to brain trauma? MacDonald and colleagues (2006) have addressed this question recently using the adult *Drosophila* olfactory system as a model to study glial responses to axon injury *in vivo* (MacDonald *et al.*, 2006). The cell bodies of factory receptor neurons (ORNs) reside outside the CNS in the third antennal segments and maxillary palps, and send axonal projections into the antennal lobes of the brain to form synapses within specific glomerular structures (Fig. 2C). If ORNs are severed by simple surgical ablation of either the antennae or maxillary palps, injured ORN axons rapidly undergo Wallerian degeneration (Fig. 2C) (MacDonald *et al.*, 2006). Within hours of cutting ORN axons, glia exhibit dramatic changes in morphology, extend their membranes to cover severed axons and begin to engulf degenerating axonal debris (MacDonald *et al.*, 2006). Thus, *Drosophila* glia can indeed sense axon injury, and such injuries result in robust activation of glial immune responses that are reminiscent of mammalian reactive gliosis.

The unique histology of the antennae versus the maxillary palps allows analysis of glial responses to two very different injury events. Surgical ablation of antennae severs ~600 antennal ORN axons that collectively project to ~44 of the 50 glomeruli in the *Drosophila* antennal lobe (Stocker *et al.*, 1990; Stocker, 1994; Vosshall *et al.*, 2000). This ablation represents a ‘traumatic’ type of injury to the antennal lobe because ~90% of all innervating axons are severed. Antennal lobe glia exhibit a conspicuous expansion of glial membranes in response to antennal ablation (Fig. 2C), which is likely to represent glial hypertrophy similar to that observed in mammals. However, it is possible that increases in glial membranes might represent a redistribution of glial processes from other areas of the brain toward sites of axon injury. In addition, glia increase expression of engulfment-related molecules (e.g. Draper, see below) after ORN axon injury (MacDonald *et al.*, 2006), which indicates that glial cells upregulate the cellular machinery that is used to clear degenerating axons from the CNS. Together these data show that *Drosophila* glia respond rapidly to axon injury with dramatic changes in morphology and gene-expression profiles.

By contrast, surgical ablation of maxillary palps results in injury to a relatively small number of ORN axons (~60). These axons innervate only six of the 50 antennal lobe glomeruli, which are uniquely identifiable in the brain based on their positions in the antennal lobe (Vosshall *et al.*, 2000). Maxillary palp ablation can therefore be used to examine glial responses to injury of a small subset of axons in an otherwise healthy CNS. Within hours of maxillary palp ablation, glial processes begin to accumulate specifically on degenerating ORN axons (Fig. 2C) and, over the next several days, clear these degenerating axons from the CNS (MacDonald *et al.*, 2006). This highly localized recruitment of glial processes to severed axons indicates that *Drosophila* glia can discriminate, perhaps with

single-axon resolution, between healthy and severed neurons. Consistent with this notion, maxillary palp ORN projections remain intact after antennal ablation and, conversely, maxillary palp ablation does not affect the morphology of antennal ORN axons (MacDonald *et al.*, 2006). Moreover, if ORN axon degeneration is blocked by expression of the mouse neuroprotective molecule Wallerian degeneration slow (Wld^s) (Lunn *et al.*, 1989) in a subset of ORNs, glia are not recruited to Wld^s-expressing severed axons, whereas adjacent Wld^s-negative severed axons become covered with glial processes and are cleared from the CNS (MacDonald *et al.*, 2006). Thus, *Drosophila* glia can discriminate between degenerating and intact neurons and engulf only degenerating axons, which implies that axons undergoing Wallerian degeneration are cell-autonomously tagged with yet-to-be-identified 'eat-me' cues, much like cell corpses. The observation that glial processes extend rapidly toward severed ORN axons (MacDonald *et al.*, 2006) opens the door to the possibility that degenerating axons may also secrete 'come-get-me' cues that recruit glia.

The fate of glial engulfment targets

After engulfment targets are recognized and internalized by glia, they must ultimately be destroyed. During metamorphosis, glia appear to digest apoptotic cells and pruned neurites through classic phagolysosomal pathways (Fig. 1). Early EM analysis has shown that glia in the pupal CNS contain engulfed cell corpses in lysosome-like structures (Cantera and Technau, 1996). More recent studies reveal that prominent multivesicular bodies (MVBs), which are a structural hallmark of active phagocytes, are detected in the glial cells that invade the MB lobes to engulf pruned gamma neuron axons (Mullins and Bonifacino, 2001; Watts *et al.*, 2004). Fluorescent labeling has also confirmed that these glia contain abundant endosomal and lysosomal compartments (Awasaki *et al.*, 2004; Watts *et al.*, 2004). Future studies are essential to determine whether similar mechanisms are used by mature *Drosophila* glia to destroy degenerating axons in the adult brain, as appears to be the case in mammals (Bauer *et al.*, 1994).

Do embryonic glia also destroy cell corpses once they are engulfed? Lysosomal-like structures have not been described in embryonic glia, although this has not been extensively studied. It has been proposed that embryonic surface glia, rather than destroy neuronal cell corpses, might instead transfer them to macrophages at the surface of the CNS for subsequent destruction (Fig. 2A) (Sonnenfeld and Jacobs, 1995a). This model is largely based on two observations. First, genetically-labeled neuronal debris is sometimes observed within embryonic macrophages (Sonnenfeld and Jacobs, 1995a). Second, when macrophages are eliminated genetically from embryos, cell corpses accumulate in the ventral surface glia and just outside the CNS (Sonnenfeld and Jacobs, 1995a). However, cell corpses that accumulate outside the CNS in macrophage-depleted embryos by EM studies might be nonneuronal corpses that are cleared normally by macrophages. In addition, cell corpses or neuronal fragments in the PNS might be engulfed by macrophages, thereby explaining the appearance of neuronal debris within embryonic macrophages. It is unlikely that macrophages directly engulf corpses in the nervous system because macrophages or cellular projections from macrophages have not been observed in the embryonic, pupal, and adult CNS (Abrams *et al.*, 1993; Sonnenfeld and Jacobs, 1995a; Sonnenfeld and Jacobs, 1995b; Cantera and Technau, 1996; M.A.L. and M.R.F., unpublished results). Future studies that include real-time imaging to follow the fate of neuronal corpses in the embryonic CNS should provide significant insights into the dynamics of corpse clearance, reveal precisely which glia perform engulfment functions in the embryo, and resolve whether surface glia do indeed transfer engulfed corpses directly to macrophages outside the CNS. In addition, given that glia provide trophic support to neurons, glial engulfment might also serve as an efficient way to recycle cellular materials from either dead neurons or degenerating axons to

support healthy neurons in the CNS. Glial cells are therefore uniquely positioned to act as both destructive phagocytes and providers of recycled nutrients.

Molecular mechanisms of engulfment: insights from *C. elegans*

Genetic studies in *C. elegans* have been fruitful in defining some of the molecular mechanisms that drive cell corpse engulfment during development. At least two, partially redundant, signaling pathways that are involved in recognition and engulfment of apoptotic cells have been identified (Fig. 3) (reviewed in Reddien and Horvitz, 2004; Mangahas and Zhou, 2005). In one pathway, a transmembrane receptor, CED-1, acts as a phagocytic receptor that recognizes an unknown ligand presented by corpses and then activates downstream pathways to drive corpse internalization (Zhou *et al.*, 2001a). The unidentified ligand for CED-1 is thought to encode the 'eat-me' cue presented by cell corpses, based on experiments that show CED-1 accumulates preferentially within engulfing cells at sites of cell corpse contact even if the intracellular domain of CED-1 is removed (Zhou *et al.*, 2001a). This indicates strongly that the CED-1 ligand is presented directly by cell corpses and recognized by the CED-1 extracellular domain (Zhou *et al.*, 2001a). CED-1 then transduces engulfment signals through a pathway that involves the adaptor protein CED-6 (Zhou *et al.*, 2001a; Mangahas and Zhou, 2005). Intriguingly, the ABC transporter CED-7 is required both in the corpse and in the engulfing cell for proper corpse engulfment (Wu and Horvitz, 1998). ABC transporters regulate the translocation of various substances, including proteins and phospholipids, across cell membranes in an ATP-dependent manner (Hamon *et al.* 2000; Jones and George, 2004). Intriguingly, CED-1 receptor clustering around cell corpses is suppressed in *ced-7* mutants, which indicates that CED-7 controls the presentation of the 'eat-me' signal in dying cells and thereby affects CED-1 receptor clustering or activation in the phagocyte (Zhou *et al.*, 2001a).

A second engulfment pathway includes CED-2, CED-5 and CED-12. Biochemical evidence indicates that these three proteins function in a complex in engulfing cells to induce cytoskeletal remodeling via the Rac GTPase CED-10, which is presumably required for internalization of cell corpses after recognition (Zhou *et al.*, 2001b; Wu *et al.*, 2001; Brugnera *et al.*, 2002; Mangahas and Zhou, 2005). The primary cell corpse-recognition receptor that acts upstream of the CED-2/CED-5/CED-12 complex has not been identified. One study implicates the *C. elegans* phosphatidylserine receptor (PS-R) as an activator of this pathway (Wang *et al.*, 2003). Phosphatidylserine (PS) is a membrane phospholipid that is translocated from the inner to the outer leaflet of the lipid bilayer on apoptotic cells and, therefore, is a prime candidate for the 'eat-me' signal (Fadok *et al.*, 1992; Fadok *et al.*, 2000; Wu *et al.*, 2006). However, the engulfment defect in PS receptor mutants is significantly weaker than the phenotype of CED-2, CED-5 or CED-12 mutants, which indicates that a second, unidentified, receptor is either largely responsible for activation of this engulfment pathway or acts redundantly of the PS receptor (Wang *et al.*, 2003). Recent evidence indicates that the CED-1/CED-6/CED-7 pathway also converges on CED-10 to promote actin remodeling and subsequent engulfment of corpses (Kinchin *et al.*, 2005). Therefore CED-10 represents a molecule that drives the functional output for both of the identified engulfment pathways in *C. elegans*.

Genes that initiate and execute apoptotic cell death have been highly conserved during evolution (Bangs and White, 2000; Lettre and Hengartner, 2006). Thus, it seems likely that the molecular components of the cell corpse engulfment machinery are also well conserved across species, and this does indeed appear to be the case. MEGF10, the likely human homolog of CED-1, both enhances the ability of HeLa cells to phagocytose apoptotic cells in an *in vitro* assay and partially rescues the corpse engulfment phenotype of *ced-1* mutant worms when expressed under the control of the *ced-1* promoter (Hamon *et al.*, 2006). Overexpression of the human homolog of CED-6 (GULP) also enhances the engulfment of

apoptotic cells by mammalian macrophages in culture, which indicates that CED-6 represents a highly conserved signaling molecule that regulates phagocytosis positively (Smits *et al.*, 1999). The mammalian counterparts of CED-2, CED-5 and CED-12 (CrkII, Dock180 and ELMO1, respectively) also function cooperatively to promote engulfment activity in cultured mammalian cells (Gumienny and Hengartner, 2001; deBakker *et al.*, 2004). Finally, ABC1, the mammalian homolog of CED-7, enhances the translocation of the phosphatidylserine from the inner to the outer leaflet of the lipid bilayer on apoptotic cells *in vitro* (Hamon *et al.*, 2000) and is implicated in engulfment activity of mammalian phagocytes (Hamon *et al.*, 2000; Jehle *et al.*, 2006; Kiss *et al.*, 2006).

Draper/CED-1 is essential for engulfment by glia in *Drosophila*

Recent studies to identify the molecular factors involved in *Drosophila* glial engulfment functions have revealed that a CED-1-like signaling pathway is essential for glial phagocytic activity. Draper is the *Drosophila* ortholog of the *C. elegans* cell corpse engulfment receptor CED-1 and appears to be expressed in nearly all *Drosophila* glia throughout development and in the adult brain. During CNS development, Draper protein accumulates around neuronal cell corpses as they form in the embryonic CNS, which indicates that it also binds cell corpses. In addition, twice as many cell corpses are found in the CNS at late embryonic stages in *draper*-null mutants compared to control animals (Freeman *et al.*, 2003). This phenotype is strikingly similar to that of *ced-1*-mutant worms, where unengulfed cell corpses accumulate throughout the animal (Zhou *et al.*, 2001a). The only other receptor implicated in the clearance of cell corpses in the *Drosophila* embryo is Croquemort, which is expressed in embryonic macrophages (Franc *et al.*, 1996) and is required for proper macrophage engulfment of apoptotic cells (Franc *et al.*, 1999). However, Croquemort is not expressed in embryonic glial cell types (M.A.L. and M.R.F., unpublished observations), and is therefore unlikely to be involved in glial engulfment of embryonic neuronal cell corpses. Thus, Draper appears to be a central component of the glial cell corpse engulfment machinery and mediates glial phagocytosis of neuronal cell corpses during embryonic CNS assembly.

Draper is also used by glia to recognize and engulf degenerating axons in at least two contexts: developmental axon pruning and Wallerian degeneration. During metamorphosis, Draper is required for glia to infiltrate the MB lobes at the time of pruning of gamma neuron axons and for the timely removal of pruned MB axonal and dendritic debris (Awasaki *et al.*, 2006; Hoopfer *et al.*, 2006). dCED-6, the *Drosophila* ortholog of *C. elegans* CED-6 and a downstream effector of CED-1 signaling, is also necessary for *Drosophila* glia to remove pruned axons efficiently from the CNS (Awasaki *et al.*, 2006). Similarly, when adult ORN axons are severed in *draper*-null mutants, glia fail to extend cellular processes toward degenerating axons and the majority of degenerating ORN axonal debris is not cleared from the CNS (MacDonald *et al.*, 2006). Together, these studies demonstrate that Draper is a core component of a glial engulfment pathway that is used to identify degenerating axons. Furthermore, they indicate that glial engulfment of axonal debris and the extension of glial processes toward degenerating axons are tightly coupled because both events are suppressed in *draper* mutants.

Notably, the Draper/CED-1 pathway is also used by macrophages as they clear degenerating neuronal debris from the PNS during metamorphosis. Dendritic arborizing (da) sensory neurons send extensive dendritic arbors beneath the epidermis of the larval body wall, and these arbors are believed to be directly engulfed by macrophages during developmental dendritic pruning (Williams and Truman, 2005). Draper is expressed strongly in *Drosophila* macrophages (Freeman *et al.*, 2003), and in *draper* mutants da sensory neuron dendrites are severed from their parental arbors on schedule but are not removed from the body wall by macrophages (Williams *et al.*, 2006). Thus, Draper is also required for macrophages to

recognize neuronal debris in the PNS. These observations, coupled with the glial data, indicate that Draper signaling is essential for engulfment of debris generated from all compartments of the neuron: soma, axon and dendrite.

In *C. elegans* the CED-1/CED-6/CED-7 pathway is partially redundant with CED-2/CED-5/CED-12. It appears likely that additional pathways have a role in glial clearance of neuronal debris targets in the *Drosophila* CNS, based on two observations. First, in the embryo a conservative estimate from recent studies (Rogulja-Ortmann *et al.*, 2007) indicates that, on average, >150 cell corpses are generated per hemisegment over the course of embryonic CNS development. At late embryonic stages ~25 cell corpses are observed per hemisegment in the wild-type CNS, whereas in *draper*-null mutants there are ~50 (approximately twice as many) (Freeman *et al.*, 2003). Far more corpses, perhaps approaching 150 cell corpses per hemisegment, might be expected if glial engulfment activity is blocked completely in *draper*-null mutants. However, the possibility that maternal contribution of Draper is responsible for the persistent clearance of cell corpses cannot be ruled out. Second, although the clearance of pruned MB gamma axons is clearly delayed in *draper*-null mutants during pupal stages, nearly all of these axons disappear from the CNS within the first two weeks of adulthood (Awasaki *et al.*, 2006). Thus, ultimately, glial clearance of pruned axons appears to reach completion even in the absence of Draper function.

Glial recognition of engulfment targets

Functional analysis in *Drosophila* shows clearly that Draper is essential for the proper removal of cell corpses, pruned neurites and degenerating axons from the CNS by glia. Studies from *C. elegans* argue strongly that CED-1 and, probably therefore, Draper act directly in engulfment target recognition. We envision the following as plausible models for how Draper might modulate glial phagocytic action in the CNS. First, each glial engulfment target (i.e. cell corpses, pruned neurites and degenerating axons) might present the same 'eat-me' cue, which is a ligand for the Draper receptor. Glial-expressed Draper would then recognize, but not discriminate between, engulfment targets through binding to this generic engulfment cue. Second, each engulfment target might present a distinct 'eat-me' cue. In this situation, unique isoforms of Draper, which are generated through alternative mRNA splicing (Fig. 4) (Freeman *et al.*, 2003), might recognize a specific ligand presented by engulfment targets, and Draper-ligand interaction would then drive subsequent phagocytosis of engulfment targets by glia. Alternatively, the ability of glia to recognize and engulf specific targets that present distinct 'eat-me' cues might be regulated by unidentified Draper co-receptors. Finally, it is possible that Draper acts as a non-specific engulfment receptor that promotes the phagocytosis of any target, whereas additional glial-expressed receptors modulate specific target-recognition events. Future experiments aimed at resolving which Draper isoform(s) are sufficient for glial engulfment of corpses versus pruned or severed axons should shed light on these important questions about glial recognition of engulfment targets. Ultimately, to understand how targets are autonomously tagged for clearance by glia it will be necessary to identify the ligand(s) for Draper and to determine how, when and where these molecules are presented by cell corpses and degenerating neurites.

Draper/CED-1 signal transduction

The emerging model for the initiation of Draper/CED-1 receptor signaling in engulfing cells is that the extracellular domain of the receptor recognizes ligands present on apoptotic cells or degenerating axons, which results in receptor clustering at sites of direct contact with either the dying cell or degenerating axon. Receptor clustering around apoptotic cells is independent of the act of engulfment: clustering of CED-1 around corpses precedes engulfment and the intracellular domain of CED-1 is dispensable for receptor clustering (although it is required for internalization of corpses) (Zhou *et al.*, 2001a). It is likely that

clustered CED-1/Draper receptors recruit signaling molecules that reorganize the cytoskeletal network of the engulfing cell to form a phagocytic cup around the dying cell and, eventually, internalize the corpse. A similar mechanism is predicted to apply for degenerating axons and dendrites. The adaptor protein CED-6 interacts directly with the intracellular domains of CED-1 and Draper, and is important for CED-1 function (Zhou *et al.*, 2001a; Su *et al.*, 2002; Awasaki *et al.*, 2006), but how CED-6 promotes engulfment remains unclear. At least two motifs in the CED-1 intracellular domain, which are also present in Draper, are important for engulfment (Fig. 4). In *C. elegans*, mutation of either the CED-6-binding site NPXY (Su *et al.*, 2002) or a YXXL domain within the intracellular domain of CED-1 partially inhibits CED-1-mediated engulfment activity *in vivo* (Zhou *et al.*, 2001a). However, if both the NPXY and YXXL motifs are mutated, CED-1 engulfment is blocked completely (Zhou *et al.*, 2001a). The YXXL motif is a potential binding site for proteins that contain Src homology 2 (SH2) domains (Pawson and Scott, 1997), but no molecules that interact directly with this conserved domain have been identified.

CONCLUSIONS

Glial phagocytic activity is a widespread cellular event during nervous system development in *Drosophila* and other higher organisms. The rapid clearance of neuronal corpses and cellular debris removes potentially harmful material from the CNS and eliminates superfluous axonal connections. What would happen to the CNS if glial engulfment were blocked completely? Would lingering cell corpses cause neurodegeneration? Would the remnants of pruned axons that are not cleared from the CNS affect the function of closely associated neural circuits? These remain open questions because there is currently no way to completely (and specifically) inhibit glial engulfment activity in either *Drosophila* or other organisms.

Despite the prevalence of glial phagocytic activity in the CNS there is a paucity of molecular information about this process. Key questions that remain unanswered include: what are the 'eat-me' cues presented by cell corpses and degenerating neurites? Are they ligands for Draper receptor? What molecular pathways lead to the exposure of 'eat-me' cues? What are the signaling molecules downstream of Draper that drive phagocytosis? What other molecular pathways drive neuron-glia interactions after neuronal death and trauma? *Drosophila* offers the opportunity to combine both forward and reverse genetic methods with a simple, well-defined, highly accessible nervous system. The CED-1/Draper signaling pathway used by *Drosophila* glia to engulf neuronal corpses and degenerating axons appears well conserved from worms to humans. Thus, mammalian glia might use a similar pathway (i.e. MEGF10) to phagocytose cellular debris during either developmental axon pruning or as reactive glia respond to degenerating axons after CNS trauma or disease. Studies of glial function in *Drosophila* will provide new insights into the role of CED-1/Draper in glial engulfment, and uncover additional aspects of the cellular and molecular features that modulate the phagocytic functions of glia.

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Fig. 1. Recognition and engulfment of target cells

(a) Some engulfment targets secrete chemoattractants to entice migratory phagocytes to 'come-get-me'. (b) Phagocytes recognize targets for engulfment through activation of phagocytic immune receptors that bind 'eat-me' tags presented on the surface of engulfment targets. (c) These immune receptors then activate downstream signaling cascades that allow phagocytes to extend pseudopodial projections and, ultimately, envelop and completely internalize the target cell into a phagosome compartment. (d) Engulfed material is then digested efficiently within the confines of the phagolysosome.

Fig. 2. Schematic models of glial engulfment in the *Drosophila* nervous system

(A) Glial cells engulf apoptotic neuron corpses in the embryo: Cartoon of a cross-section through a late stage (stage 15/16) embryonic CNS showing only surface glial subtypes (green). Most apoptotic corpses (tan) occur in surface glia (green), particularly within ventral regions of the CNS. After corpses are engulfed, they may be destroyed by glia through lysosomal destruction or possibly transferred to macrophages (pink) that reside outside of the CNS (indicated by question mark). See text for more details. (B) Glia engulf pruned neurites during metamorphosis: At late larval stages, mushroom body (MB) gamma neurons project a single axon extension that bifurcates into the dorsal and medial lobes of the MB. By 6 hours after puparium formation (APF), gamma neuron axons begin to display

signs of 'blebbing' (arrowheads), and glial cell bodies (green) have accumulated around the dorsal and medial lobes of the MB. Over the next 12 hours, glial membranes invade the MB lobes and engulf fragments of degenerating gamma neuron axons as they are pruned. Glia also engulf degenerating gamma neuron dendrites during this time. (C) Mature glia engulf degenerating olfactory receptor neuron (ORN) axons after injury: ORN cell bodies reside in either the third antennal segments (purple) or maxillary palps (orange) and send axonal projections into the brain where they synapse on antennal lobe (AL) glomeruli. Antennal or maxillary palp ORN axons can be selectively severed by surgical ablation of these olfactory organs. Within hours after injury, severed ORN axons fragment into smaller pieces, and glial cell membranes accumulate specifically on AL glomeruli that contain severed axons to begin engulfing fragmented axon debris.

Fig. 3. Current model of corpse recognition and engulfment in *C. elegans*

Studies in *C. elegans* have identified two, parallel, partially redundant pathways that are involved in the recognition and engulfment of apoptotic cells. One pathway drives engulfment activity through the receptor CED-1, the adaptor protein CED-6 and the ABC-transporter CED-7, whereas a second pathway signals via the CED-2, CED-5 and CED-12 complex. The phosphatidylserine receptor (PS-R) is placed genetically upstream of the CED-2/5/12 complex (indicated with dotted arrow), but there are probably other recognition receptors that also function within this pathway (indicated by ?). Each engulfment pathway drives phagocytosis through active remodeling of the actin cytoskeleton. See text for more details.

Fig. 4. Multiple isoforms of Draper are present in *Drosophila*

C. elegans CED-1 and all *Drosophila* Draper isoforms are single-pass transmembrane proteins. The extracellular domain (ECD) of CED-1 contains 16 EGF-like repeats (ovals) and might bind 'eat-me' signals presented by cell corpses. Two tyrosine phosphorylation sites within the intracellular domain of CED-1 (NPXY and YXXL) are required for engulfment activity in worm phagocytes. CED-6 interacts with the NPXY motif (Su *et al.*, 2002), but it is unknown how the YXXL domain is involved in transducing CED-1 signaling (indicated by ?). The longest isoform of Draper (Draper I) is most similar to CED-1 and has 15 EGF-like repeats (ovals) in the extracellular domain, whereas the shorter isoforms contain five EGF-like motifs.